Evaluating the effect of LRP/LR antibodies in colorectal carcinoma mouse models and assessing the molecular mechanism of LRP/LR on apoptotic pathways in colorectal carcinoma cells

by

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Abstract

Cancer remains one of the leading causes of death around the world with its incidence and mortality rates constantly increasing. Tumourigenic cells are characteristically seen to overexpress the 37kDa/67kDa laminin receptor (LRP/LR) compared to their normal cell counterparts. LRP/LR is involved in promoting tumourigenic processes such as cellular viability maintenance and apoptotic evasion. Thus, the first main aim of this study was to investigate the role of LRP/LR in cellular viability of early (SW-480) and late stage (DLD-1) colorectal carcinoma cells and thereafter assess the molecular mechanism of LRP/LR on apoptotic pathways upon siRNA-mediated down-regulation of LRP/LR *in vitro*. The data from the study was analysed using a one-way ANOVA, followed by a two-tailed student's *t*-test with a confidence interval of 95%. To further validate the data, the Bonferroni post-hoc test was applied, with p-values of less than 0.05 considered to be significant. Results from the *in vitro* study showed upon siRNA-mediated down-regulation of LRP/LR, the viability of early (SW-480) and late (DLD-1) stage colorectal cancer cells was significantly reduced through increased levels of apoptosis apparent by compromised membrane integrity as determined by Annexin-V/PI assays and confocal microscopy, respectively. Caspase-3 activity assays were performed to confirm apoptosis, where both cell lines exhibited significantly high caspase-3 activity. In addition, down-regulation LRP/LR resulted in a significant increase in caspase-8 and -9 activity in both colorectal carcinoma cell lines. Cell cycle analysis showed that LRP/LR down-regulation resulted in a significant increase in the sub G0/G1 apoptotic phase of DLD-1 cells, when compared to non-transfected cells, confirming the occurrence of apoptosis. To evaluate the mechanistic role of LRP/LR, proteomic analysis of pathways involved in proliferation and apoptosis were further investigated with the MAPK and Apoptotic Proteome Profiler Antibody Arrays™ as well as SWATH-MS (Sequential Window Acquisition of All Theoretical Mass Spectra). Upon siRNA-mediated down-regulation of LRP/LR, various pro-apoptotic proteins including Bax, cleaved caspase-3 and p53 as well as specific proteins in the MAPK cell signalling pathway such as Chk-2 and AMPKα2 were significantly up-regulated in the DLD-1 cells. In addition, several anti-apoptotic proteins were seen to be significantly down-regulated upon siRNA-mediated down-regulation of LRP/LR including xIAP, Claspin and Survivin. The results obtained from the Proteome Profiler Antibody Arrays™ were confirmed through qPCR, by investigating mRNA expression levels of specific proteins. SWATH-MS analysis showed that siRNA-mediated down-regulation of LRP/LR led to significant changes in the proteome of DLD-1 colorectal cancer cells, revealing new roles of the protein as well as confirming its

existing roles. Moreover, it showed that LRP/LR may alter components of the MAPK, p53-apoptotic as well as autophagic signalling pathways to aid colorectal cancer cells in continuous growth and survival. Telomerase activity and telomerase-related proteins were also investigated using qPCR and western blotting, respectively. A significant decrease in telomerase activity as well as decreases in phospho-TERT and telomeric repeat-binding factor 2 (TFR-2) protein expression levels were observed in the DLD-1 cells upon down-regulation of LRP/LR. In addition to its role in cell viability and apoptotic evasion, LRP/LR has also been found to be involved in enhancing tumour cell adhesion and invasion, key points of metastasis. However, previous studies revealed that the application of the anti-LRP/LR specific antibody IgG1- iS18 led to a significant reduction in the metastatic potential of colorectal carcinoma cells *in vitro*. Therefore, the second aim of the present study was to investigate the significance of blocking LRP/LR with the IgG1-iS18 antibody and to determine whether it will be effective in the treatment of colorectal carcinoma *in vivo* using an MF-1 nude mice model. Results from several *in vivo* pilot studies performed showed that all mice were able to successfully develop tumours upon intraperitoneal injection of HT-29 colorectal cancer cells. However, it was found that despite various changes in the concentration as well as the route of administration of the IgG1-iS18 antibody, there was no significant effect seen in tumour growth/shrinkage and metastasis. Due to the data obtained from the pilot studies and time constraints, the full study was not performed. Although the mechanism of action of LRP/LR is far from being understood, findings from this study show that LRP/LR is critically implicated in apoptosis and cell viability maintenance. The current study therefore suggests that siRNA-mediated down-regulation of LRP/LR could be a possible therapeutic strategy for the treatment of colorectal cancer.

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List of Symbols

α	Alpha
A	Amps
β	Beta
cm^3	Cubic centimetre
mm^3	Cubic millimetre
ºC	Degrees Celsius
γ	Gamma
g	Acceleration due to gravity
µl	Microlitre
ml	Millilitre
µg	Microgram
mg	Milligram
M	Molar
mM	Micromolar
min	Minutes
MW	Molecular weight
nm	Nanometre
kDa	Kilodalton
V	Volts
%	Percentage

List of Abbreviations

AD	Alzheimer's Disease
AIDS	Acquired immune deficiency syndrome
AIF	Apoptosis-inducing factor
Apaf-1	Apoptotic protease activating factor-1
ALPS	Autoimmune lymphoproliferative syndrome
AMPK	AMP-activated protein kinase
APC	Adenomatous polyposis coli
APS	Ammonium persulfate
ATCC	American type culture collection
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine triphosphate
Bad	BCL2 antagonist of cell death
Bak	BCL2 antagonist killer
Bax	BCL2 associated X protein
Bid	BH3 interacting domain death agonist
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma protein 2
BH3	Bcl-2 homology-3
BNIP	BCL2/adenovirus E1B 19kDa interacting protein
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CAD	Caspase-activated DNase
CAM	Cell adhesion molecule
Caspases	Cysteine-dependent aspartate-specific proteases
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CEA	Carcinoembryonic antigen
CHK-2	Checkpoint kinase 2
cIAP1	Cellular inhibitor of apoptosis 1

CO_2	Carbon dioxide
CREB	Cyclic AMP-response element binding protein
CT	Computed tomography
DD	Death domain
DDR	DNA damage response
DED	Death effector domain
DIABLO	Direct IAP binding protein with low PI
DISC	Death induced signalling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRAM	Damage-regulated autophagy modulator
dsRNA	Double-stranded ribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence Activated Cell Scanning
FADD	Fas-associated death domain
FAK	Focal adhesion kinase
FasL	Fas ligand
FITC	Fluorescein isothiocyanate
FCS	Fetal calf serum
FDA	Food and Drug Administration
GPX1	Glutathione peroxidase 1
HEK293	Human embryonic kidney cells
HIF-1	Hypoxia inducible factor-1
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HNRNPA1	Heterogeneous nuclear ribonucleoprotein A1

Hsp	Heat shock protein
HtrA2	High-temperature requirement protein A2
IAP	Inhibitor of apoptosis
IARC	International Agency for Research on Cancer
IgG	Immunoglobulin G
JNK	c-Jun N-terminal kinase
LRP/LR	Laminin receptor precursor/high-affinity laminin receptor
MAP	Mitogen activated protein
Mcl1	Myeloid cell leukaemia sequence 1 (BCL2-related)
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MMP	Matrix metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mTOR	Mammalian target of rapamycin
NTC	Non-template control
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCA	Protocatechuic acid
PCD	Programmed cell death
PDCD4	Programmed Cell Death Protein 4
PCR	Polymerase chain reaction
PI	Propidium iodide
PI3K	Phosphoinositide-3 kinase
PrP^c	Prion protein
PS	Phosphatidyl serine
Puma	p53 upregulated modulator of apoptosis
PVDF	Polyvinylidene fluoride
qPCR	Quantitative real-time PCR
RB	Retinoblastoma
RISC	RNA-induced silencing complex

RNA	Ribonucleic acid
RNase	Ribonuclease
RNAi	Ribonucleic acid interference
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RPSA	Ribosomal protein SA
SDS	Sodium dodecyl sulphate
Smac	Second mitochondrial activator of caspases
SMI	Small molecule inhibitor
siRNA	Small interfering RNA
SUMO	Small ubiquitin-like modifier
SWATH-MS	Sequential Window Acquisition of All Theoretical Mass Spectra
TAE	Tris-Acetic acid-EDTA
TEMED	Tetramethylethylenediamine
TLK-1	Tousled-like kinase 1
T_m	Melting temperature
TNFR	Tumour necrosis factor receptor
TRAIL	TNF related apoptosis inducing ligand
TRF-1/2	Telomeric repeat-binding factor-1/2
Tris	2-Amino-2-(hydroxymethyl)-1,3-propandiol
TSE	Transmissible Spongiform Encephalopathies
VEGF	Vascular endothelial growth factor
XIAP	X-linked mammalian inhibitor of apoptosis protein

Chapter 1: Literature Overview

1.1.Epidemiology, diagnosis and treatment

Cancer is a collective term used for the group of diseases best defined by the abnormal growth of cells, which have the potential to invade and ultimately, spread to numerous sites in the body [1]. There are several key contributors to the transformation of a normal cell into a tumourigenic cell. This includes both internal factors such as genetic mutations and epigenetic alterations, as well as external factors including tobacco intake, obesity, and exposure to radiation or infectious organisms [1].

In 2018, the World Health Organization (WHO) reported cancer to be the second leading cause of death globally [2]. Recently, the International Agency for Research on Cancer (IARC) estimated that 18 million new cases of cancer were diagnosed and 9.6 million cancer deaths occurred in 2018 [2]. In addition, it has been reported that the total number of cancer incidences will rise by over 70% within the next two decades in both economically developed countries where it has been found to be the primary cause of death, as well as economically developing countries, where cancer is identified as the second leading cause of death [1]. Recent studies have indicated that South Africa is ranked 50th with regards to the highest global cancer incidence rates and should expect a 78% increase in cancer prevalence by 2030 [3]. This has a direct correlation with the high prevalence of HIV/AIDS as cancer is known to target immuno-compromised systems [4]. Furthermore, due to South Africa being an economically developing country, factors such as shortage of medical facilities, diet change and population growth all contribute to the rise in cancer incidences [1].

The current study focuses on a cancer type known as colorectal cancer. In South Africa, colorectal cancer was found to be the fourth most common cancer in 2018, and the sixth most fatal [5]. However, globally, it has been ranked as the 3rd most common cancer type and is among the top five most frequent locations for cancer development in men and women - with over 1.8 million new cases in the year 2018, contributing to 10.9% of the total number of cancer cases diagnosed (Fig.1), including 881 000 cancer related deaths [2]. Due to the increasing prevalence and mortality rates of colorectal cancer, it is crucial to develop a novel treatment strategy to combat this disease.

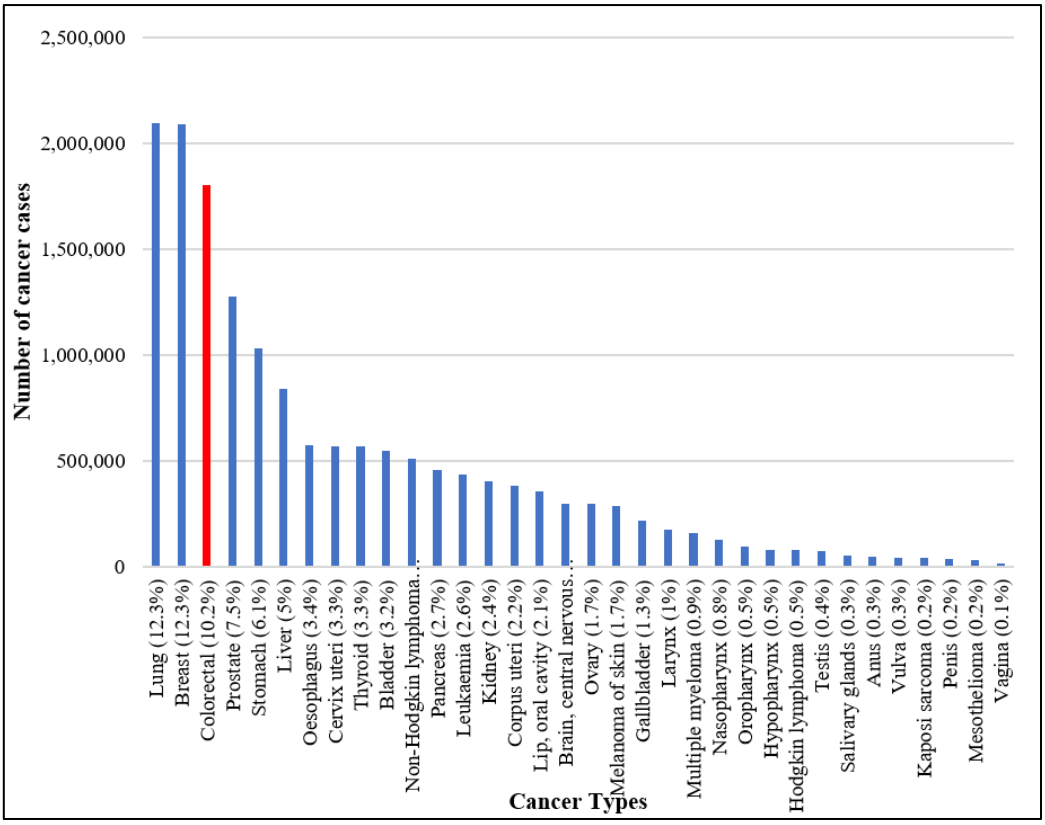


Figure 1: Estimated global cancer incidence rates for 2018. Colorectal cancer was found to be the third most common cancer diagnosed, accounting for 10.9% of the global cancer incidence in 2018 (Adapted from https://www.wcrf.org/dietandcancer/cancer-trends/worldwide-cancer-data).

Research indicates that almost one third of cancer deaths are a consequence of poor lifestyles – including behavioural and dietary risks such as physical inactivity, poor nutrition as well as smoking and alcohol abuse – thus deaths occurring from these factors are seen as preventable [6]. In addition, some cancers related to infectious organisms could be prevented by using protective antibiotic and vaccination treatments. Protection of the skin from extreme sun exposure and avoiding indoor tanning could prevent several of the many skin cancer cases that are diagnosed annually [7]. Moreover, early detection and diagnosis is also of great importance since it allows for elimination of the cancer cells, before metastasis can occur – where curative treatment is no longer as effective [6].

1.2.Diagnosis

There are several tools used to diagnose cancers, all starting with tissue biopsies and genetic testing [8]. Imaging technology is routinely utilised, which includes magnetic resonance imaging (MRI), computed tomography (CT) scans, X-rays as well as ultrasounds [8]. Other methods to diagnose cancers include common screening tests particular to cancer types such as mammograms for breast cancer or colonoscopies [9] for colon cancer as well as serology for specific cancer biomarkers including CEA (carcinoembryonic antigen) – specific for colon cancer [10]. However, there are limitations to existing diagnostic tools such as insensitivity and non-specificity to several cancer biomarkers, making these tools not as reliable. Moreover, most cancer deaths are unfortunately caused by metastatic tumours thus, the lack of effective diagnostic and therapeutic options is an ever-motivating factor for many research efforts worldwide.

Once diagnosed, treatment strategies can be employed which commonly include surgery, chemotherapy, hormonal therapy, radiation therapy, or at times a combination of these strategies [11]. In addition, studies have shown that due to cancer cells having specific functions and characteristics, they can be targeted through other therapeutic strategies such as antibody therapy, viral therapy as well as gene therapy [12].

1.3.Existing therapeutics for cancer

As mentioned above, the most widespread treatment strategy for cancer is surgery. However, if the cancer has already reached the late stages, additional treatments such as chemotherapy and radiotherapy, or a combination of the two is recommended. Unfortunately, chemotherapy and radiation may damage epithelial cells and cause swelling to soft tissue [13]. Furthermore, these treatments are often ineffective, cause severe side effects which may lead to other conditions, and potential relapse may still occur from remaining cancer cells, thus, other therapeutic approaches have been explored [14].

Several strategies including hormone therapy and targeted therapy have been considered to reduce cancer progression. Targeted therapy, which can be either natural or drug-based, is particularly used specifically to deliver agents that block essential routes needed for cancer cells to survive [15]. In particular, agents such as monoclonal antibodies and small interfering RNAs (siRNAs) are mostly used to target tumours while anti-angiogenic drugs are employed to target angiogenesis of tumour cells.

1.3.1. Monoclonal antibodies

Monoclonal antibodies have become a prominent source of treatment as they act mainly through blocking specific functions or modifying signalling pathways involving a target molecule [16]. The Food and Drug Administration (FDA) has approved several antibodies for treating solid and haematological cancers. Well-known antibodies such as Iressa® and Erbitux® target the epidermal growth factor receptor (EGFR) while Avastin® targets vascular endothelial growth factor (VEGF), and are used for the treatment of colorectal carcinoma and non-small cell lung cancer. The Herceptin® and Kadcyla® antibodies on the other hand, are commonly used by binding to the HER2 receptor and inhibiting the receptor's signalling, ultimately leading to the inhibition of cell signalling pathways for the treatment of breast cancer [17]. Antibodies can also be conjugated to nanoparticles, radioactive particles or chemotherapeutic agents, allowing the antibody to enter the cancer cells directly [18].

The advantages of monoclonal antibodies as therapeutic agents are that they have a long half-life in the body, especially if they are full-length antibodies, and their intravenous administration guarantee a direct and rapid action. However, disadvantages of monoclonal antibodies include their high cost to be produced and the fact that they are only able to act on the cell surface [13].

1.3.2. Small interfering RNAs (siRNAs)

The use of RNA interference (RNAi) to down-regulate or to silence genes involved in cancer development is becoming an increasingly popular route regarding cancer treatment. RNA silencing is used by double-stranded RNA molecules called siRNAs which aid in degrading sequences found in the target mRNA as well as those sequences that are complimentary to it [19]. These double-stranded molecules play key roles in inducing and activating the RNAi process since siRNAs are able to cleave inducer molecules as well as degrade target mRNA [19]. Thus, most siRNAs aim to interfere with metastasis and angiogenesis of cancer cells.

Since siRNA technology is a relatively new route of targeting cancer cells, most siRNA-based drugs are only at the clinical stage. This is due to a serious obstacle in siRNA development involving controlled and effective delivery of siRNAs *in vivo*. For siRNAs to work effectively, they have to first undergo degradation by nucleases followed by hydrolysis in lysosomes [20]. Therefore, several siRNAs require chemical modification or highly specific delivery systems in order to improve their efficiency.

There are several advantages of using siRNAs including their unrestricted target specificity and high efficacy. However, there are also disadvantages to this method including the

instability of siRNAs at physiological conditions and thus, the need for efficient packaging into vesicles for effective delivery to cancer cells. Nanoparticles protect the siRNA from degradation within the circulatory system and represent an effective delivery method to targeted tumour cells [20]. One such example is the siRNA known as Atu027, which uses lipid-based nanoparticles to down-regulate the expression of protein kinase N3 (PKN3), an important protein that regulates growth, adhesion and survival in the Phosphoinositide-3-kinase (PI3K) cell signalling pathway. Animal studies showed that Atu027 effectively down-regulated the PKN3 gene, ultimately inhibiting metastasis [21]. Thus, in conjunction with a delivery system, the use of siRNA technology is a promising avenue for cancer therapy.

Since all cancers behave differently and exert effects through several signalling pathways, various treatment strategies have been investigated in attempt to combat this complex disease.

1.4.Colorectal cancer

1.4.1. Characterization

Colorectal cancer has been found to be difficult to treat as its formation is linked to several signalling pathways including: the Ras/Raf, MEK/ERK pathway, Wnt pathway, PI3K/Akt/ PTEN/ mTOR pathway, apoptotic pathways and cell cycle regulatory pathways [22]. Most colorectal cancers arise from adenomatous polyps in the colon which appear as benign tumours *in situ* [23]. However, if the polyp starts to spread and invade beyond the basement membrane of the colonic lining, it is known as an invasive adenocarcinoma [23]. The term adenocarcinoma is used for a cancer arising in cells that form glandular structures which produce mucus for lubrication as is the case inside of the colon and rectum [23]. If left untreated, colorectal cancer can spread to other sites in the body via the bloodstream. The most common site for colorectal cancer to spread to is the liver since blood drains directly from the bowel to the liver [24]. Additionally, studies have shown that most colorectal cancers develop gradually, over a period of 10 to 20 years [25].

1.4.2. Signs, symptoms and risk factors

When colorectal cancer is in its early stages, there are no apparent symptoms; hence the necessity for early screening tests such as colonoscopies and serum CEA. However, as tumours progress, they may cause obstruction or even bleeding to the intestine, causing blood to appear in the stool [26]. Other symptoms include unintentional weight loss, loss of appetite, fatigue, cramping or discomfort in the lower abdomen, dark black stools or a change in the stool shape [26]. There are several known factors that increase the risk of colorectal cancer, including

family history, diabetes, existing intestinal conditions such as Crohn's disease as well as high consumption of processed meat [27].

1.4.3. Staging

According to the American Joint Committee on Cancer (AJCC) fifth and sixth edition staging systems, colorectal cancer is classified into four primary stages [25]. Furthermore, Haggit et al. (1985) proposed that the progression of early stage, also known as Duke's Type A colorectal adenocarcinoma includes four levels, where each level depends on the invasion of the polyp's structure (Fig. 2) [28]. Although this stage of colorectal cancer has many levels, it is easily treatable. However, it is found that subsequent stages are not as easily treatable. Stage two (Duke's Type B) colorectal cancer can be classified as the spread of cancer cells through the serosa – the outermost layer of the colon wall, while stage three (Duke's type C) is when the cancer cells have spread to local as well as regional lymph nodes [29]. Lastly, stage four (Duke's type D) is when the cancer cells have not only spread to the lymph nodes but to various other organs inside the body [29]. This stage is known as distant metastasis, where fatality is more probable than remission or eradication of the cancer. Metastasis is the ability of malignant tumours to migrate from one location in the body to another, thus allowing for secondary and tertiary tumours to become established at different sites within the body [30]. Most cancer deaths are caused by the metastatic disease and the lack of effective therapeutic options is an ever-motivating factor for many research efforts the world over.

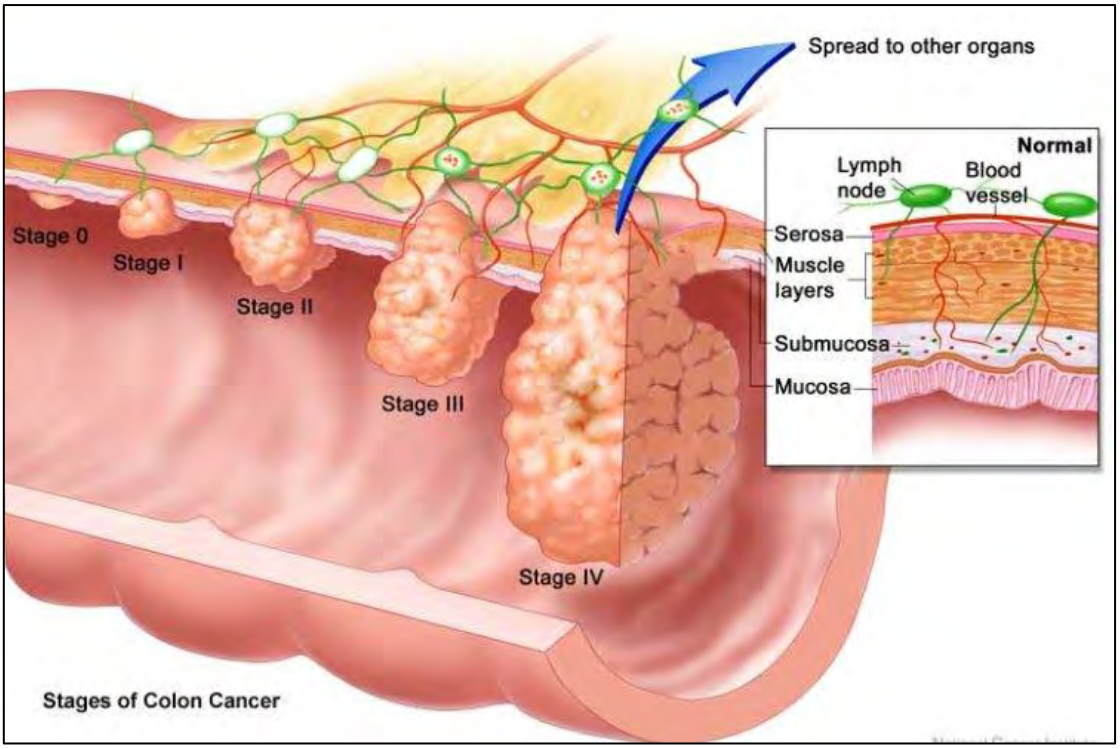


Figure 2: The primary stages of colorectal cancer. Stage zero involves polyp structures which are confined to the mucosa and the surface of the submucosa, while stage one invades through the submucosa [29]. Stages two occurs by invasion through muscle layers and serosa, whereas stage three occurs at the lymph nodes. Lastly, stage four involves the spread of the tumour to new organs through a process known as metastasis [29] (Image obtained from http://alamocitycancercouncil.org/cancer/colorectal-cancer/diagnosis/staging/).

1.4.4. Treatment and prevention

The most effective prevention strategy regarding this type of cancer is early detection through multiple screening tests including stool occult blood tests, sigmoidoscopies, colonoscopies, serum CEA as well as various genetic tests [31]. Other important factors which prevent colorectal cancer include maintaining a healthy diet. This would consist of reducing fat and processed meat intake while increasing fibre intake, in order to rid the intestines of potential carcinogens [32]. The most effective treatment regime for colorectal cancer will depend on the stage of the cancer as well as the state of the individual's health and immune system. The most common treatment for colorectal cancer is surgery, especially for polyps found in the early stage which have a high risk of becoming malignant [26]. If the cancer has already reached the late stages, additional treatments such as chemotherapy and radiation therapy is usually recommended [26]. However, since incidence and mortality rates for colorectal cancer are rising each year, novel therapeutic strategies are needed to combat this disease.

1.5. The molecular mechanism of cancer

In order to develop an anti-cancer treatment strategy, an insight into its molecular mechanism is required. Cancer is a disease which is found to alter cell cycle checkpoint regulators as well as DNA repair systems, resulting in uncontrolled cell proliferation [33] and decreased levels of programmed cell death [34]. There are several intrinsic and extrinsic factors which contribute to the process of tumourigenic transformation. Due to the complexity and diversity of neoplastic diseases, the collective term known as "the hallmarks of cancer" came about in order to provide understanding of this disease [34]. These hallmarks show that tumour cells acquire several capabilities that their normal counterparts do not have, including: self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis and apoptosis evasion (Fig.3) [34].

Cells are also largely dependent on the extracellular matrix (ECM), which is the non-cellular component of all tissues and organs that provides a physical scaffold to cellular components and assists with initiation of essential biochemical processes needed for proper tissue differentiation, homeostasis and morphogenesis [35]. It is noteworthy that these above-mentioned cell-functioning processes largely depend on cell-ECM interactions, rather than solely cell-cell interactions.

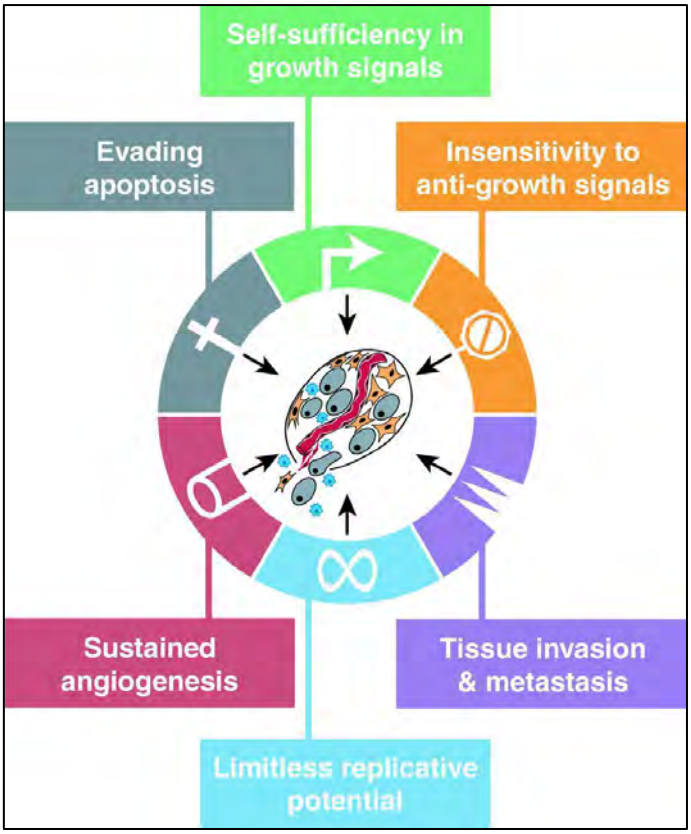


Figure 3: The six hallmarks of cancer. Tumourigenic cells acquire these six alterations in order to sustain a neoplastic state. Image obtained from Hanahan and Weinberg, 2000 [34].

1.5.1. The hallmarks of cancer

For transformation of normal cells into tumourigenic cells to occur, cells need to acquire six vital characteristics (Fig.3). These include self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis and evading apoptosis. Once these characteristics are obtained, tumourigenesis is able to thrive.

1.5.1.1.Self-sufficiency in growth signals

All cells depend on growth signals in order to propagate [35]. Normal cells are able to regulate the production of these growth signals and release them in a controlled manner which is in proportion to the progression of cells through the cell cycle, thus ensuring a homeostatic balance is maintained regarding cell structure, function and more importantly, cell number [35]. In contrast, tumourigenic cells are found to reduce their dependence on growth stimulation by also producing their own growth signals, resulting in the simulation of autocrine proliferation.

Alternatively, tumourigenic cells are able to stimulate normal cell growth within the tumour-associated stroma, resulting in the supply of growth factors to these cancer cells [34]. This results in the homeostatic balance being disrupted, allowing tumour cells to proliferate and survive without restriction [35]. In addition, somatic mutations in several proteins including Phosphoinositide 3- kinase (PI3-kinase) and B-Raf may lead to the disruption of normal signalling through the PI3K [36] and Mitogen-activated protein (MAP) - kinase [37] signalling pathways, respectively – ultimately leading to uncontrolled and continuous tumourigenic cell growth.

1.5.1.2.Insensitivity to anti-growth signals

In order for the maintenance of homeostasis and cellular quiescence, normal cells possess several anti-proliferative signals [34]. These anti-growth signals are able to block proliferation by driving cells out of the proliferative state into the G0 (quiescent) state in the cell cycle [34]. Moreover, for growth inhibition processes to be achieved, the need for several tumour suppression genes is critical. The tumour suppressor genes known as p53 and retinoblastoma (Rb) have been identified as key players in negatively regulating cell proliferation by activating cell senescence or apoptosis [34].

The tumour suppressor gene p53, is known to induce apoptosis or halt cell-cycle progression upon stressful and abnormal conditions such as DNA damage within the cells [38]. It is found that tumour cells have the ability to evade this action of the p53 gene, resulting in continuous cell survival and proliferation [38]. On the other hand, the Rb protein essentially functions as a cell cycle regulator by integrating intra- and extracellular signals to determine whether cells qualify for cell cycle progression [34]. However, tumourigenic cells are known to block the Rb pathway, making these proteins ineffective. As a result, this leads to defective regulatory functions supplied by the RB pathway, leading to uncontrolled cellular proliferation due to the lack of cell cycle regulation [34]

1.5.1.3.Tissue invasion and metastasis

Once normal cells undergo transformation into tumourigenic cells, they acquire the ability to modify the expression of particular molecules [39]. Research has shown that tumour cells selectively down-regulate E-cadherin, a protein part of the cell adhesion molecules (CAMs) family which is normally ubiquitously expressed on epithelial cells [34]. In contrast, tumourigenic cells are found to up-regulate specific proteases known as collagenases due to

them being able to degrade the extracellular matrix (ECM). These mechanisms allow for the degradation of the basal lamina to occur, leading to tumourigenic cells being able to migrate and invade new sites [40].

1.5.1.4.Limitless replicative potential

It is known that normal cell proliferation is finite where cells are only able to undergo limited growth and cell divisions [41]. Once this limit has been reached, cell proliferation is discontinued either by undergoing two processes: senescence, which is the irreversible entry into a non-proliferative but viable state, or crisis, which involves cell death [34]. On the other hand, tumourigenic cells acquire the ability to undergo limitless cell proliferation, resulting in the process known as immortalization [42]. It has been found that telomeres – short tandem repeats which protect the ends of chromosomes from DNA damage – facilitate the immortalization process [34]. Telomerase is a specialized ribonucleoprotein which functions in adding TTAGGG repeats to the ends of chromosomes, in order to prevent telomere erosion [43]. Moreover, the length of each telomere governs how many replicative cycles a cell is able to undergo before the protective function of the telomere is lost and erosion occurs [43]. In cancer cells, telomeres are found to be maintained through elevated expression levels of telomerase [44]. As a result, this leads the decrease of telomere shortening, allowing cells to proliferate uncontrollably, through the avoidance of senescence or apoptosis [44].

1.5.1.5.Sustained angiogenesis

In order for growth and survival, all cells require a constant source of blood, nutrients and oxygen [34]. In addition to these requirements, cells need metabolic wastes as well as carbon dioxide to be removed [45]. During the transformation process of normal cells into tumourigenic cells, normal vasculature begins to produce new vessels continuously in order to endure the development of neoplastic growths. This process is known as the angiogenic switch, which is controlled by several angiogenic regulatory proteins that are able to bind to inhibitory or stimulatory cell surface receptors [45]. Thus, tumour cells are found to deregulate the expression of vascular endothelial growth factor (VEGF) [45]. VEGF is an angiogenic initiator, aiding in the formation of new blood vessels that supply tumourigenic cells with oxygen and nutrients, which results in sustained tumour growth and progression [45].

1.5.1.6.Evasion of apoptosis

To maintain the homeostatic balance within the cell microenvironment, programmed cell death – also known as apoptosis – occurs due to either cellular stress or damaged DNA which can cause detrimental mutations [46]. Tumourigenic cells, however, acquire the ability to evade apoptosis, resulting in uncontrolled proliferation and continuous cell survival. As mentioned above, deregulation of the p53 tumour suppressor allows for cancer cells to avoid the apoptotic process and continuously proliferate, leading to aggressive tumour formation [34]. In addition, tumour cells have been found to alter the expression of molecules which are either pro-apoptotic or anti-apoptotic, depending on their function [47]. Evasion of apoptosis is seen as a major factor contributing to the progression and aggressiveness of several cancer types [34]. Thus, targeting the mechanisms of this hallmark for cancer therapeutics, can provide insight on how cancer can be combated – the key focus of the present study.

1.6.Apoptosis

The process known as apoptosis is defined as a form of physiological cell death performed in a highly stringent, ordered and programmed manner [48]. Apoptosis is essential for several other processes within organisms including: tissue homeostasis maintenance, normal development preservation, as well as damaged cell elimination – all involving cells actively committing suicide [49]. This highly-specific and ordered process occurs throughout the lifespan of an organism, however if apoptosis is deregulated, several damaging conditions can occur such as cancer [50], neurodegenerative diseases [51], autoimmune diseases [52] and viral-related infections [53] – the former being central to the present study.

There are several triggers which can induce the apoptotic process – this includes external triggers such as oxidative stress and ionizing radiation [54] as well as internal triggers such as DNA damage and replication errors [55]. Apoptotic induction may lead to two types of alterations, namely: cellular morphological alterations and biochemical alterations [54]. The main cellular morphological alterations of early apoptosis occur in the nucleus such as nuclear fragmentation, chromatin condensation and cell shrinkage (Fig.4) [56]. During the late stage of apoptosis, most morphological changes seem to occur in the cytoplasm, resulting in structural changes to cytoplasmic organelles, membrane blebbing, weakened membrane integrity and loss of adhesion to the extracellular matrix (Fig.4) [57]. With regards to biological alterations, changes such as enzymatic DNA and protein breakdown, caspase activation and membrane alterations which lead to phagocytosis, are all observed in apoptotic cells [58].

Another hallmark of apoptosis is the "flipping-out" mechanism which involves the phospholipid known as phosphatidyl serine (PS). In normal cells PS is confined to the inner membrane, however in apoptotic cells, it translocates to the outer membrane and becomes exposed [58]. The exposed PS enables phagocytes to recognise these apoptotic cells, resulting in rapid engulfment [58].

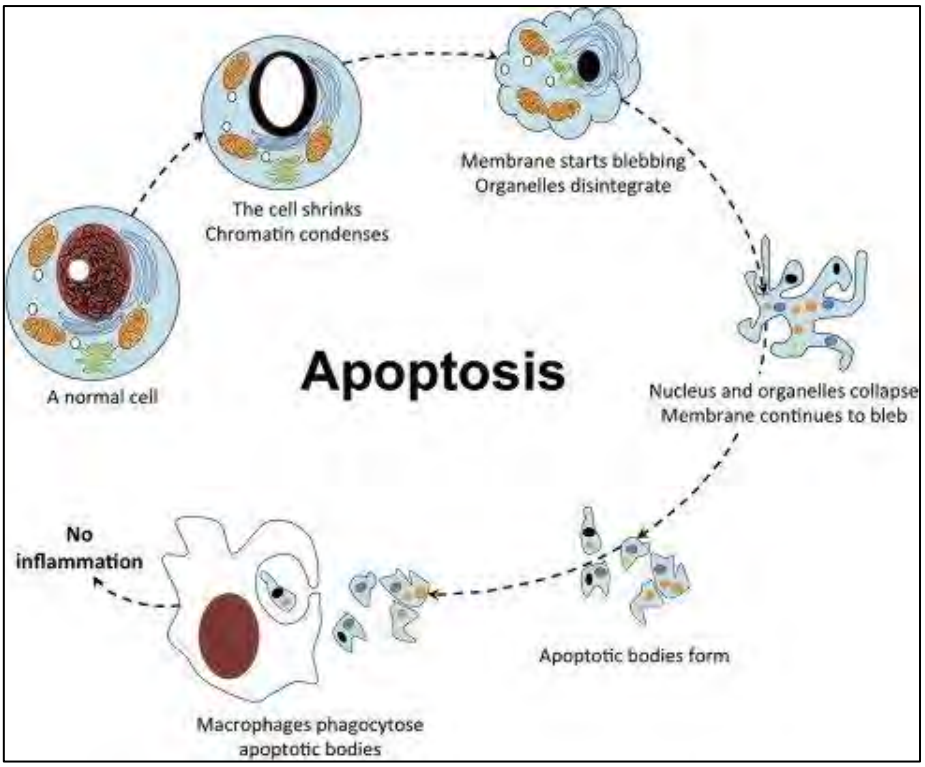


Figure 4: Morphological and biochemical changes apoptotic cells undergo. The cell membrane of an apoptotic cell starts to show blebbing, resulting in numerous apoptotic bodies forming and ultimately leads to phagocytosis by surrounding cells. (https://worldwide.promega.com/~/media/files/resources/paguide/a4/chap3a4.pdf?la=en).

The apoptotic process has been shown to largely depend on the action of caspases. Caspases are part of a family known as aspartate-directed cysteine proteases which initiate and execute the apoptotic process [59]. Research has shown that the initiator caspases (caspase 8, 9 and 10) as well as the effector caspases (caspase 3, 6 and 7) are essential for the mechanism of apoptosis to take place [60]. Caspases are initially synthesized as inactive homodimer procaspases that require proteolytic cleavage to become activated [61]. These caspases can be activated through two key pathways known as the mitochondrial (intrinsic) pathway and the death receptor (extrinsic) pathway [54].

1.6.1. Mitochondrial (intrinsic) pathway

The mitochondrial (intrinsic) pathway can be activated through both internal and external stimuli [62]. Internal stimuli such as hypoxia, oxidative stress, DNA damage as well as high concentrations of calcium within the cytosol are all able to form within the cell [62]. External stimuli mostly include death signals from the extrinsic death receptor pathway [62]. Both these stimuli are then known to induce permeabilization of the outer mitochondrial membrane, which in turn facilitates the release of pro-apoptotic proteins into the cytoplasm. It is understood that these pro-apoptotic molecules belong to the Bcl-2 family and include cytochrome *c*, AIF (apoptosis inducing factor), Smac/DIABLO, Omi/HtrA2 as well as endonuclease G [63]. This family of proteins are known to regulate the intrinsic pathway [46]. Apart from pro-apoptotic proteins, another group of Bcl-2 molecules exist known to be anti-apoptotic [64]. These anti-apoptotic molecules regulate the apoptotic process through preventing the mitochondrial release of cytochrome *c*, while pro-apoptotic molecules regulate apoptosis by undergoing specific post-translational modifications which aid in the release of cytochrome *c* that will act in the mitochondria [64]. Hence, the intrinsic pathway depends on the balance between both pro-apoptotic and anti-apoptotic molecules in order for apoptosis to be initiated [64].

Once released into the cytoplasm, cytochrome *c* binds to a molecule known as Apaf-1 (apoptotic protease activating factor-1) [65]. This monomeric molecule then experiences a conformational change resulting in oligomerisation, all occurring in the presence of ATP. This leads to the activation of caspase-9 (the most common mitochondrial apoptotic initiator) as well as triggering of the formation of the apoptosome [65]. In the apoptosome, activated caspase-9 leads to the activation of procaspase-3 in order to activate caspase-3 [66]. Activated caspase-3 molecules are responsible for initiating several downstream events, ultimately leading to cell death (Fig. 5B).

1.6.2. Death receptor (extrinsic) pathway

The death receptor (extrinsic) pathway has been found to be activated by cell surface receptors binding to their specific death ligands [64]. These receptors, known as death receptors, belong to the gene superfamily known as tumour necrosis factor receptor (TNFR), which includes death receptors known as TNFR-1, TRAIL-R1 (TNF-related apoptosis-inducing ligand receptor 1) and Fas/CD95 [67]. Once each ligand binds to their respective death receptor, a conserved amino acid sequence known as the death domain (DD) becomes exposed [67]. The death domain (DD) is seen to be a main characteristic of death receptors.

The association between Fas and the adaptor molecule called Fas-associated death domain (FADD) is promoted by the binding of the Fas ligand to its Fas receptor through a receptor trimerization process [60]. In addition, FADD has also been known to contain a death effector domain (DED) which allows for the recruitment and interaction of procaspase-8 through DED-DED interactions [67]. This binding of FADD, together with the receptor death domains, results in the formation of the death inducing signalling complex (DISC). Procaspase-8 proteins are then able to become highly concentrated in the DISC, leading to transactivation of the proteins – producing active caspase-8 [64]. The activated caspase-8 molecules are then known to hydrolyse inactive procaspase-3 to form active caspase-3 molecules [67]. This allows for active caspase-3 to cleave specific downstream substrates, ultimately leading to cell death (Fig. 5A).

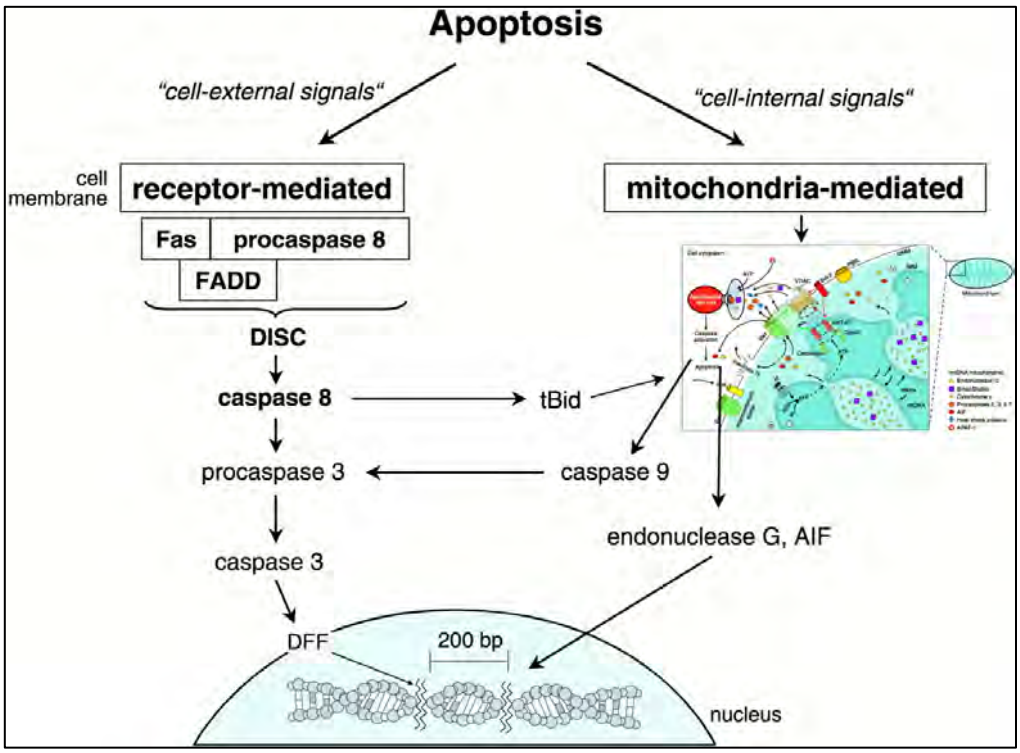


Figure 5: The two principle pathways of apoptosis. The apoptotic process is induced either by the mitochondrial intrinsic pathway or the death receptor extrinsic pathway. A) The extrinsic pathway relies on the binding and interacting of Fas with FADD proteins of the Fas receptor through a receptor trimerization process. This leads to DED of Fas associating with the DED of procaspase-8 resulting in the DISC complex. DISC enables procaspase-8 molecules to become transactivated, resulting in active caspase-8 proteins. Active caspase-8 allows for the cleavage of downstream substrates leading to cell death. B) The intrinsic pathway is activated by the mitochondrial release of cytochrome *c*, allowing the molecule to bind to Apaf-1. This results in the recruitment of procaspase-9 molecules, leading to the formation of the apoptosome. Within the apoptosome, caspase-9 is activated resulting in the activation of procaspase-3. Subsequently, activated caspase-3 initiates downstream processes which lead to cell death. Image obtained from Ziegler and Groscurth, 2000 [67].

Despite the apoptotic pathways being stringently regulated, deregulation may occur resulting in numerous pathological conditions which include cancer [50] and neurodegenerative disorders [68]. A large distinction between these two conditions is that cancer exhibits low levels of apoptosis, thereby facilitating tumourigenesis – the focal point of the present study.

1.7.Relationship between apoptosis and cellular proliferation

As mentioned previously, apoptosis is a process that eradicates damaged cells which in turn leads to a decreased number of cells [69]. In contrast, cellular proliferation can be defined as cell division yielding an increased cell number [70]. Thus, it can be said that there is a close relationship between these two processes with regards to maintaining tissue homeostasis when concerning cell number regulation.

All eukaryotic cells need to undergo the tightly controlled cell cycle in order to complete the growth process. The cell cycle is a sequence of events involving cell replication followed by cell division. In particular, the eukaryotic cell cycle is divided into four main phases including: Gap phase 1 (G1); DNA synthesis (S); Gap phase 2 (G2) cell division preparation; and mitosis (M) where chromosomes are able to separate and the cell divides (Fig.5) [69]. Important proteins known as cyclin-dependent kinases (CDKs) are upregulated in order to drive cell transition and progression through the several key stages of the cell cycle [69]. Moreover, it has been found that several genes are involved in regulating the cell cycle. In particular, genes such as c-myc, c-fos and c-jun are responsible for inducing apoptosis, allowing for healthy proliferation [69]. However, mutations in these genes may result in the creation of oncogenes, leading to uncontrolled proliferation and ultimately tumourigenesis [69]. The previously mentioned tumour suppressor gene, p53 is also important in the regulation of apoptosis. The p53 gene is mostly responsible for the association between apoptosis and cellular proliferation since it allows for cells at the G_1-S and G_2-M checkpoints to maintain genomic integrity, preventing the propagation of damaged cells [69]. This is necessary for the detection of possible genome alterations which can cause the cell cycle to come to a halt. As a result, mutations in p53 may lead to the improper functioning of the G_1-S and G_2-M checkpoints [69]. This results in uncontrolled cellular proliferation and survival of damaged cells.

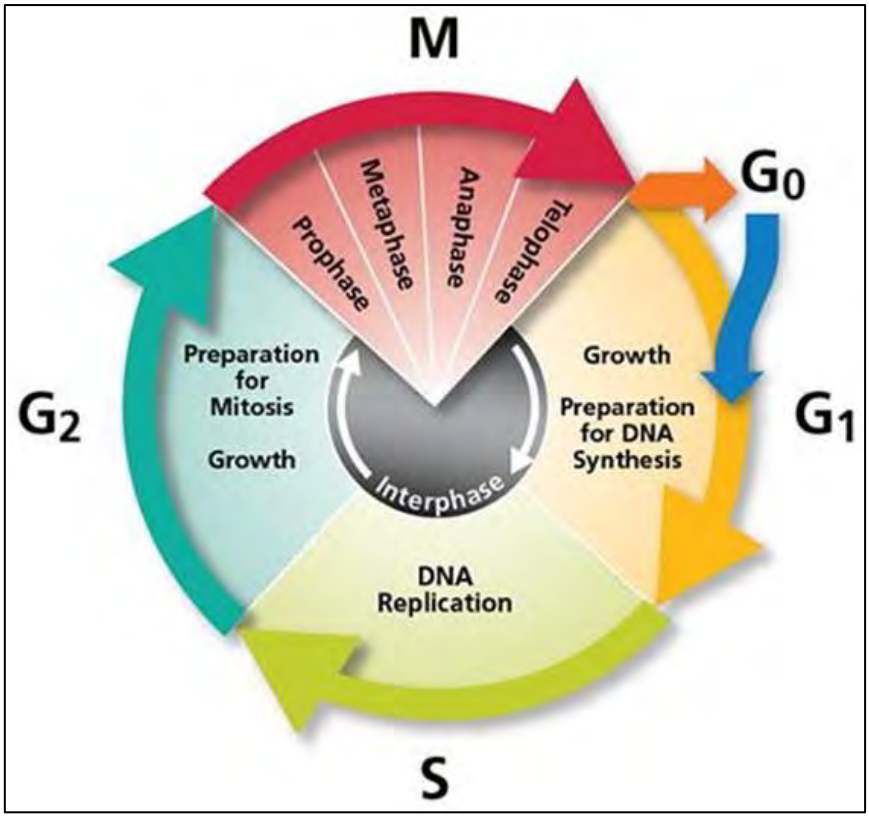


Figure 6: Main stages of the cell cycle. Stage 0/Gap 0 is when the cells are metabolically active but quiescent. For inactive quiescent cells to enter the G1 phase, stimulated entry by growth factors and nutrients must be employed in order to generate extracellular signals. Cells then progress to the S phase where they undergo DNA replication which permits entry into the G2 phase- in which cells increase in size as well as synthesize proteins required for the mitotic (M) phase. In the M phase the cells duplicate via mitosis to form identical daughter cells. There are 2 crucial check-points within the cell cycle that ensure the orderly progression of cells through the distinct phases in the cell cycle and these are the G1-S and G2-M check-points [69]. (Image obtained from Kamal, 2014 [71]).

Once an apoptotic stimulus is detected, the p53 gene is activated through the inhibition of MDM2 (a well-known p53 inhibitor) [72]. The activated p53 results in pro-apoptotic genes such as Bax to become activated. The p53 gene has also been seen to transactivate apoptotic effector machinery components, including the Apaf-1 gene, resulting in the co-activation of caspase-9 – ultimately leading to apoptosis [72]. Alternatively, p53 may induce apoptosis though the mitochondrial release of cytochrome *c,* through the intrinsic pathway [73]. This leads to Smac activation through Bcl-2 inhibition, and as a result, inhibitors of IAPs (inhibitors of apoptosis) become inactivated [73]. Once cytochrome *c* and Smac interact, a cascade of caspases is then activated which lead to apoptosis induction (Fig. 7).

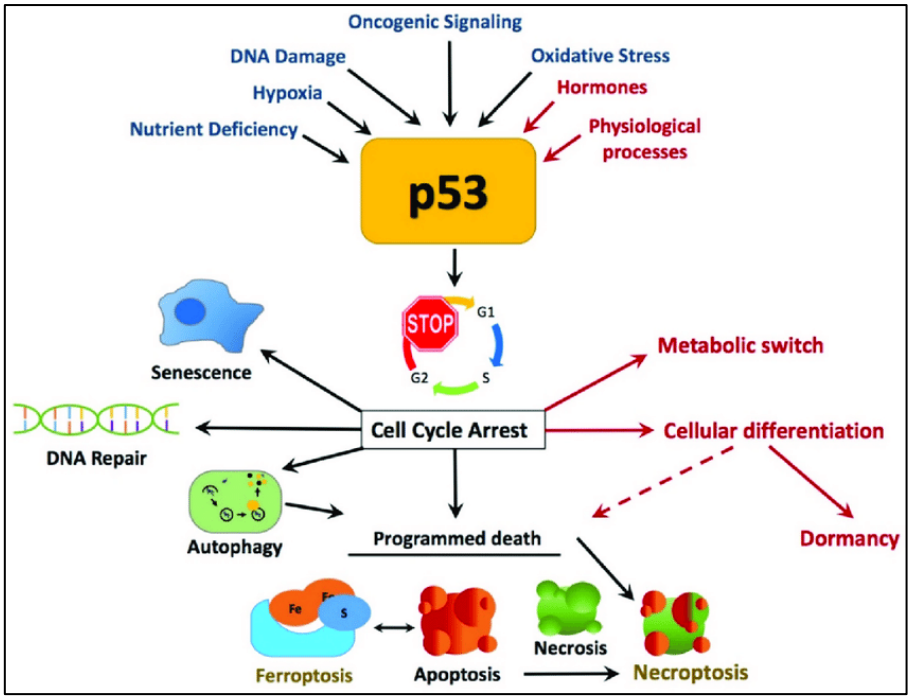


Figure 7: The role of p53 apoptosis induction. p53 can be activated by several stress signals including hypoxic conditions, DNA damage, nutrient stress and oxidative stress. Activated p53 can result in cell cycle arrest placing the cells into a senescent state or apoptosis is induced. p53 can also result in other modes of cell death including autophagy, necrosis, necroptosis and ferroptosis. Image obtained from Moulder et al., 2018 [74].

1.8.Role of apoptosis in tumour progression

Since the apoptotic process is largely dependent on several genes, there is a high probability that genetic mutations may occur. As a result, these abnormal genes also known as oncogenes, are involved in the deregulation of apoptosis and homeostatic imbalances, allowing uncontrolled cellular proliferation and survival – promoting the tumourigenic phenotype [69]. There are several families of proteins involved in these processes which are discussed below.

1.8.1. Bcl-2 family of proteins

Members of the B cell CLL/lymphoma-2 (Bcl-2) family of proteins are responsible for controlling and regulating the permeability of the apoptotic-associated mitochondrial membrane [75]. This family of proteins comprises of pro-apoptotic and anti-apoptotic molecules and can be divided into four groups [76]. The first group consists of anti-apoptotic proteins known as Bcl-X_L, Bcl-2, Mcl-1, which are localized in the cytosol but have also been associated with the endoplasmic reticulum and the mitochondrial outer membrane [76]. Upon apoptotic induction, these proteins fuse with the mitochondrial membrane to preserve the outer membrane integrity by directly hampering the pro-apoptotic Bcl-2 proteins [76]. Specifically,

Bcl-xL and Bcl-2 are able to prevent cytochrome c release from the mitochondria and obstruct caspase activation, thereby enhancing cell survival and inhibiting apoptosis [77].

The second and third groups are made up of the pro-apoptotic proteins. The second group consists of pro-apoptotic effector proteins known as Bak and Bax. These proteins become active once apoptosis commences resulting proteolipid pores forming in the mitochondrial outer membrane, ultimately leading to permeabilization [76]. The third group are direct activator pro-apoptotic proteins including Bid, Bim and Puma which are able to induce Bak and Bax oligomerization directly, resulting in the permeabilization of the mitochondrial outer membrane [76]. All Bcl-2 pro-apoptotic proteins become activated during the apoptotic process and are found to undergo conformational changes that lead to the exposure of the pro-apoptotic Bcl-2 homology-3 (BH3) domain [78]. Activated Bid and Bim promote the oligomerization of Bak and Bax, mitochondrial outer membrane permeabilization and the release of cytochrome c [76, 79]. Puma is suggested to also promote the activation of Bak and Bax, however indirectly [80, 81].

The last group of the Bcl-2 family comprises of sensitizers and de-repressor pro-apoptotic proteins (BMF, Bad, Bik, HRK, Puma and Noxa). Sensitization is a process that lowers the threshold for mitochondrial outer membrane permeabilization and Bak and Bax activation, but does not induce apoptosis itself [76]. Once a BH3-only sensitizer protein is in contact with an anti-apoptotic protein, it is able to inhibit the anti-apoptotic protein, resulting in the release of Bak or Bax from them [76]. Sensitizer and direct activator pro-apoptotic protein groups are only able to contain the BH3 domain, while de-repressor proteins are able to release direct activator proteins that are bound by anti-apoptotic proteins [76]. While this occurs, the direct activator promotes the permeabilization of the mitochondrial outer membrane [76].

Research has shown that several cancer types are found to have altered expression of these proteins [54]. More specifically, a study showed that cancers such as prostate and breast [63] have shown to exhibit over-expression of Bcl-2, thus promoting apoptosis evasion. In addition, over-expressed Bcl-2 allows tumour cells to become drug resistant, consequently aiding in apoptosis prevention and tumourigenesis promotion [82].

1.8.2. Inhibitor of apoptosis (IAP) family of proteins

Research has shown that the IAP family has been implicated in oncogenesis and anti-tumour treatment resistance [83]. There are several proteins making up the IAP family such as Survivin, Livin, XIAP (X-chromosome binding IAP) as well as cIAP1 and cIAP2 (cellular

inhibitor of apoptosis 1 and 2) [83]. These IAP proteins have been shown to inhibit the pro-apoptotic function of specific caspases by binding to them at a ratio of either 1:1 or 2:1 [83]. In addition to interacting with caspases, IAPs associate with Smac/DIABLO and HtrA2 and as a result, caspases are activated leading to the induction of pro-apoptotic processes [19]. Research has shown that IAP expression is deregulated in in several cancer types, however how these IAPs are expressed is still unknown [19].

1.8.3. Tumour necrosis factor (TNF) family of proteins

As mentioned previously, the death (extrinsic) pathway involves the use of death receptors belonging to the tumour necrosis factor family [64]. Inhibitors of the death receptor pathway, including the caspase homolog cFlip, have been shown to be involved in various cancer types [82]. Furthermore, these inhibitor proteins are known to compete with caspases for binding to FADD, and as a result forms the death-inducing signalling complex (DISC) that leads to apoptotic inhibition and a Fas-resistant tumour [82]. This resistance is advantageous for tumour cells as it aids in apoptotic evasion once it encounters the Fas ligand, an important TNF protein. In addition, Fas-resistance allows the Fas ligand to be expressed without causing damage to the tumour cells, consequently aiding in cancer progression [82]. Moreover, several studies have shown that mutations in the Fas ligand result in other complex immune disorders such as autoimmune lymphoproliferative syndrome (ALPS) [36].

1.8.4. Heat-shock proteins (HSPs)

Heat-shock proteins, also known as molecular chaperones, are mainly expressed during high levels of stress. In particular, HSP27, HSP70 and HSP90 have been found to be strongly induced upon stresses such as irradiation, oxidative stress, DNA damage and anti-cancer drugs [84]. HSPs are hardly expressed in normal cells, therefore suggesting that they are crucial prognostic factors in cancer development [85]. Moreover, HSPs have been found to associate with primary stress signalling and apoptotic molecules, thereby preventing caspase activation and promoting cell survival [84]. HSPs have been found to inhibit both intrinsic and extrinsic apoptotic pathways by interacting with specific proteins at three stages: 1) upstream of the mitochondria, resulting in signalling pathway modulation, 2) at the mitochondrial stage, thereby controlling apoptogenic molecule release and 3) at the post-mitochondrial stage, where they are able to robustly block the apoptotic process [84].

It is noteworthy that these above-mentioned cell-functioning processes largely depend on cell-cell interaction with their extracellular matrix (ECM) environment, rather than solely alone.

1.9.The family of laminins

Research has shown that cells interact and communicate with other cells through their environment known as the extracellular matrix (ECM) [35]. The ECM is a network of secreted proteins and carbohydrates which co-ordinates several critical signals needed for determining the fate of its surrounding cells [35]. This includes signals for adequate cell functioning regulation, cell contact facilitation as well as controlled cell migration [39]. The ECM consists of two components, the basement membrane and the interstitial/condensed matrix [39]. The interstitial matrix is composed of essential elements such as fibronectins, matrix metalloproteinases (MMPs), elastin, collagens, integrins and laminins [39].

In particular, laminins are shown to play a large role in forming the ECM. These large (400-900 kDa) heterotrimeric, non-collagenous glycoproteins consist of α, β and γ subunits, forming 15 different isoforms (Fig. 7) [42]. These subunits are linked by disulphide bonds and assemble in several combinations in order to bind to the ECM and settle in the basement membrane [86]. Therefore, laminins function as key players in enhancing biological processes which include cell adhesion, homing [46], migration, polarity, proliferation, differentiation [42], as well as neutrite outgrowth [47]. Specific to this study is laminin-1, an isoform that has been shown to have bind to both integrin and non-integrin receptors in order for its function to be carried out [42]. In particular, laminin-1 has been shown to bind to the 37kDa/67kDa non-integrin laminin receptor precursor/ high-affinity laminin receptor (LRP/LR), where overexpression of LRP/LR is a consequence of its increased interaction with laminin-1 [50]. Research has shown that overexpressed LRP/LR is implicated in cellular processes such metastasis, angiogenesis and cell survival; hence the binding to laminin-1 is seen as enhancement of tumour growth and progression [49].

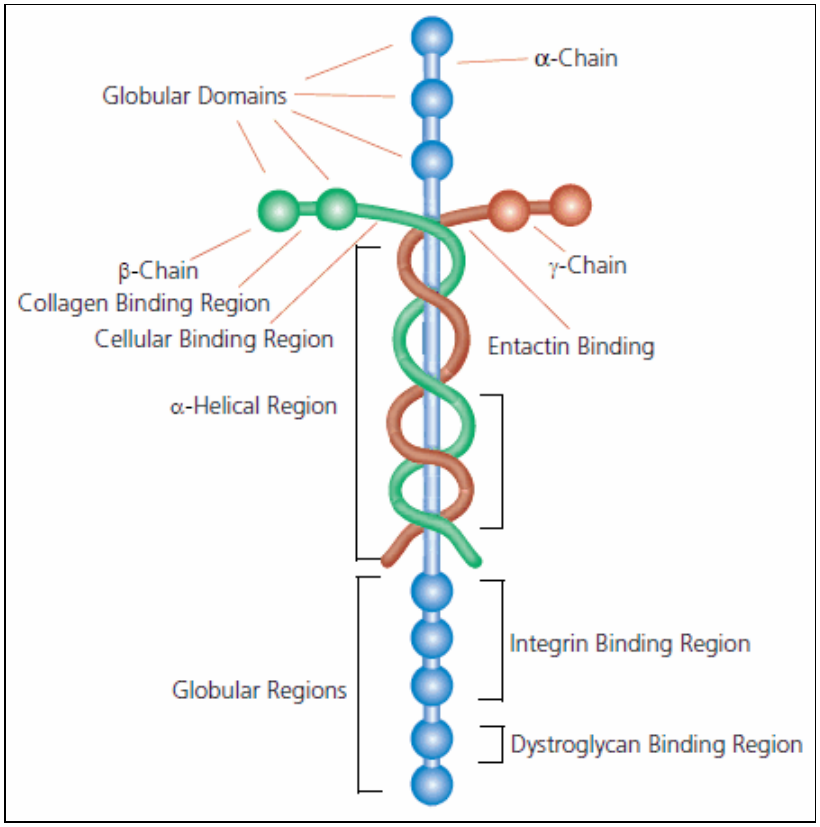


Figure 9: The complex structure of laminin. Laminin consists of α, β and γ chains which are stabilized by disulphide bonds. In addition to binding to several ECM molecules such as entactin, heparin and collagen IV, laminin also binds to cell surface receptors, enabling this protein to perform its many functions [42]. (Image obtained fromSitterly, 2008 [87]).

1.10. 37kDa high-affinity laminin receptor/67kDa laminin receptor (LRP/LR)

1.10.1. Structure of LRP/LR

The 37kDa/67kDa laminin receptor (LRP/LR) is a non-integrin, cell surface receptor located in the extracellular matrix of mammalian cells. The gene for this receptor is known as *RPSA* and is located on the short arm of chromosome 3 and consists of 1700 base pairs, encoding a protein of 295 amino acids with a mass of 33 kDa that appears as ~37 kDa when viewed by SDS-PAGE [87, 88]. This is supported by the high sequence conservation in 37 kDa LRP across phyla [87]. LRP/LR has been found to be a Type II transmembrane receptor comprising of two domains: an RPS2-like globular N-terminal domain and an intrinsically disordered C-terminal domain which contain binding sites for immunoglobulins, carbohydrates, microorganisms and elastin, and most importantly, laminin-1 [87]. Although, some controversy surrounds the identity of the laminin-1 interaction interface, attempts to map the laminin-1

binding surface have identified several possibilities: a study showed that a buried peptide (peptide G) and a surface peptide at amino acids 161-180 were directly involved in the interaction of LRP/LR with laminin-1 [89]; while mutagenesis studies of recombinant 37 kDa LRP suggest that laminin-1 binding involved Phe32 (N-terminal), Glu35 (N-terminal) and Arg155 (C-terminal) [90]; and finally, other studies suggested that direct LRP/LR-laminin-1 binding occurs on the C-terminus of the receptor at amino acids 205-229 [91, 92]. Thus, the receptor's structure has been extensively studied however, questions on how the precursor becomes a mature cell surface receptor remain.

It has been hypothesised that since the amino acid sequences of the 37 kDa and 67 kDa forms are the same [93], homodimerization could be forming the mature receptor, however, this theory was disproved by Hundt et al. (2001) [94]. It is also known that 37 kDa LRP is acylated with oleate, palmitate and stearate, which are thought to promote fatty acid-fatty acid interactions between 37 kDa LRP monomers or between 37 kDa LRP and other acylated partner proteins, resulting in the observed higher molecular weight mature receptor [87]. In addition, recent studies have suggested that SUMOylation of LRP may result in the maturation of the 67 kDa form from 37 kDa LRP and that the receptor may therefore be a target for small ubiquitin-like modifier (SUMO) proteins that have the potential to form oligomeric chains [84, 85]. Thus, the composition of the 67 kDa LR is still a point of contention as *in vitro* studies show that the translated 37 kDa LRP is monomeric and the expression of RPSA cDNA does not show any 67 kDa LR production [91]. To date, the maturation of the 37 kDa LRP to the 67 kDa LR has yet to be resolved.

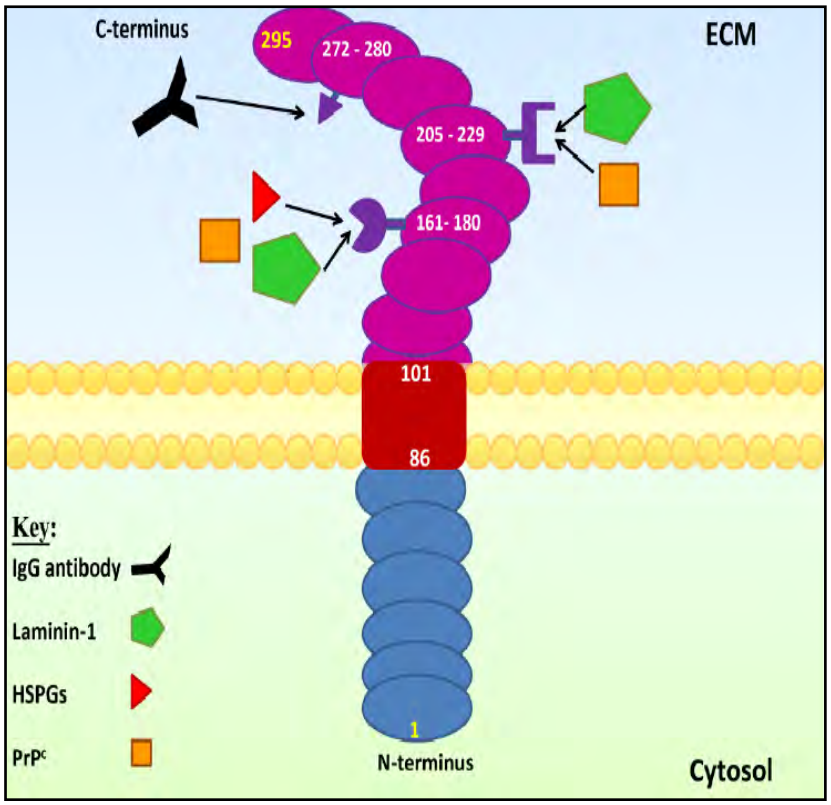


Figure 10: Structure of 37kDa/67kDa laminin receptor precursor (LRP). LRP/LR is a 295 amino acid transmembrane receptor which is located in the lipid region of the plasma membrane [95]. The receptor comprises of three domains, the intracellular N-terminal domain (blue), the transmembrane domain (red) and the extracellular C-terminal domain (purple). Laminin binds to the receptor at amino acid regions: 1) 161-180 2) 205-229 and 3) the C-terminal 53 residues. There is also an immunoglobulin G binding domain which is located in region 272-280 of the amino acid sequence [95]. (Image obtained from Jovanovic et al. , 2015 [95]).

1.10.2. Functions and pathological roles of LRP/LR

Despite many unknowns, LRP/LR has interestingly been found to play several physiological roles within the cytoplasmic, perichromosomal and perinuclear areas as well as on the plasma membrane of cells [87]. As mentioned above, LRP/LR aids ribosomal processing and protein translation in the cytoplasm [96]. In addition, the receptor has been seen to interact with histones in the nucleus, thereby aiding in nuclear structure maintenance [97, 98]. LRP/LR is also found to interact with components of the cytoskeleton such as actin and tubulin, by acting as a linker for ribosomes to microtubules, resulting in functional protein synthesis [99]. Moreover, the receptor is known to be predominantly located in the plasma membrane, specifically in lipid rafts, where it acts as a receptor for various viruses including Dengue, Sindbis [100], Adenoviruses and Venezuelan Equine Encephalitis (VEE) virus [101]. It also acts as binding partner for PrP^{c} [102-104] and as a receptor for prion proteins PrP^{c} and PrP^{sc}

[105, 106], respectively, bacteria including the cytotoxic necrotizing factor-1 expressing *Escherichia coli* K1 [107] as well as several other proteins part of the extracellular matrix such as laminin-1, elastin and heparin sulphate proteoglycans [95]. Recently, it has been found that LRP/LR has a link to ageing, since it to co-localises with telomerase, an enzyme involved in regulating the ageing process [108]. Specifically, increased LRP/LR has been found to enhance telomerase activity and reduce senescence markers, making LRP/LR a potential novel telomerase activator and anti-ageing drug [108]. In addition, LRP/LR is involved in several diseases including cancer [33, 50, 109-113], micro-organism infections [114], Transmissible Spongiform Encephalopathies (TSEs) [95], prion disorders [102, 103, 105, 115], Alzheimer's Disease (AD) [51, 95, 116-121] and age-related disorders [108, 122]. However, the receptor's primary roles have been identified as enhancing adhesion of tumour cells to the basement membrane and disseminating these tumours, which are both fundamental events of metastasis [33, 50, 109, 111-113, 123], promoting tumour angiogenesis [124] and finally, allowing for cancer cells to survive through apoptotic evasion [125-130].

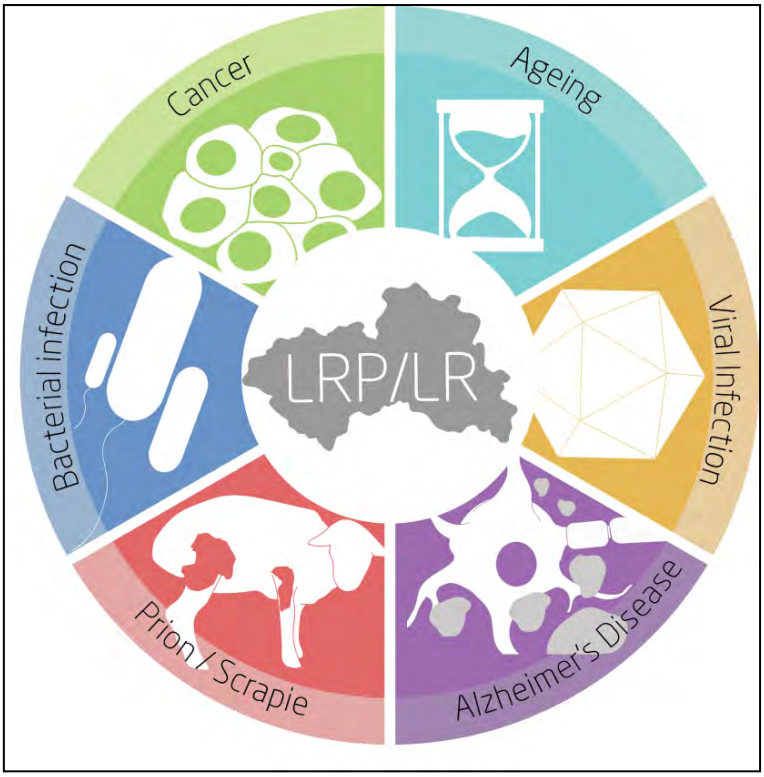


Figure 11: The pathological roles of the 37 kDa/67 kDa laminin receptor (LRP/LR). Over the years, research has shown that LRP/LR is involved in several pathologies. This is due to LRP/LR having several binding sites thus, serving as a receptor for numerous viruses, bacteria and infectious prions. Furthermore, LRP/LR has been found to associate with enzymes responsible for the formation of the neurotoxic amyloid-beta peptide as well as internalization of the peptide in AD [51, 95, 117]. It has also been shown to play a role in ageing and other age-related disorders due to its interaction with the enzyme, telomerase. In addition, overexpression of LRP/LR is a key characteristic of cancerous cells which has a correlation with angiogenic enhancement [124], metastasis [33, 109, 111, 112] and cell survival [110, 125, 126, 128] of neoplastic cells. (Image obtained from Vania et al. (2019) [130]).

1.11. Role of LRP/LR in metastasis

As mentioned above, LRP/LR has been found to play primary roles in cancer and cancer progression. Specifically, it was revealed that LRP/LR is over-expressed on the surface of several cells including various cancers such as pancreatic (AsPC-1) cancer cells [131], lung (A549), prostate (LNCaP) cervical (HeLa) [33], oesophageal (WHCO1) breast (MDA-MB231) [110] liver (HUH-7) cancer cells [109] as well as early (SW-480) and late (DLD-1) stage colorectal cancer [111] and malignant melanoma (A375SM) cells [113]. Furthermore, a correlation between LRP/LR expression and tumour development has been reported [49]. This over-expression of LRP/LR might be a consequence of its interaction with the laminin-1 glycoprotein. As previously mentioned, laminin-1 is found in the basement membrane of cells

and is normally involved in biological processes such as cell adhesion [42], differentiation, growth and migration [132]. Thus, it is no surprise that laminin-1 is said to promote the invasive phenotype of tumourigenic cells. Studies have shown that the LRP/LR-laminin-1 interaction is essential in promoting two main steps in the metastatic cascade – adhesion and invasion [33, 110-112, 133]. Upon the binding of LRP/LR, laminin-1 undergoes a conformational change and becomes more efficient in interacting with integrins of the basement membrane, which leads to increased adhesion [95]. This as a result, increases the sensitivity of laminin-1 to proteolytic enzymes such as type IV collagenase which hydrolyses collagen in the basal lamina, leading to degradation of the basement membrane and invasion of the tumourigenic cells [134]. In addition, the LRP/LR-laminin-1 interaction may also contribute to angiogenic enhancement by providing these cancer cells with oxygen and nutrients, making it possible for the cells to metastasise to new sites of the body [124]. Hence, it is proposed that up-regulated LRP/LR may serve as molecular marker for metastasis in many cancer types. However, recent studies have shown that the anti-LRP/LR specific antibody IgG1-iS18 is able to significantly impede adhesion and invasion of several cancer cell types including lung (A549), prostate (LNCaP) cervical (HeLa) [33], oesophageal (WHCO1) breast (MDA-MB231) [110] liver (HUH-7) cancer cells, early (A375) and late (A375SM) stage malignant melanoma cells [113] and of particular interest, early (SW-480) and late (DLD-1) stage colorectal carcinoma cells [111]. Thus, IgG1-iS18 may serve as a potential therapeutic tool for the treatment of metastatic cancer.

1.12. Role of LRP/LR in tumour angiogenesis

Alongside metastasis, LRP/LR is also found to play a role in angiogenesis. Angiogenesis is an essential process needed for growth and development whereby new blood vessels grow from pre-existing ones [135]. Additionally, it plays a vital role in wound healing as well as granulation tissue formation [136]. This is exploited by cancer cells to aid in the conversion of tumours from benign to malignant, because as they grow and move away from blood vessels, they become deprived of nutrients and oxygen. The cancer cells perform this by emitting signals known as angiogenic factors e.g. VEGF to stimulate the growth of new blood vessels to receive oxygen and nutrients [137] which results in rapid growth of the tumour cells, allowing for the cells to become malignant. Thus, angiogenesis favours tumourigenic enhancement as well as aiding in the migration and metastasis of cancer cells through the circulatory system.

As reported before, 67 kDa LR has been shown to be vital in the neovascularisation and migration of haemopoietic stem cells (HSCs) which are known to encourage angiogenesis by producing new endothelial cells [138]. It has also been shown that high levels of LRP/LR on the surface of human umbilical vein endothelial (HUVE) cells could play a role in enhancing their invasive potential [124]. Consequently, this may allow them to move towards pro-angiogenic stimuli by degrading important constituents of the extracellular matrix, and ultimately lead to the development of tubular structures needed for the formation of blood vessels. Moreover, it was found that when HUVE cells were treated with the anti-LRP/LR specific antibody, W3, tubular formation was fully halted [124]. Thus, LRP/LR specific antibodies such as W3 may be a potential therapeutic tool for the treatment of cancer through impediment of tumour angiogenesis.

1.13. Role of LRP/LR in telomere biology

It has very recently been observed that in addition to its various functions and interactions in cancer and neurodegenerative disorders, LRP/LR plays a role in mediating telomerase activity in breast cancer cells [122]. However, to understand the receptor's relationship with telomerase, a deeper understanding into telomerase and telomere biology is needed.

1.13.1. Telomerase and telomere biology

1.13.1.1. Telomerase

Telomerase is a ribonucleoprotein principally responsible for maintaining and elongating telomeres; tandemly repeated TTAGGG repeats found on the ends of chromosomes which help prevent chromosomal degradation [139]. Telomerase is found to have reverse transcriptase activity essentially functioning to conserve the structural integrity of telomeres, thereby increasing the proliferative potential of a cell [139]. As a result, additional cell divisions which exceed the cell's predetermined limit are made possible. Telomerase is frequently found in germline, immortalized and importantly, tumourigenic cells thus, stimulated telomerase is said to enhance tumourigenesis by avoiding cellular senescence and apoptosis induction while allowing for unrestricted DNA replication [140]. The enzyme is made up of two essential components to enable telomere replication, namely, in humans: the reverse transcriptase catalytic component, hTERT, which is responsible for the addition of telomeric repeats; and

the functional RNA component, hTERC, which serves as the telomeric template [139]. Studies have shown that TERT is needed for telomerase activity regulation in cells [141]. Moreover, TERT is said to be a limiting factor of telomerase activity since TERT expression levels have been found to directly influence the amount of telomerase activity in cells [141]. In addition, it has been found that all mammalian cells express the TERC subunit, whereas TERT is only actively transcribed in epithelial and embryonic cells – further signifying its role as a limiting factor [142]. Moreover, TERT expression was also found to be strictly regulated in human somatic cells, resulting in undetectable expression levels which corresponded to undetectable levels of telomerase activity [142]. Another study showed that exogenous TERT exposure to human or murine cell lines caused the reactivation of telomerase, resulting in telomere extension [142, 143]. These findings highlight the role of TERT as the core regulator of telomerase activity, and consequently, proliferative potential. Thus, telomerase is said to maintain normal cellular processes and pre-senescent functioning, while increasing the proliferative capacity of cells.

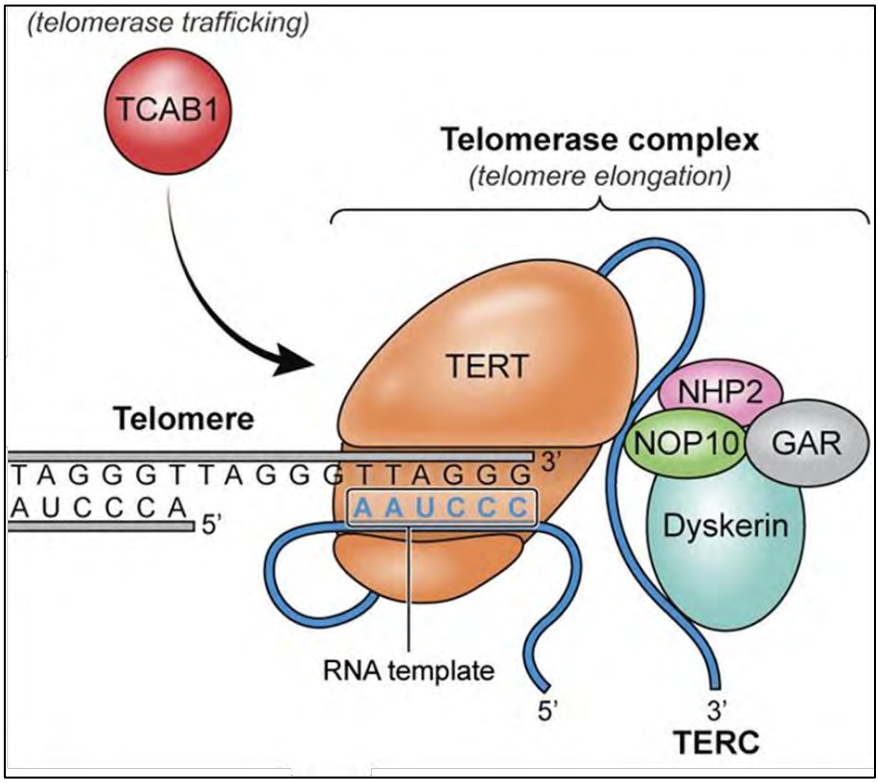


Figure 12: Schematic illustrating the telomerase complex performing its primary function of telomere extension. Telomerase is made up of two main subunits among other proteins to form a multi-protein complex. TERT and TERC are the core subunits which function to extend telomeres, while the other subunits are used for stabilization. The

stabilization proteins include NOP10, NHP2, Dyskerin and GAR, which together with TERT and TERC aid in telomere synthesis at the ends of chromosomes (Image obtained from Townsley et al., 2014 [144].

1.13.1.2. Extra-telomeric functions of TERT and telomerase

Studies have shown that telomerase has various other functions, in addition to telomere extension. These functions, also known as extra-telomeric functions, all favour cell viability preservation and cancer [145]. A major extra-telomeric role of TERT involves the activation and regulation of cellular pathways. More specifically, TERT acts as a transcriptional activator for several proliferative genes – which are usually activated by the c-Myc and Wnt pathway [146]. Thus, it can be said that there is an intricate regulation system shared between TERT, c-Myc and Wnt. Moreover, Li et al. (2011) showed that TERT physically occupied promoter regions of cyclin D1 and c-Myc – both responsible for cell cycle regulation [147]. These findings confirm that TERT is not only able to regulate cell cycle initiation but also drive proliferation [148]. Another pathway that TERT has been involved in regulating is the inflammatory pathway, serving as a transcriptional activator for NFκB [149]. Aside from regulating the inflammatory response pathway, NFκB has also been shown to play a role in the Wnt pathway for proliferation [149]. Therefore, the activation of the NFκB pathway allows for an interlinking of pathways between NFκB, TERT, Myc and Wnt [149]. Thus, TERT's involvement in pathway regulation and transcriptional activation, highlight its crucial importance in regulating proliferation and cell viability.

In addition to regulating pathways, TERT also plays several roles in the mitochondria. Specifically, TERT is found to translocate to the mitochondria under hypoxic conditions where it actively lowers reactive oxygen species (ROS), thereby aiding in the increase of mitochondrial membrane potential [148]. Saretski (2014) showed that TERT is implicated in promoting apoptosis resistance as well as initiating a DNA damage response (DDR) by possibly aiding in the recruitment of DNA repair enzymes, thereby revealing its role in DNA repair [145]. In addition, studies have shown that the inhibition of telomerase, and the resultant loss of telomere maintenance, resulted in the induction of apoptosis [150]. The loss of telomerase activity caused progressive telomere shortening and resulting in the inactivation of the telomere capping function of the shelterin complex, eventually leading to cell death [150]. Moreover, a recent study has shown that downregulating TERT through RNA silencing, activated pro-apoptotic protein Bax, which triggered a mitochondrial cell death pathway [151].

Furthermore, this did not occur as a result of telomerase inhibition, as there was no telomere erosion, ultimately suggesting a role of TERT in preventing apoptosis [151].

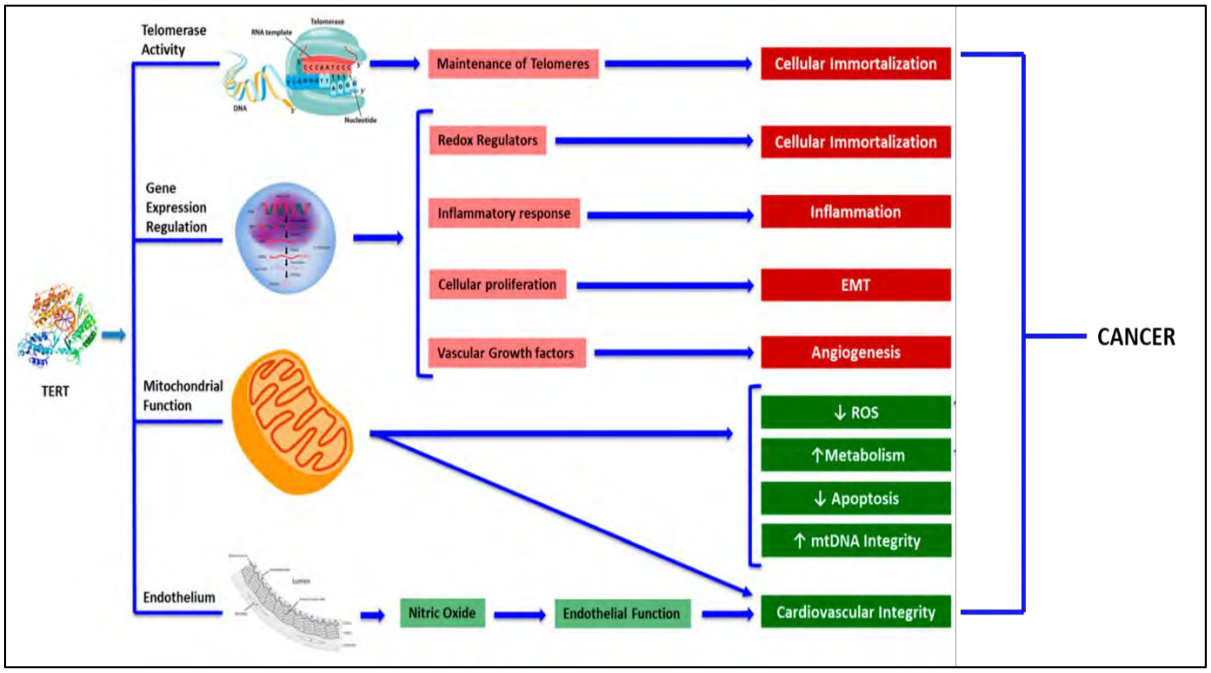


Figure 13: Schematic representation of the extra-telomeric roles of TERT. The above diagram illustrates the numerous functions and pathways where TERT plays a role in, besides telomere extension. TERT's extra functions all appear to aid in promoting the continuation of normal cell viability and proliferation. In addition, it can be observed that TERT operates by regulating gene expression to affect molecular pathways involved in cell viability and proliferation, inflammation, angiogenesis and the Epithelial to Mesenchymal transition process (EMT). Moreover, its localization to mitochondria aids in influencing the reduction of ROS production, mitochondrial metabolism and respiration and anti-apoptosis functions. These functions are left unregulated within cancer cells are found to drive the tumourigenic process, thereby highlighting TERT's necessity in promoting cell viability in normal and tumourigenic cells. (Image adapted from Quryshi et al., 2018 [152].

1.13.1.3.Telomeres

As mentioned above, telomerase plays a role in maintaining the terminal ends of linear chromosomes, also known as telomeres. Telomeres are made up of non-coding tandem repeat sequences of TTAGGG as well as several telomere related proteins [153]. These tandem repeats are extended in a 5′ to 3′ direction from genomic DNA ending in a single stranded region comprised of guanine, resulting in an overhang forming. These repeats together with telomere-related proteins work concurrently to protect and "cap" genomic DNA by preventing damage and degradation [154]. In particular, telomere-related proteins include: TRF-1, TFR-

2, RAP-1, POT-1, TPP-1, and TIN-2, forming the complex known as “shelterin” which has a high specificity for telomeres [155]. The shelterin proteins functions include additional telomere protection such as: the maintenance of telomere length, development of t-loop formation, recruitment of telomerase to the ends of telomeres and lastly, protection of chromosomal ends as being DNA damage [155]. The development of the t-loop not only allows for telomeric ends to be inaccessible to DNA damaging agents, but also prevents the telomeric ends from being read by DNA repair machinery known as double-stranded breaks [155]. The shelterin complex is also able to maintain telomere length by regulating telomere extension i.e. by preventing telomerase from binding to the telomeres, resulting in the inhibition of telomerase activity [155].

Telomeres have also been found to shorten with each successive cell division, due to a process well-known as the “end replication problem” [156, 157]. To this effect, when telomeres shorten to a critical length, the dysfunctional telomere is recognised as damaged DNA and cellular senescence is triggered [157]. Cells can remain in this senescent state for years, provided no other changes occur and as such, this is considered to be somewhat of a tumour suppressor mechanism in humans. However, cancerous cells are found to overcome inhibitory pathways induced by DNA damage, as well as senescence, through the upregulation of telomerase activity [158]. The initiation and maintenance of the senescent state involves cell cycle checkpoint proteins, including p53/p21 and pRB/p16. When these proteins are inactivated, cells are able to bypass senescence and subsequently proliferate, causing genetic instability, ultimately leading to cell death. However, cells can survive this by activating telomerase, where the cells then gain the capability to maintain the length of these unstable telomeres, resulting in the formation of cancer [158]. Interestingly, it has been shown that cancer cells that escape senescence, can be reversed to the senescent state by restoring the tumour suppressor, p53 [159]. Therefore, it is generally believed that telomeres and telomerase are the connection between cellular senescence and immortalization [160].

1.13.1.4.Telomeric repeat-binding factor-1 and -2 (TRF-1 and TRF-2)

The shelterin complex plays a crucial role in protecting telomeres. Two important proteins making up the shelterin complex are the telomeric repeat-binding factor-1 (TRF-1) and -2 (TRF-2). They are both found to directly bind to the double-stranded DNA region of telomeres and play crucial roles in the formation of the t-loop structure [161]. It has been suggested that

TRF-1 negatively regulates telomere length by limiting telomerase's access to the telomere [162]. In addition, TRF-1 is also found to play roles in DNA remodelling as well as DNA replication regulation [163]. TRF-2 on the other hand has been found to have no influence on telomerase and primarily functions in stabilizing t-loop formation thereby protecting telomeric ends from DNA damage but has been found to play a role in chromatin assembly [164]. Thus, telomere length regulation and homeostasis require the action of both TRF-1 and TRF-2.

However, in cancerous cells, a dynamic balance exists in the expression of these proteins in order to maintain telomere length and undergo unlimited cellular proliferation. Some studies have shown that TRF-1 and TRF-2 expression is regulated by primary cancer signalling pathways such as the Wnt [165] and PI3K/AKT [166] and that the up-regulation of both proteins lead to lung [167] and gastric cancers [168]. Conversely, other studies show that down-regulated expression levels of these protein were found in malignant hematopoietic cells [169] as well as breast [170] and gastric cancers [169]. Thus, the expression of these proteins and their role in tumourigenesis has not been clearly understood, making them important points of interest in relation to LRP/LR, a receptor known to contribute to cancer development.

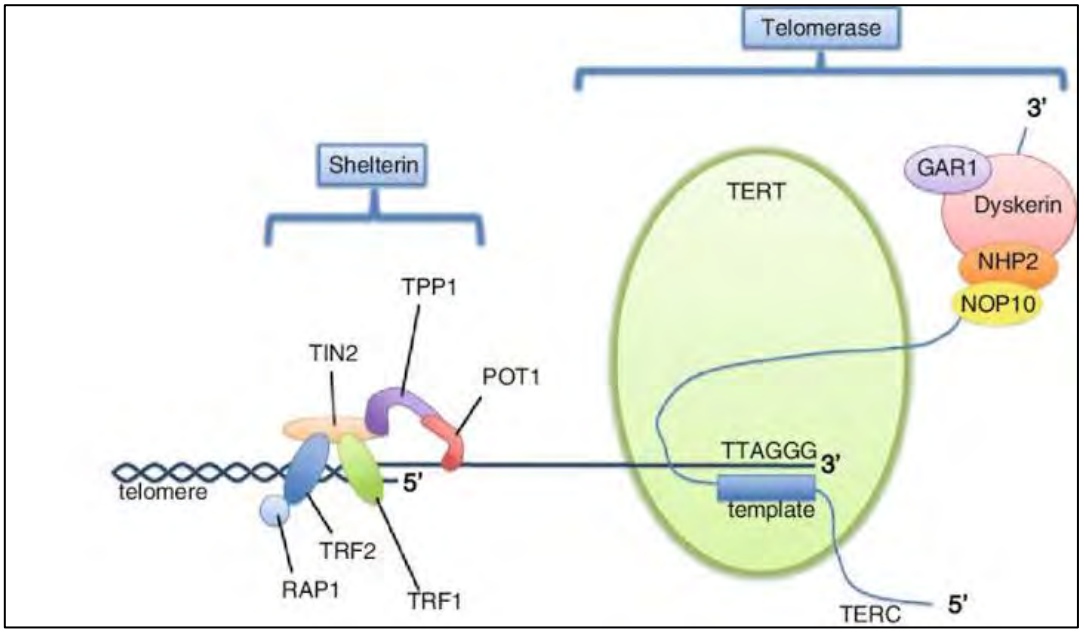


Figure 14: Schematic illustrating telomerase with the shelterin complex. The telomerase multi-protein complex consists of TERT, TERC, Dyskerin, NOP10, NHP2, and GAR1. TERT functions by adding new telomeres (TTAGGG repeats) onto chromosomal ends by using the template provided by TERC. The shelterin complex consists of six proteins namely: TRF1, TRF2, RAP1, POT1, TPP1, and TIN2 which functions to protect telomeres and regulate telomerase. (Image obtained from Nishio et al., 2010 [171]).

1.13.2. Relationship between LRP/LR and telomerase

The link between LRP/LR and telomerase has not clearly been elucidated; however, studies have shown that both proteins have similar functions in several cellular processes, including cell viability maintenance [52, 75]. More importantly, the two proteins are also found to have interlinked roles in various diseases such as cancer. Naidoo et al. (2015) showed that telomerase and LRP/LR co-localize in the perinuclear compartment of breast cancer cells [122] and it has recently been found that LRP/LR enhances telomerase activity [108, 121]. Furthermore, the knockdown of LRP/LR with LRP-specific siRNA was found to significantly reduce telomerase activity and should this state be maintained it could effectively prevent the preservation of the shorter telomeres characteristic of tumorigenic cells [122]. This would therefore lead to widespread telomere erosion, which could not only increase the susceptibility for the occurrence of crisis in the cells but also further enhance chemosensitivity [172]. This enhanced chemosensitivity would arise from the loss in genomic protection that the telomeres convey, thereby allowing chemotherapeutic drugs to induce widespread genomic instability and damage more effectively. Therefore, this suggests that the knockdown of LRP/LR may be a potential therapeutic intervention for cancer through the resultant down-regulation of telomerase activity, owed to the relationship between LRP/LR and TERT.

In addition to telomeric dysfunction, the knockdown of LRP may further affect TERT levels affecting its ability to carry out extra-telomeric functions, especially regulation of pathway and gene expression [145]. This could lead to a profound effect on the cancer cell's physiology, as shared pathways between LRP and TERT would become affected. One such pathway that could be affected is the p53-dependent apoptotic pathway, where both proteins have been found to be profoundly influenced by p53 [173, 174]. In this regard, p53, a protein commonly dysregulated or mutated in cancers has been found to influence expression of both TERT and LRP by transcriptional repression to limit proliferation [173, 174]. Moreover, p53 has been found to not only affect TERT but also down-regulate telomerase activity for apoptotic induction or cell cycle arrest [175]. Consequently, a decrease in both of these proteins (which share an antagonistic relationship with p53) may allow the elevation of p53 and subsequent induction of apoptosis in the cancerous cells. Furthermore, as both LRP and TERT have been intricately linked to proliferation pathways like the MAPK pathway [176, 177], knockdown of LRP and subsequently TERT, could severely impact the aggressive and rapid proliferation

observed in cancer cells. Thus, targeting LRP with its corresponding effect on TERT/telomerase, will inhibit several key cancer hallmarks influenced by both proteins, including inhibition of growth suppressors, invasion, replicative immortality apoptotic and importantly, evasion of apoptosis.

1.14. LRP/LR and apoptotic evasion

As mentioned above, LRP/LR has been linked to a variety of cancer promoting processes, including cell viability maintenance and apoptotic evasion. By blocking apoptosis, several diseases such as autoimmune lymphoproliferative syndrome as well as neurodegenerative diseases like Parkinson's disease and Alzheimer's disease are able to arise, and most importantly, cancer [56]. Here, changes in the apoptotic process have been reported to enhance the progression of tumours and, several cancers are thought to favour these changes and evade apoptosis [34]. One of the reported changes include mutations in the p53 tumour suppressor gene which may result in the loss of regulation in several pro-apoptotic genes such as Bax and Bid, ultimately leading to increased cell growth [34].

Moreover, since LRP/LR is not only located in the plasma membrane, but also in the cytosol, studies have shown that the receptor is able to maintain the viability of yeast cells through ribosomal maturation and processing [178]. In addition, LRP/LR is also found in the nucleus where it maintains chromosome stability via contact with the Midkine heparin-binding growth factor; well-known for promoting cell migration, proliferation and survival [179]. As a result, the growth factor assists LRP/LR in maintaining cellular viability. In addition, it has been found that LRP/LR is able to interact with focal adhesion kinase (FAK) as a result of the LRP/LR-laminin-1 interaction, leading to the activation of the PI3-kinase/AKT and MEK/ERK 1/2 cellular survival pathways as well as an increased expression of the Bcl-2 protein (Figure 10B) [180].

Up-regulated LRP/LR expression in cancer cells has therefore been implicated in maintaining the viability of these cells [98]. In addition, up-regulated LRP/LR is seen to enhance tumour progression by increasing apoptosis inhibition [98]. This was proven by Susantad et al. (2008) through siRNA-mediated down-regulation of LRP/LR in liver (Hep3b) cancer cells, which resulted in the induction of apoptosis [99]. More recently, other studies have shown that siRNA-mediated knockdown of LRP/LR resulted in a decrease in lung (A549), cervical (HeLa), breast (MDA-MB-231) [127], pancreatic (AsPC-1) [128], neuroblastoma (IMR-32)

[128] as well as early (A375) and late (A375SM) stage malignant melanoma [126] cells. Furthermore, it was found that apoptosis was induced upon siRNA-mediated LRP/LR down-regulation (Fig. 10D) [98]. The results from these studies reiterate the essential role of LRP/LR in cellular viability maintenance and targeting the receptor through siRNA technology may be a possible therapeutic tool for the treatment of cancer.

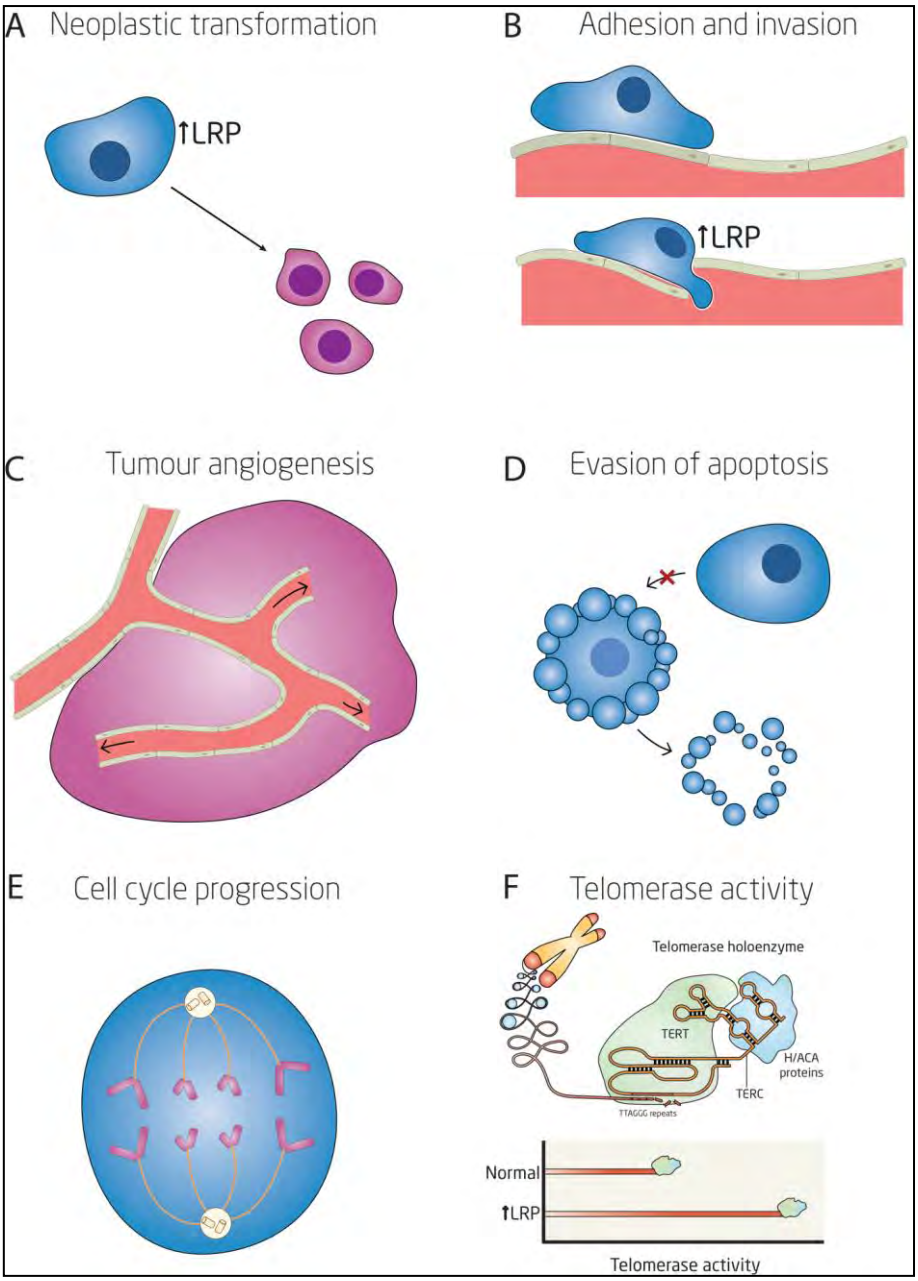


Figure 14A: Summary of roles that LRP/LR plays in cancer. A) One of the main characteristics of tumourigenic cells is that the 37/67 kDa laminin receptor (LRP/LR) is overexpressed when compared to their normal cell counterparts, thus LRP/LR is said to play a role in neoplastic transformation [33]. B) LRP/LR expression levels have been found to have

a direct correlation with the adhesive and invasive potential (primary features in metastasis) of tumourigenic cells [33, 109-113]. C) LRP/LR has been shown to play a key role in the formation of tumour angiogenesis via endothelial tube establishment [124]. D) LRP/LR has been shown to cause tumourigenic cells to have increased cell survival through apoptotic evasion [181]. E) LRP/LR is said to be involved in the cell cycle by halting the G1 phase, and as a result exposes its role in tumour protein translation and progression in specific cancer cell lines [125-128]. It has also been found that through siRNA-mediated knockdown of LRP/LR, the receptor is linked to telomerase, an enzyme involved in cancer progression [122, 129]. (Image obtained from Vania et al. (2019) [130]).

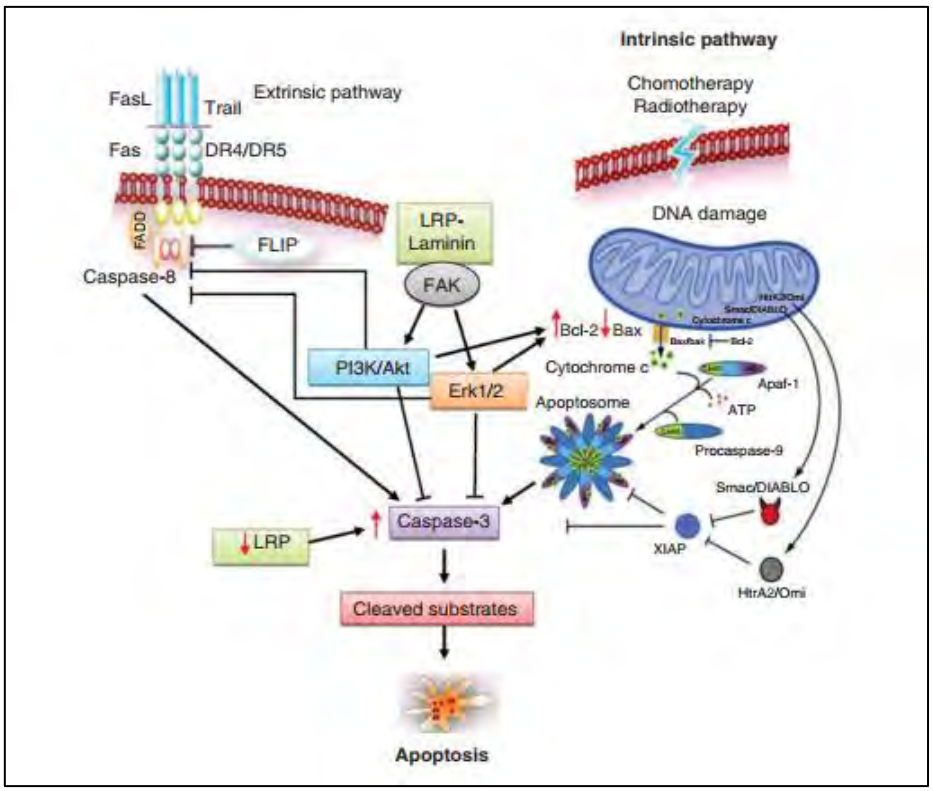


Figure 14B: Schematic illustrating the role LRP/LR has in the intrinsic and extrinsic apoptotic pathways. Specific signals trigger the activation of each apoptotic pathway, ultimately leading to caspase activation. This results in characteristic morphological changes such as chromatin condensation, cell shrinkage as well as the formation of cytoplasmic blebs and apoptotic bodies [182]. The LRP/LR-laminin-1 interaction results in the activation of the PI3K/Akt and Mek/Erk1/2 survival pathways, leading to the increased expression of the anti-apoptotic Bcl-2 protein and the inhibition of caspase-3. There are various other pathways by which apoptosis is induced via caspases however, only the intrinsic and extrinsic pathways are displayed here. (Image obtained from Jovanovic et al., 2015 [95]).

Although many anti-cancer therapeutic strategies against LRP/LR have been developed to target its roles in cancer metastasis, angiogenesis and apoptotic evasion, there is growing evidence that LRP/LR makes use of several other cell signalling pathways, including the Mitogen Activated Protein Kinase (MAPK) signal transduction pathway to exert its

tumourigenic effects [49, 177]. Thus, it is imperative to understand the receptor's role in these pathways to establish an effective and powerful therapeutic tool for the treatment of cancer.

1.15. Mitogen Activated Protein Kinases/Extracellular signal-regulated Kinases (MAPK/ERK) signal transduction pathway

1.15.1. The role of MAPK in cancer

Mitogen Activated Protein Kinases/Extracellular signal-regulated Kinases (MAPK/ERK) are a family of serine/threonine kinases that control the activity of numerous substrates, through sequential phosphorylation events [183]. This pathway is able to transmit several signals, intracellularly and extracellularly, which ultimately lead to changes in physiological processes such as: mitosis, metabolism, gene expression, cell differentiation, proliferation, migration and apoptosis [183]. The MAPK pathway consists of three main families namely: the ERK extracellular signal-regulated kinase family, p38 family and the stress-activated/c-Jun terminal protein kinase (JNK) family [49]. The ERK family response primarily to growth factors and mitogens and are found to play vital roles in cell differentiation, proliferation and survival. On the other hand, the c-Jun NH_2-terminal protein kinase (JNK)/stressed-activated protein kinase and the p38 MAPK families become activated by inflammatory cytokines as well as environmental and chemical stress, resulting in the induction of apoptosis [49]. However, cross talk between the different MAPK pathways can occur easily.

The ERK family is able to modulate several cellular activities through the regulation of transcription factors. The ERKs are mainly involved in promoting cell proliferation by activating various proteins known to enhance differentiation and proliferation [184]. The p38 MAPK family is vital in regulating apoptosis, growth inhibition and cell cycle arrest [185]. The JNK family is involved in several cell processes such as autophagy and apoptosis [185]. Once JNK becomes activated, it not only regulates transcription factors like c-Jun and p53 but is also able to phosphorylate apoptotic proteins such as Bcl-xl and Bcl-2 [186]. As a result, numerous cell events are activated including metabolism, apoptosis and proliferation. In particular, JNKs are able to regulate apoptosis by activating both the intrinsic and extrinsic apoptotic pathways, leading to apoptosis [186].

However, deregulation of this cellular pathway may occur through mutations in cytokine receptors or chromosomal translocations, leading to cancer [108]. Current treatment strategies to target these cancers involve inhibiting the MAPK pathway itself however, studies have shown that this is not efficient in robustly reducing tumour growth and metastasis [187]. This

could be explained by the release of negative feedback loops which result in the activation of alternate cancer pathways taking over [188]. Thus, there is a great need for new therapeutic avenues that not only target the MAPK pathway but also proteins and receptors involved in the pathway.

1.15.2. LRP/LR and the MAPK pathway

There has been a growing amount of evidence that cell surface receptors, including LRP/LR, activate the MAPK pathway for tumour invasion and metastasis [87]. The role of LRP/LR with MAPK was first investigated by Givant-Horwitz et al. (2004) [49]. The study revealed that exogenous exposure of laminin-1 to malignant melanoma (A357SM) cells was able to decrease the phosphorylation of MAPK, an event that was partly owed to the decreased expression of LRP/LR [49]. The study also found that the decreased activity of MAPKs correlated to the increased malignancy of cancer cells, further confirming that decreased levels of LRP/LR resulted in the phosphorylation of MAPKs [49]. The results from this study showed that LRP/LR not only played a role in laminin-induced signalling but also, in phosphatase/kinase induction. That been said, it is important to note that LRP/LR's direct relationship to the MAPK pathway remains unclear as decreased LRP/LR has also been found to induce stress in cells, to which the signalling pathway responds to [87].

Several other studies have shown additional ways on how LRP/LR may be involved in the MAPK pathway. Duan et al. (2010) showed the LRP/LR-laminin-1 interaction via the MAPK pathway, is able to increase the phosphorylation of the c-Myc transcription factor, thereby stimulating the expression of the Fas ligand (FasL) [177]. This resulted in the induction of apoptosis of immune competent cells, allowing cholangiocarcinoma cells to thrive [177]. Another study found that signals induced by laminin led to the phosphorylation of phosphoprotein enriched in astrocytes-15 (PEA-15/PED), an adapter protein known to bind to LRP/LR [189]. The phosphorylation of PEA-15/PED resulted in the activation of growth signals via the ERK MAPK pathway as well as inactivation of caspases, exposing the role of the LRP/LR-laminin-1 interaction in pro-survival signalling [189]. Further studies revealed that LRP/LR expression is regulated by the ERK and JNK MAPK pathways in hypoxic micro-environments, which results in the increase in adhesion and invasion of gastric cancer [190]. This is due to LRP/LR possessing a hypoxic response element 16bp upstream from the translational start site, which is found to be responsive to the hypoxia-inducible-factor-1 (HIF-

1). HIF-1 is a transcription factor that is activated in hypoxic environments via the ERK and JNK pathways [190]. This is relevant since hypoxia is known to promote the formation of metastasis. Finally, as mentioned previously, it has also been found that LRP/LR is able to interact with focal adhesion kinase (FAK) as a result of the LRP/LR-laminin-1 interaction, leading to the activation of the PI3-kinase/AKT and MEK/ERK 1/2 cellular survival pathways as well as an increased expression of the Bcl-2 protein (Figure 10B) [180].

These studies show several ways of how LRP/LR and the MAPK pathway interact to promote cell growth and survival however, the exact mechanism by which they can be targeted remains somewhat unclear. A promising study performed by Lu et al. (2016) demonstrated that siRNA-mediated knockdown of LRP/LR was able to reduce FAK phosphorylation, leading to the knockdown of the anti-apoptotic protein Bcl-2 while an up-regulation of the pro-apoptotic Bax protein, reiterating that the knockdown of LRP/LR induces apoptosis [191]. Thus, by targeting the MAPK signalling pathway through siRNA-mediated knockdown of LRP/LR may not only provide answers on how the receptor is linked to the pathway but also offer insights into the mechanism of LRP/LR as well as a novel therapeutic intervention to combat cancer via the induction of apoptosis.

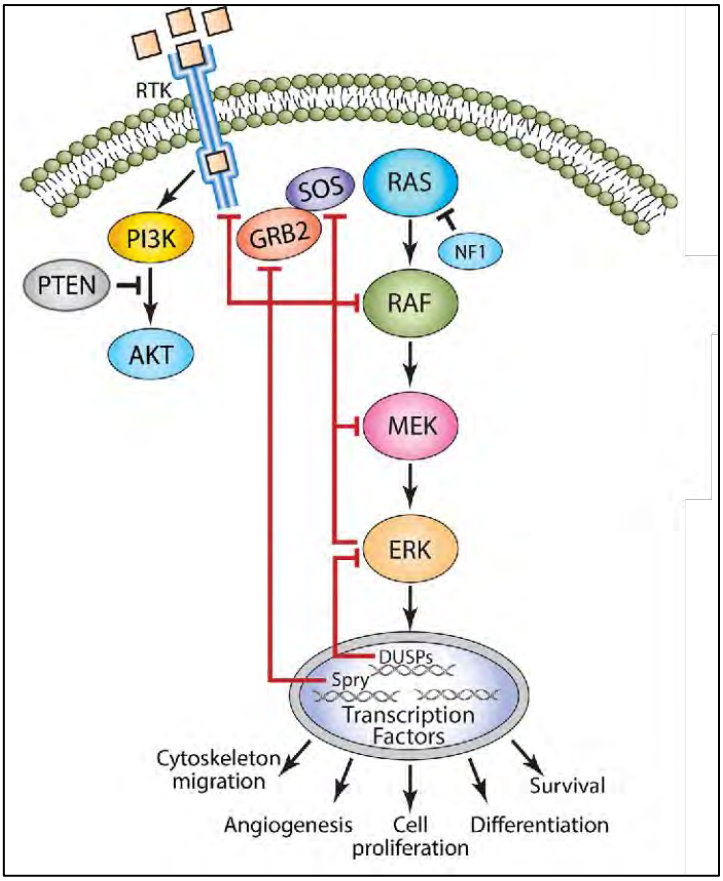


Figure 15: The MAPK/ERK signalling cascade. Activated receptor tyrosine kinases (RTKs) have been found to initiate the MAPK/ERK signalling pathway, resulting in several cell responses including proliferation, differentiation, angiogenesis, migration and survival (Image obtained from Maik-Rachline et al., 2016 [192]).

Chapter 2: Rationale, Aims and Objectives

2.1.Rationale

Cancer has become one of the leading causes of death worldwide with its incidence and mortality rates at a constant rise. The total number of cancer incidences is expected to increase by over 70% within the next two decades in both economically developed countries where it has been found to be the primary cause of death, as well as economically developing countries, where cancer is identified as the second leading cause of death [1]. Most cancer types have also been found to behave in a non-discriminative manner in that they manifest in individuals of any race, gender and age. Therefore, there is an urgent need to develop novel therapeutic strategies to combat cancer.

The 37kDa/ 67kDa laminin receptor (LRP/LR) has been found to be over-expressed on the surface of several cells including various cancers such as pancreatic (AsPC-1) cancer cells [131], lung (A549), prostate (LNCaP) cervical (HeLa) [33], oesophageal (WHCO1) breast (MDA-MB231) [110] liver (HUH-7) cancer cells, early (A375) and late (A375SM) stage malignant melanoma cells [113] and of particular interest, early (SW-480) and late (DLD-1) stage colorectal carcinoma cells [111]. The over-expression of LRP/LR has been identified as a key factor in tumour progression via the critical maintenance of tumourigenic cell viability. This has been shown through several studies where down-regulation of LRP/LR through siRNA technology results in the reduction in cellular viability in liver (Hep3B) [193], cervical (HeLa) [129], lung (A549) [129], breast (MCF-7 and MDA-MB231) [127], oesophageal (WHC01) [127], neuroblastoma (IMR-32) [128] and pancreatic (AsPC-1) [128] cancer cells as well as early (A375) and late (A375SM) stage malignant melanoma cells [126]. Moreover, this was validated through increased caspase-3 activity observed in several of the mentioned cell lines [126-129, 193], further indicating the induction of apoptosis.

The apoptotic induction observed is said to be due to the reduced interaction of LRP/LR with focal adhesion kinase (FAK) [180, 191]. In particular, a study performed by Lu et al. (2016) demonstrated that siRNA-mediated knockdown of LRP/LR reduced FAK phosphorylation, leading to the knockdown of the anti-apoptotic protein Bcl-2 and an up-regulation of the pro-apoptotic Bax protein, reiterating that the knockdown of LRP/LR induces apoptosis [191]. Moreover, another study performed by Sun et al. (2014) showed that the LRP/LR-laminin-1 interaction may allow for LRP/LR's interaction with FAK, leading to the activation of the PI3-

kinase/AKT and MEK/ERK 1/2 cellular survival pathways as well as an increased expression of the Bcl-2 protein [180]. Hence, these outcomes encouraged several questions regarding siRNA-mediated down-regulation of LRP/LR. Firstly, whether siRNA-mediated down-regulation will reduce the viability of early (SW-480) and late (DLD-1) stage colorectal carcinoma cells, and whether apoptosis is responsible for the possible cellular viability decrease. Secondly, whether siRNA-mediated down-regulation of LRP/LR influences the expression of proteins involved in apoptosis and other cell signalling pathways.

In addition to the receptor's role in maintaining tumour cell viability, LRP/LR has also been found to increase the metastatic potential of tumour cells by interacting with its primary ligand, laminin-1. This interaction is said to increase adhesion and invasion of tumour cells, ultimately resulting in an increase in the aggressiveness of tumours [33]. Furthermore, a direct correlation between LRP/LR levels and the invasive potential of several tumourigenic cells has been observed [33, 111-113, 123, 133]. However, we previously have reported that application of the anti-LRP/LR specific antibody IgG1-iS18 to early (SW-480 and HT-29) and late (DLD-1) stage colorectal cancer cells leads to a significant reduction in the adhesive and invasive potential of the cells, two main steps in metastasis [111].

Although these *in vitro* studies have demonstrated that the LRP/LR-laminin-1 interaction can be hindered in several cancer types using the anti-LRP/LR specific antibody IgG1-iS18, it is imperative to investigate the antibody's effect *in vivo*, using cancer mouse models. A previous study revealed a significant reduction in the growth of human fibrosarcoma (HT1080) tumour cells in the lungs of BALB/c *nu/nu* nude mice[194]. However, these findings were obtained using the P4G polyclonal antibody directed against the 37 kDa LRP [194]. Thus, due to the significant reductions in adhesion and invasion displayed in the afore-mentioned cancer cell lines upon IgG1-iS18 application, this prompted the question of whether the IgG1-iS18 antibody will be effective for the prevention and treatment of metastatic colorectal cancer using a colorectal cancer mouse model.

2.1.1. *In vitro* aim

- To investigate how siRNA-mediated down-regulation of the 37kDa/67kDa laminin receptor (LRP/LR) influences the cellular viability of early (SW-480) and late (DLD-1) stage colorectal carcinoma cells.

- To determine whether siRNA-mediated down-regulation of LRP/LR influences the expression of specific apoptotic proteins and signalling pathways.

2.1.2. *In vitro* objectives

1. To effectively down-regulate the expression of LRP/LR in the cell lines afore-mentioned through cell transfection with siRNAs targeted to 37 kDa LRP mRNA.
2. To determine the endogenous levels of LRP/LR in the cell lines mentioned above and also to confirm siRNA-mediated LRP/LR down-regulation through the use of western blotting and qPCR.
3. To assess the effect of siRNA-mediated down-regulation of LRP/LR on the cellular viability of the above-mentioned cell lines by using MTT assays.
4. To assess nuclear morphological changes that arise due to siRNA action targeting LRP/LR in the afore-mentioned cell lines using confocal microscopy and Airyscan™ processing.
5. To determine potential apoptosis-inducing effects of siRNA-mediated LRP/LR down-regulation on the cell lines mentioned above using Annexin-V/PI assays as well as caspase-3 assays.
6. To evaluate apoptotic pathways used by the afore-mentioned cell lines for siRNA-mediated apoptotic induction using caspase-8 and caspase-9 assays.
7. To determine if siRNA-mediated down-regulation of LRP/LR induces cell cycle arrest in late stage (DLD-1) colorectal cancer cells.
8. To evaluate the effect of LRP down-regulation on the proteome of late stage (DLD-1) colorectal cancer cells using SWATH-MS/mass-spectrometry based proteomics.
9. To evaluate how specific apoptotic and signal transducing proteins are affected in the cell when LRP/LR is down-regulated in late (DLD-1) stage colorectal cancer cells using Proteome antibody profiler arrays™.
10. To evaluate mRNA expression levels of cancer-related proteins upon LRP/LR down-regulation in late (DLD-1) stage colorectal cancer cells using qPCR.
11. To determine the effect of LRP/LR down-regulation on telomerase-related proteins in late (DLD-1) stage colorectal cancer cells using western blotting.
12. To determine the effect of LRP/LR down-regulation on telomerase activity in late stage (DLD-1) colorectal cancer cells using qPCR.

2.1.3. *In vivo* aim

- To investigate the significance of blocking LRP/LR with the IgG1-iS18 antibody and its effectiveness in the prevention and treatment of metastatic colorectal cancer *in vivo.*

2.1.4. *In vivo* objectives

1. To assess the viability of the IgG1-iS18 antibody for the prevention/treatment of metastatic colorectal cancer using an MF-1 nude mouse model (pilot and full study).
2. To analyse the effect of IgG1-iS18 on tumour cell shrinkage/growth and metastasis reduction.

Chapter 3: Materials and Methods

Note: Please refer to Appendix B for a detailed list of all reagents, suppliers and equipment used for experiments

3.1. *In vitro* study

3.1.1. Cell culture

3.1.1.1. Cell lines

- SW-480 (obtained from Fox Chase Cancer Centre): Early stage human colorectal adenocarcinoma which was isolated from a 50-year-old Caucasian male diagnosed with Dukes' type B colorectal adenocarcinoma.
- DLD-1 (obtained from Fox Chase Cancer Centre): Late stage human colorectal adenocarcinoma which was isolated from a male adult diagnosed with Dukes' type C colorectal adenocarcinoma.

3.1.1.2. Cell cultivation method

Colorectal cancer cell lines SW-480 and DLD-1 were cultured in Roswell Park Memorial Institute (RPMI) 1640 culture medium, supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) non-essential amino acids and 1% (v/v) Penicillin/Streptomycin/ in 75 cm^3 culture flasks. Cells were sub-cultured twice a week by discarding the media and washing the cells in PBS to remove any residual media. This was followed by an incubation in 1 X Trypsin/EDTA at 37 °C with 5% CO2 in a humidified atmosphere for 5 minutes. The Trypsin/EDTA was inactivated with culture medium and cells were seeded at appropriate concentrations and dilution factors when necessary.

3.1.1.3. Cryopreservation of cells

Cryopreservation is a process required to preserve the structure of cells by freezing them in an appropriate cryopreservation media. In addition, this process reduces the risk of losing cells due to reaching the passage limit or contamination. Cells were washed with PBS and incubated in 1X Trypsin/EDTA in order to facilitate detachment. Thereafter, detached cells were resuspended in media and centrifuged at 150 x g for 10 minutes. This was followed by resuspension of the cells in 80% culture medium, 10% DMSO and 10% FCS, which all together served as freezing media. The cells were then separated into 1ml aliquots before an hour

incubation at -20ºC, following a further incubation at -80°C overnight. Cells were stored in liquid nitrogen.

When a cryopreserved stock of cells was needed, 1 ml of pre-warmed culture medium was used to thaw the frozen stock. This was followed by centrifugation at 150 x g (Eppendorf 5417C centrifuge) for 10 minutes and re-suspension of the cell pellet in fresh culture media as well as 10% FCS. The cells were then placed in a 75 cm^3 culture flask and incubated at 37ºCand 5% CO2.

3.1.1.4. Quantification of cultured cells

Several of the experiments performed in this study required specific cell numbers and densities, since this allows for effective comparisons between cell lines. In order to determine the specific cell density and viability of each cell line, a TC 20™ automated cell counter was used.

The cells were washed with PBS and detached using 1X Trypsin/EDTA, Thereafter, 10 µl of the cell suspension was aliquoted into a microcentrifuge tube together with 10 µl of Trypan blue. The cells were then resuspended and 10 µl of cell suspension placed into the chamber of the cell counter. The cell counter provided the cell concentration in the form of cells/ml.

3.1.2. siRNA-mediated down-regulation of the laminin receptor (LRP/LR)

3.1.2.1. Transfection procedure

Before transfections could take place, cells were seeded on 6-well or 24-well tissue culture plates at specific concentrations, depending on the experiment being performed. Cells were allowed to reach 50-70% confluency prior to transfection. Thereafter, serum-free Opti-MEM® media was used to dilute the experimental Dharmacon™ ON-TARGETplus SMARTpool Human RPSA and MISSION® esiRNA-RPSA* siRNAs (both targeted to LRP/LR) as well as the negative control MISSION® esiRNA-RLUC siRNA (see Appendix A). In order for the transfection procedure to take place, the cells were also supplemented with appropriate amounts of DharmaFECT® and MISSION®* transfection reagents**.** This was followed by incubation at 37°C for 72 hours in order to allow the transfection to take place. This procedure was performed before any downstream experiments take place.

*: This treatment was used for SDS-PAGE/western blotting and MTT assays only, as a means of eliminating any off-target effects that may arise upon transfection.

3.1.3. Preparation of cell lysates

The attached cells were washed with 1 X PBS, detached using 1 X Trypsin/EDTA and harvested. This was followed by centrifugation at 2700 x g for 10 minutes. The supernatant was discarded and 100-300 µl of 1 X RIPA buffer (10mM Tris-Cl (pH 8), 10mM EDTA, 300mM NaCl, 1% sodium deoxycholate (DOC), 1% Triton-X, 0.1% SDS) was added to the pellet, followed by incubation at 4ºC for 15 minutes. Thereafter, the suspension was centrifuged at 20000 x g for 3 minutes. The supernatant containing the extracted protein was retained and the pellet discarded. The supernatant was stored at - 20ºC and thawed at 4ºC when required.

3.1.4. BCA protein assay

This protein assay was used to quantify total protein concentrations within the cells in the form of a lysate. Bovine serum albumin (BSA) standards of concentrations including 0, 0.2, 0.4, 0.6, 0.8 and 1 mg/ml were prepared. Thereafter, 25 µl of each standard was loaded into wells of a 96-well plate in triplicate. Likewise, this was performed with 25 µl of cell lysates which was diluted with dH_2O (1:5) and loaded in the 96-well cell culture plate in duplicate. This was followed by the addition of 200 µl BCA reagent (copper II sulphate and bicinchoninic acid in a ratio of 1:50) to the wells containing the standards and the lysates. After a 30-minute incubation at 37°C, the absorbance was then measured at 562 nm on an ELISA reader. This was allowed for a standard curve to be constructed, where protein concentrations can be determined.

3.1.5. Sodium dodecylsulphate- polyacrylamide gel electrophoresis (SDS-PAGE)

In order to perform western blotting, LRP/LR, hTERT, pTERT, TRF-1 and TRF-2 proteins were separated using sodium dodecylsulphate- polyacrylamide gel electrophoresis (SDS-PAGE) The cell lysates were heated in the presence of SDS, which allowed for the proteins to be denatured; however, they were also subjected to β-mercaptoethanol which facilitated protein denaturation through the reduction of disulphide bridges situated within the proteins. SDS does not only allow for protein denaturation but it also imparts a uniform negative charge along the length of the proteins thereby allowing molecular weight to be the principal determinant in the separation procedure. The negatively charged proteins migrated towards the anode of the electrical field and the proteins were separated on the basis of molecular size by the use of a polyacrylamide gel. With respect to the present study, a 12% polyacrylamide gel [containing Stacking Tris buffer (0.5M Tris, pH 6.8), Separating Tris buffer (3M Tris, pH 8.8), 10% ammonium persulfate (APS) in distilled water, TEMED (N, N, N', N'-

tetramethylethylenediamine] was prepared and cast. After the lysate samples were heated at 95ºC for 5 minutes in the 2 X loading buffer (2X loading buffer [100mM Tris/HCl pH 6.8, 4% SDS, 2% β-mercaptoethanol, 20% glycerol, 0.02% bromophenol blue), equal amounts (10-50 µg) of each protein sample was loaded on the gel, together with a Pre-stained protein molecular weight marker (New England Biolabs Inc.). The gel was resolved at 150 V for 45-60 minutes in 1X SDS running buffer (10% of 250mM Tris, 192mM glycine in distilled water, 10% SDS).

3.1.6. Western blotting

In order to determine the levels of LRP/LR, hTERT, pTERT, TRF-1 and TRF-2 proteins upon siRNA-mediated down-regulation of LRP/LR, western blotting was performed. This technique allows for the separated proteins resulting from SDS-PAGE to be transferred onto a PVDF membrane via electroblotting. Whatman filter papers were soaked in transfer buffer (20% methanol in 192mM glycine and 25mM Tris) for 5 minutes while simultaneously, the Polyvinyldifluoride (PDVF) membranes were soaked in 100% methanol for 2 minutes followed by an additional soak in transfer buffer for 5 minutes. Thereafter, the membranes were placed in between the soaked Whatman papers which were already placed onto the electroblotting device, and the gels were then placed on top of the membranes. The Trans-Blot® Turbo™ Transfer System was used for electroblotting with set parameters at 25V for 30 minutes. Following this, the membranes were blocked for an hour using 3% (w/v) BSA (Bovine Serum Albumin) in 1 X PBS-Tween (0.1% (v/v) Tween 20 and PBS) in order to prevent non-specific binding of the primary and secondary antibodies. Thereafter, the membranes were incubated for overnight in the appropriate primary antibody diluted in blocking buffer [IgG1-iS18 (1:1000), anti-β actin peroxidase, (1:1000), anti-rabbit TRF-1 (1:1000), anti-rabbit TRF-2 (1:1000), anti-rabbit pTERT (1:1000) and anti-mouse hTERT (1:1000)]. The membranes then underwent three 10-minute washes in 1X PBS-Tween before a 1-hour incubation in the appropriate anti-mouse (1:2000) or anti-rabbit (1:2000) secondary antibody with a horseradish peroxidase (HRP) conjugate was added to the membrane and diluted in 3% (w/v) BSA in 1X PBS-Tween at toom temperature. This was followed by three more 10-minute washes in 1X PBS-Tween before the chemiluminescent substrate was added and proteins were detected using the Chemidoc™ imaging machine. In addition, the 42 kDa β-actin antibody was used for the detection of β-actin which served as a loading control. Since β-actin is directly conjugated to HRP, it thus does not require a secondary antibody. The Bio-Rad Image Lab 5.1 Image acquisition and analysis software was used to analyse the blot features and capture the image data.

3.1.7. Cellular viability assessment – The MTT assay

The MTT assay is known to be a colorimetric assay which uses coloured compounds in a solution to determine concentration of viable cells. To achieve the above-mentioned reaction, 100 000 cells were seeded per well in 6-well tissue culture plates for each colorectal cancer cell line before the cells were transfected. After transfection, cells were incubated at 37°C for 72 hours. This was followed by 100 µg of MTT being dissolved in 1 X PBS and added to each well (1 mg/ml), followed by a further incubation at 37°C for 2 hours. Thereafter, the media containing MTT was discarded from each well while the residual formazan crystals were dissolved in 500 µl of DMSO. The resultant absorbance of the formazan solution was then measured at 570 nm using an ELISA plate reader. This procedure was performed for controls as well which included: untreated cells, cells treated with 8mM protocatechuic acid (PCA) as positive control and cells transfected with esiRNA-RLUC as negative siRNA control.

3.1.8. Assessment of nuclear morphological changes – Confocal microscopy with Airyscan

In order to evaluate the nuclear morphological changes that take place once down-regulation of LRP/LR occurs through the use of siRNA technology, confocal microscopy was used. A total of 100 000 cells were seeded onto coverslips per well in 6-well tissue culture plates, followed by cell transfection after 72 hours. The cells were then fixed in 4% paraformaldehyde (PFA) for 15 minutes prior to three washes with 1 X PBS. Once excess 1X PBS was blotted off after washing, cells were permeabilized by adding 2 ml 0.1%Triton-X into each well for 20 minutes, followed by two washes with 1X PBS. This was followed by an incubation with the Hoescht 33342 nuclear stain diluted in PBS (1:100) for 5 to 10 minutes in the dark. Once stained, the coverslips which contained the Hoescht-stained cells were washed 2-3 times in 1 X PBS prior to each coverslip being mounted facedown onto a clean microscope slide using Fluoromount mounting fluid. The microscope slides were then left to set for 45 minutes in the dark, after which they were stored at 4ºC.

Airyscan is a processing tool founded on confocal laser scanning microscopy. This detector is able to significantly improve signal by utilizing light that otherwise is rejected by the confocal pinhole. Thus, the increased signal-to-noise ratio can be used to retrieve high resolution information [195]. It has been shown that total resolution is improved by a factor of 1.7 in all spatial directions i.e. a resolution of 140 nm laterally and 400 nm axially for 480 nm can be achieved [195]. Airyscan was used to further analyse the nuclear morphological changes apoptotic cells undergo once LRP/LR was down-regulated.

3.1.9. Assessment of apoptotic induction

3.1.9.1. Annexin-V/ PI assay

One of the main features of apoptosis the translocation of the lipid phosphatidyl serine (PS) from the inner membrane to the outer membrane. This assay is based on the calcium-dependent protein known as Annexin V which specifically binds to the phosphatidylserine (PS) protein (Fig.13). Due to the externalisation of PS to the outer membrane allowing Annexin V to bind in the presence of calcium, detection of apoptosis is possible. This is performed by fluorescent-labelling the Annexin V protein, enabling flow cytometric detection of the translocated PS, and in turn apoptotic cells. The fluorescent-labelling in this assay is achieved by using propidium iodide (PI) which is a vital dye that can stain DNA by intercalating between base pairs. PI cannot permeabilise live cells and apoptotic cells but is able to stain dead cells binding tightly to the nucleic acids in the cell. The Annexin V protein is conjugated to FITC, which is able to bind to the exposed PS. Further identification can also be made between early apoptotic (Annexin V positive, PI negative) and late apoptotic (Annexin V positive, PI positive) or necrotic cells. Hence, viable cells will show no fluorescence, whereas apoptotic cells will fluoresce green (from FITC staining) and dead cells will fluoresce green and red (from PI staining).

For the Annexin V/ PI assay to be performed, 500 000 cells were seeded per well for both cell lines in 6-well tissue culture plates, prior to cell transfection for 72 hours. The annexin-V/PI assay was performed as per the supplier's instructions (BD Biosciences). Cells were then washed with cold 1X PBS prior to them being subjected to 1X trypsin/EDTA, resulting in cell suspensions. This was followed by centrifugation at 500 x *g* (Eppendorf 5417C centrifuge) for 5 minutes, after which the supernatant was discarded, while the pellet was resuspended in 1X annexin-binding buffer. Thereafter, 10 µl of Annexin V-FITC solution and 20 µl of PI viability dye were added to each cell suspension, followed by 15 minutes of incubation on ice in the dark. Following this, 400 µl of cold 1X annexin binding buffer was added to the samples for 30 minutes, after which flow cytometric analysis took place. The FL1 laser was used for the detection of Annexin V-FITC staining, while the FL2 laser detected the PI stain since this dye was detected in the orange range of the spectrum using a 562-588 nm band pass filter. Compensation and quadrants were set up using controls which included: unstained cells, Annexin V-FITC stained cells, PI stained cells, cells stained with both Annexin V-FITC and PI, and PCA-treated cells stained with both Annexin V-FITC and PI.

3.1.9.2. Caspase-3, -8 and -9 assays

These caspase assays are colorimetric assays, all which depend on the caspase-mediated hydrolysis of *p*-nitroaniline (p-NA), a chromophore which is coupled to a peptide substrate. Each caspase assay has a specific peptide substrate which is recognized and cleaved. This leads to the liberation of *p*-NA chromophore, which is known to have a high absorbance at 405 nm. Thus, the concentration of *p*-NA released can be used as an indicator of caspase activity and hence, the amount of apoptosis.

A total number of 100 000 SW-480 and DLD-1 cells were seeded per well in 6-well tissue culture plates prior to transfection for 72 hours. Thereafter, the cells were pelleted by centrifugation at 240 x g for 10 minutes followed by resuspension in 100 µl of cold 1X cell lysis buffer and incubated for 10 minutes on ice. This was followed by the cells being further centrifuged at 10000 x g for 5 minutes, after which the supernatant was placed immediately on ice. Thereafter, the protein concentration of the supernatant was determined by performing a BCA assay (as described in section 3.2.4). Following this, 200 µg of protein was prepared according to manufacturer's instructions (see Appendix B). The prepared assay mixture for each sample was then added to appropriate wells of a 96- well plate and incubated for 2 hours at 37˚C. The absorbance was measured at 405 nm using an ELISA plate reader.

3.1.10. Cell cycle analysis – Flow cytometry

Cell cycle analysis is made by measuring the DNA in cells stained with a DNA specific dye known as propidium iodide (PI). This dye is a nuclear and chromosome counterstain which binds to DNA by intercalating between the bases with little sequence preference. The fluorescence intensity of the stained cells at specific wavelengths therefore correlates to the amount of DNA the cells possess. Since the DNA content of cells is duplicated in S phase, the relative number of cells before the S phase (cells in the G_0 and G_1 phases) as well as cells after the S phase (G_2 and M phases) can be determined. This is because the fluorescence of cells in the G_2/M phase will be twice as high as that of cells in the G_0/G_1 phase. These results can then be analysed using flow cytometry since this method allows for rapid measurement of cellular DNA content.

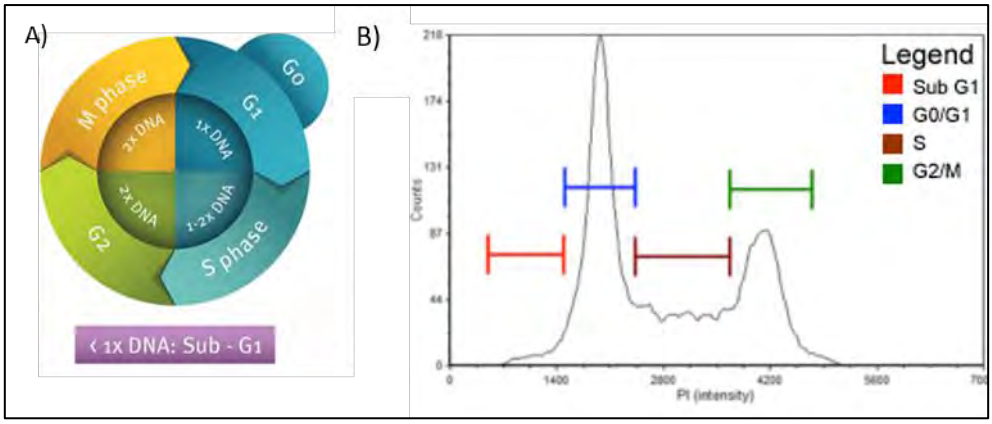


Figure 16: Summary of cell cycle analysis. A) There are four main stages within the cell cycle. The G2 and M phases have double the amount of DNA compared to the G0/G1 and S phases have since cells undergo DNA replication in the S phase. The sub G0/G1 phase represents cells that have undergone DNA fragmentation, an indicator of apoptosis, which results in a loss in DNA content (< 1 x DNA). B) Analysis of the cell cycle is performed through flow cytometry by looking at the percentage of cells in each phase of histogram post treatment. Image adapted from: https://chemometec.com/cell-counting-assays/cell-cycle-analysis/ and https://www.nexcelom.com/fluorescence-based-cell-cycle-analysis-drug-study-part-2/)

A total of 100 000 DLD-1 colorectal cancer cells were seeded per well in 6-well tissue culture plates prior to transfection for 72 hours. The cells were then harvested and washed in 1X PBS. Thereafter, cells were fixed in cold 70% ethanol in a dropwise manner while vortexing, in order to ensure all cells are fixed. Fixation took place for at least 24 hours at 4°C. Following this, cells were washed twice in 1 X PBS after which they were centrifuged at 420 x g (Eppendorf 5417C centrifuge) for 10 minutes in order to rid the cells of the ethanol. Following the supernatant being discarded, cells were treated with 200 µl of Guava® cell cycle reagent containing Propidium Iodide and incubated for 30 minutes in the dark. These resulting suspensions were evaluated using the BD Accuri C6 flow cytometer and software, whereby the forward scatter (FS) and side scatter (SS) identifies single cells. Pulse processing was employed for the exclusion of cell doublets, using the pulse area vs. pulse width/height. Since the maximum emission of PI is in the orange range of the spectrum, the FL2 laser using a 562-588 nm band pass filter was utilized to detect the dye. Thereafter, the percentage of cells within each cell cycle was determined using markers set within the analysis program.

Note: For cost effectiveness, this experiment together with subsequent experiments were only performed on the late stage (DLD-1) colorectal cancer cell line.

3.1.11. Quantification of mRNA expression levels – Quantitative reverse transcriptase polymerase chain reaction (RT-qPCR)

Quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) is not only an effective, but also an extremely sensitive and reliable method to quantify gene expression, including mRNA which is known to have low expression levels [196].

3.1.11.1. RNA Extraction

In order to quantify mRNA expression levels, total RNA must be extracted from samples after which the mRNA is converted into complementary DNA (cDNA) through a method known as reverse transcription [196]. This cDNA is then used as the template for the qPCR reaction. A total of 750 000 DLD-1 cells were seeded per well in 6-well tissue culture plates prior to transfection for 72 hours. To determine mRNA expression levels, RNA was first extracted from the DLD-1 samples. The extraction procedure followed the Quick-RNA™ MiniPrep kit (Zymo Research) manufacturer's protocol. Briefly, cells were trypsinized and collected by centrifugation (Eppendorf 5417C centrifuge). The pellets were then resuspended and lysed in the provided lysis buffer, followed by centrifugation. The remaining supernatant from the centrifugation contained the RNA, while the pellet contained the DNA. Thereafter, the supernatant containing the RNA was further purified by incubating the samples in DNase buffer for 15 minutes in order to eliminate any trace contaminants of DNA. The samples were then loaded onto spin columns specific for RNA extraction and washed twice using 400 µl wash buffer. Lastly, the purified RNA samples were eluted using 80 µl nuclease-free water. The resultant sample concentrations were determined using Nanodrop spectroscopy (Nanodrop® ND-1000).

Thereafter, a 1% (w/v) agarose gel containing a nucleic acid gel stain was used for gel electrophoresis (agarose, in 1 x TBE buffer consisting of Tris, boric acid and EDTA) to evaluate the RNA integrity. The samples were loaded with Fermentas 6 x loading dye and Fermentas Low range DNA ladder was used as a molecular weight marker. The gel was run at 100 V for approximately 30 min in TBE buffer. The RNA was visualized using Biorad Chemidoc system order to view total/intact RNA as well as any DNA contamination (Refer to Appendix A).

3.1.11.2. cDNA synthesis

Once the absence of DNA contamination was confirmed, the samples were converted into cDNA. The SensiFAST™ cDNA synthesis kit (Bioline) was used as per the manufacturer's instructions whereby a mastermix was prepared containing appropriate volumes of: 5X TransAmp buffer, reverse transcriptase and nuclease-free water which was aliquoted into PCR tubes. Thereafter,1 µg of mRNA was added each PCR tube prepared. A no reverse transcriptase control was also prepared for each sample, containing everything except reverse transcriptase. Thereafter, the samples were run on the CFX Maestro™ thermo cycler with the following parameters: One cycle of 25°C (primer annealing) for 10 minutes, one cycle of 42°C (reverse transcription) for 15 minutes, one cycle of 48°C (additional reverse transcription for highly structured RNA) for 15 minutes and one cycle of 85°C (inactivation) for 5 minutes. The resultant samples were stored at -20°C for mRNA quantification.

3.1.11.3. mRNA expression level quantification – Quantitative reverse transcriptase polymerase chain reaction (RT-qPCR)

In order quantify mRNA expression levels of proteins, qPCR was performed using the SensiFAST SYBR™ No-ROX kit (Bioline) as per the manufacturers protocol whereby mastermix was prepared for gene containing: 2X SensiFAST SYBR™ No-ROX mix, 10 µM of the forward primer (refer to Appendix A), 10 µM of the reverse primer (refer to Appendix A), nuclease-free water and lastly, 1 µg of cDNA. This mixture was pipetted into the necessary wells of a 96-well tissue culture plate, ensuring that the mixture was thoroughly combined. Thereafter, the samples were run on the CFX Maestro™ thermo cycler with the following parameters: One cycle at 95°C for3 minutes, 45 cycles at 95°C for 15 seconds, one cycle T_m of gene for 30 seconds, melt curve: one cycle at 95°C for 10 seconds, 65°C for 1 minute and one cycle at 72°C for 30 seconds. Refer to the Appendix A for the melt peaks of each gene.

Thereafter, the average C_q value from technical repeats was used for calculations. In addition, to acquire reliable comparisons of gene expression levels between samples, corresponding qPCR reactions must be performed for reference genes known to be invariant expression. Reference genes are known to encode housekeeping enzymes, required for the functioning of all cells [197]. When data are analysed for the gene of interest, the calculation also incorporates measurements for the reference gene. The reference genes used in this study include *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) and *ACTB* (β-actin) since these genes are present in all cells. These reference genes were used since these genes are present in all cells. Moreover, the reference genes were used because when tested, the mRNA levels of these genes were not

affected between each treatment. (Refer to Appendix A). Normalisation with stably expressed reference genes as internal controls, known as the comparative C_q or the $\Delta\Delta C_q$ method, was used for the normalisation of mRNA data. In addition, Reverse transcriptase controls (RTC's) were subtracted from all samples to remove any doubt of DNA contamination (for further normalisation). The comparative C_q method normalises the C_q value of a target gene to internal reference genes before comparisons are made between samples. First, the difference between C_q values (ΔC_q) of the target gene and the mean of two reference genes is calculated for each sample, and then the difference in the ΔC_q ($\Delta\Delta C_q$) is calculated between samples. The fold-change in expression of the two samples is calculated as $2^{-\Delta\Delta Cq}$, where 2 derives from 1 + efficiency and efficiency is assumed to be 1 (i.e., 100% efficiency) [196]. Dissociation (melting) curve analysis was performed to verify PCR specificity (See Appendix B).

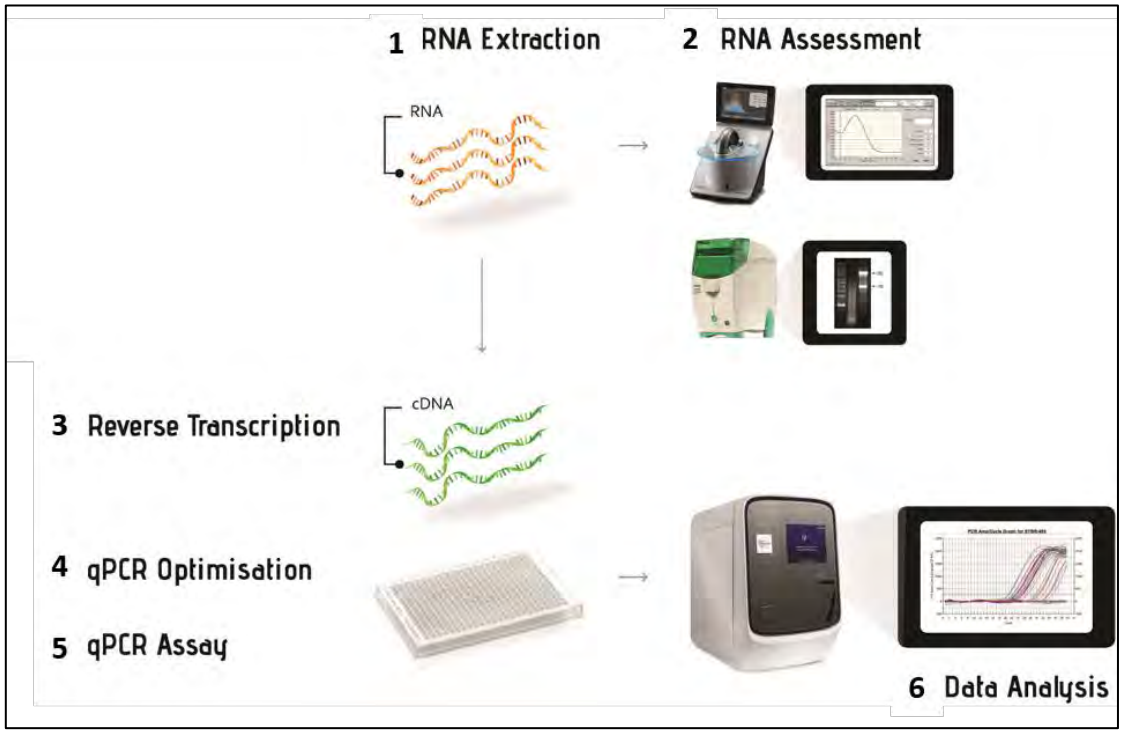


Figure 17: Sequential steps of quantitative real-time qPCR workflow. RNA is extracted assessed for concentration and quality through an agarose gel. Thereafter, the RNA is converted into cDNA, which is used as template in the final qPCR reaction. The conditions of the final qPCR assay must be optimised for each experiment followed by data analysis Adapted from Kuang et al., 2018 [196].

3.1.12. Telomerase activity assay - Quantitative polymerase chain reaction (qPCR)

Telomerase activity in DLD-1 cell samples were quantified with the use of the TRAPeze® RT Telomerase Detection Kit. The kit provides a highly sensitive assay for fluorometric detection

and allows real time quantification of telomerase activity. The principle of the assay is based on the telomeric repeat amplification protocol (TRAP) as initially defined by Piatyszek et al., (1995) [198]. The TRAPeze® kit makes use of fluorescence energy transfer primers (Amplifluor® primers) which provide a way in which to detect and quantify telomerase activity by directly measuring real time fluorescence emission. The initial stage of the reaction allows telomerase in an extracted sample to add telomeric repeats to the 3′ end of the substrate over a given time period. Hereafter, the extended products are amplified by Taq polymerase, utilising the Amplifluor® primers. These primers only produce a fluorescent signal once they have been incorporated into the telomeric repeat amplification products. The amount of TRAP products produced are then directly proportional to the fluorescence emission produced. This kit, therefore, takes into consideration the real time measurement of fluorescence produced which directly allows for relative quantification of telomerase activity through the addition of telomeric repeats.

3.1.12.1. Sample preparation

A total of 750 000 DLD-1 cells were seeded per well in 6-well tissue culture plates prior to transfection for 72 hours. The cells were then harvested and centrifuged at 1500 x g (Eppendorf 5417C centrifuge) for 10 minutes. The resultant pellets were resuspended in 250 µl of CHAPS ((3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate)) lysis buffer (Sigma) was added followed by 30-minute incubation on ice. Thereafter, samples were then centrifuged for 20 minutes at 15000 x g at 4°C in a microcentrifuge where the resulting supernatant was snap frozen on dry ice. The protein concentration was then quantified using Nanodrop spectroscopy (Nanodrop® ND-1000). Once the proteins were diluted to 500 ng/µl, a portion was kept for the heat-treated control.

3.1.12.2. Telomerase activity detection

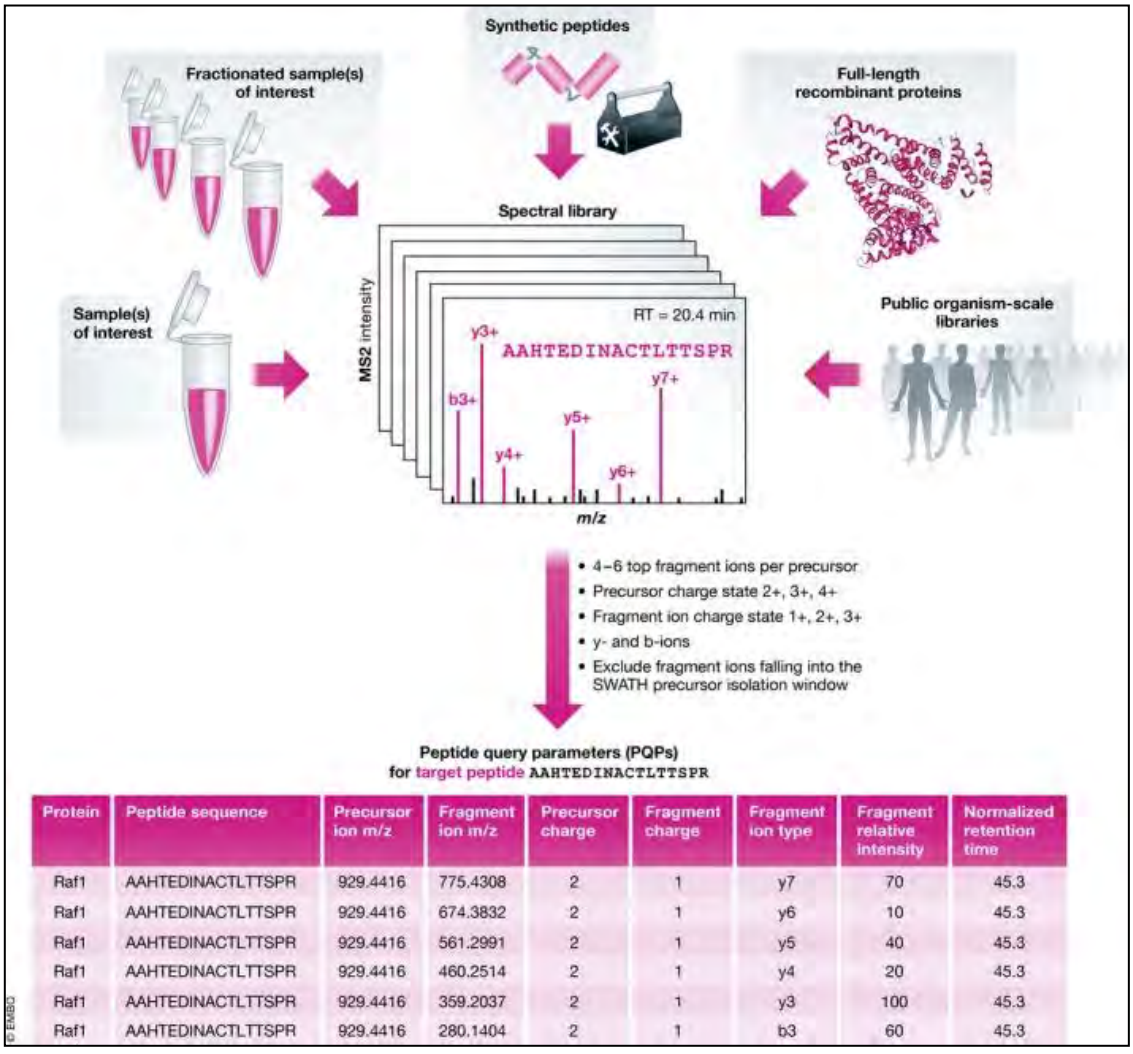
Relative telomerase activity was quantified by the TRAPeze® RT1 Telomerase detection Kit (Merck Millipore), following the manufacturers protocols. Briefly, cell samples were prepared as mentioned in section 3.2.12.1 and washed in 1X PBS, after which the samples were lysed using CHAPS lysis buffer. Protein and RNA extractions were then collected in the supernatant where protein concentrations were standardized to 500 ng/µl for all experimental and control reactions. All samples were then subjected to experimental analysis by qPCR accompanied by a positive control (human embryonic kidney (HEK293) cell extracts with confirmed telomerase

activity) and three negative controls: a minus telomerase control, consisting of only CHAPS Lysis Buffer was used to ensure the buffer had not been contaminated and had no telomerase activity; a no template control (water) consisting of only Nuclease free/PCR Grade Water was used so as to normalise against primer dimer formation of the Amplifluor primers in the absence of telomerase activity, and lastly, since telomerase is a heat-sensitive enzyme, a heat-treated telomerase negative control was also included so as to evaluate each sample for heat sensitivity, whereby 10 µl of each 500 ng/µl sample was incubated at 85 ºC for 10 minutes prior to detection, in order to inactivate telomerase. All reactions were performed in triplicate in 96-well tissue culture plates. OneTaq® HotStart Taq Polymerase (5 U/µl) was used as it is not supplied with the kit. All samples were analysed via qPCR with the CFX Maestro™ thermo cycler with the following cycling parameters applied: One cycle of 37°C for 30 minutes, 95°C for 2 minutes and 45 cycles of 95°C for 15 seconds, 59°C for 60 seconds and 45°C for 10 seconds. Telomerase activity was thereafter calculated from the standard curve generated by 1:10 serial dilutions (20–0.0002 amoles) of the provided TSR8 control template as per Merck Millipore instructions. Negative controls were included. The data was then analysed with CFX Maestro™ software version 1.0. All values were normalised against the negative controls, whereby, all negative control values (including signals for CHAPS only, no template control and heat-treated, respectively) were subtracted from the signal of each sample and thereafter, the mean value calculated for all biological repeats.

3.1.13. Proteomic analysis - Sequential Window Acquisition of All Theoretical Mass Spectra (SWATH MS)

Proteomics is a well-known technique used for several proteins to be studied in a single analysis. In particular, Sequential Window Acquisition of All Theoretical Mass Spectra (SWATH-MS) is a proteomic platform based on a data independent acquisition (DIA) theory involving simultaneous identification and quantification of proteins [199]. In order to obtain quantitative measurements, non-labelled protein samples are digested in trypsin where the resultant peptides are analysed through liquid chromatography together with a tandem mass spectrometer [200]. Consequently, the ionized proteins within a sample that lie between a particular mass range are then fragmented in an unbiased and systematic manner [200]. An important advantage of SWATH-MS is that it allows for quantitative analyses of peptides covering thousands of proteins with high accuracy. The technique is also suited for samples that require not only precise, but also reproducible quantification for the main portion of the

expressed proteome/peptidome in each sample [200]. In addition, several studies consisting of complex mammalian samples showed proteome coverages of approximately 50% of MS-measurable proteomes in a single-shot analysis [201, 202]. As a result, this allows for novel screening of several proteins involved in siRNA-mediated down-regulation of LRP/LR allowing for insight into the mechanism of the receptor.



Protein	Peptide sequence	Precursor ion m/z	Fragment ion m/z	Precursor charge	Fragment charge	Fragment ion type	Fragment relative intensity	Normalized retention time
Raf1	AAHTEDINACTLTTSPR	929.4416	775.4308	2	1	y7	70	45.3
Raf1	AAHTEDINACTLTTSPR	929.4416	674.3832	2	1	y6	10	45.3
Raf1	AAHTEDINACTLTTSPR	929.4416	561.2991	2	1	y5	40	45.3
Raf1	AAHTEDINACTLTTSPR	929.4416	460.2514	2	1	y4	20	45.3
Raf1	AAHTEDINACTLTTSPR	929.4416	359.2037	2	1	y3	100	45.3
Raf1	AAHTEDINACTLTTSPR	929.4416	280.1404	2	1	b3	60	45.3

Figure 18: Summary of SWATH-MS procedure. Samples are typically analysed where the results are summarized in the form of one or more spectral library files. From the spectral library files, the appropriate peptide query parameters (PQPs) are extracted by filtering the identified peptide coordinates. In particular, PQPs include information about: the protein itself, peptide sequence, fragment *m/z*, precursor *m/z*, precursor and fragment charge, expected relative fragment ion intensities and normalized retention time [200].

3.1.13.1. Sample preparation

DLD-1 cells were seeded, transfected and harvested as previously described in 3.2.9.1. Cell pellets were resuspended in 200 ul of lysis buffer per pellet. Thereafter, cells were sonicated

on ice for 9 pulses (10 sec per pulse) followed by centrifugation at 15 000 x g for 10 minutes to clear cell debris. Cell lysates were then incubated with 25 units, (1ul of stock –2500 units in 100ul) of benzonase, per 0.5 million cells and at 2mM $MgCl_2$ at 37°C for 30 minutes. This was followed by another centrifugation at 15 000 x g for 10 minutes. The supernatant was collected, and the concentration determined using a BCA assay. Protein solutions were then reduced using 10 mM DTT for 30 minutes at 37°C and alkylated using 40 mM IAA for 30 minutes in the dark.

Note: The following methods regarding proteomics were performed at The Council for Scientific and Industrial Research (CSIR) centre:

3.1.13.2. Sample clean-up and digestion

All experiments were performed with a KingFisher™ Flex magnetic particle processing robot. The Fisher™ Flex system was configured for automated HILIC-protein clean-up and on-bead trypsin digest. Deep-well 96-plates were loaded in each carousel position with each plate filled as follows: 1) 96 well tip heads; 2) 10 µl, 20mg/ml hyper porous magnetic HILIC micro spheres in 20% Ethanol and 180 µl Equilibration buffer (100 mM NH_4Ac, 15% ACN pH 4.5); 3) Equilibration Buffer (500 µl); 4) Protein extract mixed 1:1 with Bind buffer (200 mM NH_4Ac, 30% ACN pH 4.5), final volume of 100 µl; 5) 500 µl 95% ACN (wash 1); 6) 500 ul 95% ACN (wash 2); 8) 200 µl 50 mM Ammonium formate pH 8.2 and Promega sequencing grade Trypsin for an enzyme:protein ratio of 1:10. The Bindit programme was then run with the magnetic pins transferring the magnetic HILIC beads from position 2 to 8 and in the process binding proteins, washing off SDS and other contaminants and finally generating peptides ready for LC-MSMS analysis post the on-bead trypsin digest.

3.1.13.3. Spectral library building (Data-dependent analysis)

Post HILIC peptide samples were vacuum dried, resuspended in 2% ACN/0.2%FA and spiked with iRT peptide standards. Three injections were then performed per sample for each of the conditions. Analysis was performed using a Dionex Ultimate 3000 RSLC system coupled to an AB Sciex 6600 TipleTOF mass spectrometer. Peptides were first de-salted on an Acclaim PepMap C18 trap column (75 µm × 2 cm) for 5.5 min at 5 µl/min using 2% acetonitrile/0.2% formic acid, than separated on Acclain PepMap C18 nanoRSLC column (75 µm × 15 cm, 2 µm particle size) using a 60 min linear gradient at a flow-rate of 0.5 µl/min (A: 0.1% formic

acid; B: 80% acetonitrile/0.1% formic acid). An electrospray voltage of 2.5 kV was applied to the fused silica emitter (New Objective: 20 µm ID x 5 cm, 10 µm tip). The 6600 TipleTOF mass spectrometer was operated in Data Dependant Acquisition mode. Precursor MS scans were acquired from *m/z* 400-1500 using an accumulation time of 250 ms followed by 80 MSMS scans, acquired from *m/z* 100-1800 at 25 ms each, for a total scan time of 2.3 sec. Multiply charge ions (2^+ - 5^+, 400 -1500 *m/z*) were automatically fragmented in Q2 collision cells using nitrogen as the collision gas.

3.1.13.4. SWATH –MS analysis

Three injections, representing each of the technical replicates, were performed per sample for each of the conditions. For SWATH–MS, the LC gradient used for spectral library building was applied. The SWATH-MS method consisted of the acquisition of 100 MS2 scans of overlapping sequential precursor isolation windows (variable m/z isolation width, 1 m/z overlap, high sensitivity mode) covering the 400 to 900 m/z mass range, as well as a single MS1 scan. The accumulation time was 300 ms for the MS1 scan and 25 ms for the MS2 scans for a total of 2.3 s total cycle time.

3.1.13.5. Protein identification and spectral library building

Raw data were searched against the human UNIPROT sequence database (reviewed entries, downloaded on 2 June 2017) supplemented with a list of common contaminating proteins as well as the sequences of the Biognosys iRT peptide retention time standards. Thereafter, data processing was performed using Protein Pilot (v 5.0.1). The following search settings were applied: trypsin as the proteolytic enzyme, IAA based alkylation, thorough search effort. False discovery rate (FDR) analysis was then performed with 1% global FDR cut-off applied at PSM, peptide and protein levels. A spectral library was constructed by importing the .group Protein Pilot output into the Skyline (v 4.1.1.18179) spectral library builder. A cut-off of 0.995, corresponding to 1% peptide FDR, was applied during import. The Biognosys iRT peptides were appended to the library in order to normalize the peptide retention time. The following filters were applied for peptide and protein import into Skyline: Tryptic peptide, size 5-36 amino acids, with up-to 1miss-cleavage and one matched cleavage site allowed; Structural modifications: Carbomedomethyl (Cys), Oxidation (Met), Acetylation (N-terminal); Precursor charge states 2-4, product charge states 1-2, product ions: b and y; Ion match tolerance of 0.1

m/z. Post protein, peptide and transition import into Skyline a decoy peptide list was generated by shuffling the sequences of all imported peptides.

3.1.13.6. SWATH-MS processing

SWATH data files were converted to mzML format as well as centroided using the Proteo Wizard MS Convert tool. The converted SWATH mzML files were imported into Skyline (v 4.1.1.18179). The following filters were applied for SWATH mzML import: Precursor charge states 2-4, product charge states 1-2, product ions: b and y; 3-6 transitions per peptide; Product m/z range 100-1800; MS1 filtering: Isotope peaks included (Count), Precursor mass analyzer (Centroid), Peaks (3), Mass accuracy (20 ppm); MS2 filtering: Acquisition method (DIA), Product mass analyzer (Centroid), Mass Accuracy (20 ppm) and an isolation scheme of 80 variable windows as per the SWATH method run in Analyst; For retention time filtering only scans within 10 min of the predicted iRT retention times were selected. Once all SWATH mzML files were imported all repeated peptides and proteins were removed and a peak scoring model was trained using mProphet and the decoy peptides generated during spectral library building. All peaks were then re-integrated using this model and only peptides with q-values of less than or equal to 0.01 were used for further processing.

3.1.13.7. Selection of differentially expressed proteins

Differentially expressed proteins were detected via the Skyline external tool, MS Stats. Thereafter, the MS Stats output list of differentially expressed proteins was further filtered so that only entries fitting the following criteria remained: Minimum fold change ≥ 2 and Maximum adjusted p-value ≤ 0.01.

3.1.14. Proteomic analysis - Proteome Profiler Antibody Arrays™

The antibody array is an antibody-pair-based assay similar to an ELISA, however, uses a membrane as a substrate instead of a plate. Specific capture antibodies are spotted/arrayed onto the nitrocellulose membrane with each pair of spots a different analyte. Once the sample is diluted, it is added to the membrane, and paired with biotinylated detector antibodies as well as streptavidin- Horseradish Peroxidase (HRP). The sample-antibody mixture is then incubated with the array. Any analyte/detection antibody complex present is bound by its cognate

immobilized capture antibody on the membrane. The antibody array can then be analysed using the same method as a chemiluminescent western blot. Comparison between samples can be performed by applying densitometry software for a semi-quantitative comparison. Proteome Profiler antibody arrays require no specialized equipment and eliminates the need to perform multiple western blot experiments.

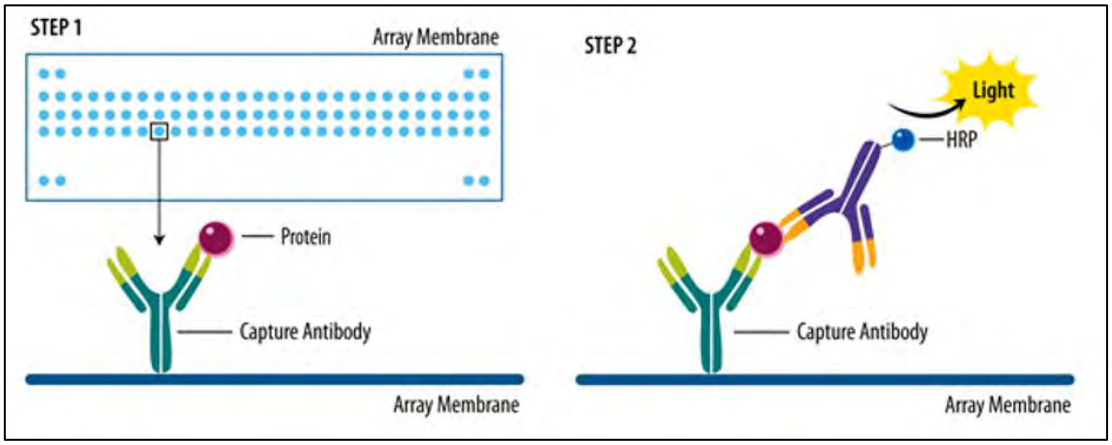


Figure 19: The proteome profiler antibody array detects several proteins in a single sample. Once experimental lysates are incubated on the membranes, the capture antibodies on nitrocellulose membranes are able to bind to their specific target proteins (Step 1). An HRP-conjugated antibody is subsequently used to specifically detect proteins (Step 2). Proteins are visualized using chemiluminescent detection reagents which produce a signal that is proportional to the amount of analyte bound (Image obtained from https://www.rndsystems.com/resources/technical/using-rd-systems-proteome-profiler-antibody-arrays).

Note: Each kit contained the necessary membranes, buffers, antibody cocktails and chemiluminescent substrates.

In order to determine whether proteins involved in the apoptotic and MEK/ERK signalling pathways are affected post siRNA-mediated down-regulation of LRP, Proteome profiler antibody arrays were performed as per the supplier's instructions (R&D systems). A total of 750 000 DLD-1 cells were seeded per well in 6-well tissue culture plates prior to transfection for 72 hours. The cells were harvested, and cell counts were performed since each sample had to contain $1x10^7$ cells. Thereafter, 300 -600 µl of cold lysis buffer (the lysis buffer for the apoptotic array contained a protease inhibitor cocktail) was added to each sample, followed by a 30-minute incubation at 4 °C. Each cell suspensions were then centrifuged at 10 000 x g for

5 minutes where the resultant supernatant was used as the experimental protein lysate. A BCA assay was performed, and each lysate was diluted, and 200-600 µg of protein was loaded on each membrane. Following this, each membrane was placed printed side up into the 8-well tray provided with each kit, followed by incubation with 1 ml of 1X Array buffer at room temperature for 1 hour, in order to ensure no non-specific binding to the membrane surface. The 1X Array buffer was then removed, and the membranes were incubated with 1 ml of protein sample overnight at 4ºC with gentle shaking. Thereafter, each membrane was placed into separate containers where they were washed three times with 20 ml 1X Wash Buffer for 10 minutes. Arrays were incubated with the detection antibody cocktail solution for 1 hour at room temperature and then washed three times with 1 X Wash buffer. This was followed by incubation with 1X Streptavidin-HRP at room temperature for 30 minutes. The arrays were washed three times with washing buffer, and protein spots were visualized using the chemiluminescence detection reagents supplied in the Array Kits. The intensity score of each duplicated array spot was measured with the ImageLab (version 5.1) software and the average intensity was calculated by subtracting the averaged background signal and PBS spots (negative control). The identity and the respective coordinates of all the antibodies on the arrays can be found in the Appendix A.

Note: For cost-effectiveness, only non-transfected and Human-RPSA siRNA-transfected samples were used. The experiments were performed in triplicate with two technical repeats to confirm results obtained.

3.1.15. Statistical evaluation

3.1.15.1. Statistical significance using one-way ANOVA and two-tailed student's *t*-test

A one-way ANOVA followed by a two-tailed student's *t*-test with a confidence interval of 95% was used as a means of analysing and confirming the data. To further validate the data, the Bonferroni post-hoc test was applied, with p-values of less than 0.05 considered to be significant. The statistical analysis was performed using the Microsoft® Excel 365 statistical programme.

3.1.15.2. Analysis of Pearson's Correlation Coefficient (r)

Pearson's correlation coefficient was also used to measure correlation between LRP/LR levels and cell viability as well as apoptotic induction. A positive coefficient is an indication of direct

proportionality between the two variables and a negative coefficient implies inverse proportionality. Measured correlation values close to 1 signifies high positive correlation.

3.2.*In vivo* study

The MF-1 nude mouse model was used to assess the effect of the anti-LRP/LR specific antibody, IgG1-iS18, with regards to transformation, invasion and metastasis of colorectal carcinoma. Human colorectal carcinoma (HT-29) cells were intraperitoneally injected into immunocompromised MF-1 nude mice (Animal Ethics Clearance Number (AESC No.) 2015-04-C).

This mice model is effective in investigating therapeutic approaches for human colorectal cancer as the cancer cell line used was isolated from human tumour tissue i.e. the HT-29 cells were isolated from a 44-year-old Caucasian female.

In order to optimize the experimental design of the study, various pilot studies were performed.

3.2.1. Acclimatization

Mice were left for 2 weeks to acclimatize to the new environment before commencement of any experimental procedures.

3.2.2. Induction of cancer

Mice were divided into three groups consisting of three mice each and then individually anesthetized with Isofor Infusion (Isoflurane-100%) and oxygen at 1,5 – 2% for approximately 3 minutes or until un-conscious, prior to intraperitoneal injection of the colorectal carcinoma cells. The cell mixture was drawn up into a syringe and carefully injected into the abdomen region of each mouse using a 27 mm^2 gauge needle. For pilot study 1, the tumours were then allowed to establish for 3 weeks before treatment began.

Tumour formation was observed and quantified via 3D calliper measurements. In addition, the mice were monitored and weighed throughout the study (before and after treatments).

3.2.3. Treatment regime

<u>Pilot study 1 (Treatment approach)</u>

The HT-29 colorectal carcinoma cell line was used in all pilot studies. This cell line was obtained from Fox Chase Cancer Centre and is a human colorectal adenocarcinoma which was previously isolated from a 44-year-old Caucasian female colorectal adenocarcinoma. Prior to the injection of HT-29 colorectal carcinoma cells into the mice, the cells were detached using 1X Trypsin/EDTA and re-suspended in full growth medium (DMEM/Hams F12 supplemented with 10% heat-inactivated FBS and 1% Penicillin-Streptomycin). Thereafter, the cells were pelleted by centrifugation at 150 x g (Eppendorf 5417C centrifuge) for 10 minutes and then washed in 1X PBS prior to a second round of centrifugation at 150 x g. The resultant pellet was re-suspended in media and the cells were counted with a TC 20^{TM} automated cell counter. A total of 5.5 x10^5 cells were injected at a volume of 110 µl.

Once the mice had visible tumour formation, group 1 was treated with 0.885 mg/ml IgG1-iS18 antibody, group 2 was treated with the PBS vehicle control solution and the last group remained untreated. The 0.885 mg/ml IgG1- iS18 antibody was administered at 2.5 mg/kg body weight twice a week, intraperitoneally over a period of 4 weeks. For the vehicle control, PBS was injected in the same manner as stated above. The injection volume was limited by possible toxicity of the substance and by the size of the mouse and was therefore kept to a treatment volume of less than 105 µl [203]. The intraperitoneal injection was performed by grasping the scruff of the neck and tail tightly and turning the mouse over so that the abdomen was exposed. The antibody was injected into the lower abdomen to avoid hitting vital internal organs. No anaesthetic was administered, and the chest movements of the mice were monitored to make sure the animal was comfortable. In addition, the tumour size was measured twice a week during the 4-week treatment period.

Pilot study 2 (Prophylactic approach)

Prior to the injection of colorectal carcinoma cells (HT-29) into the mice, the cells were detached using 1X Trypsin/EDTA and re-suspended in full growth medium (DMEM/Hams F12). Thereafter, the harvested cells were incubated either with 0.885 mg/ml IgG1-iS18 antibody or PBS (vehicle control) for 1 hour. Thereafter, the cells were pelleted by centrifugation at 150 x g for 10 minutes and then washed in 1X PBS prior to a second round of centrifugation at 150 x g. The resultant pellet was re-suspended in media and the cells were

counted with a TC 20^{TM} automated cell counter. A total of 5.5 x10^{5} cells were injected at a volume of 110 µl.

Since this study was a prophylactic approach, group 1 received an intraperitoneal injection of 0.885 mg/ml IgG1-iS18 at 3 mg/kg of body weight while group 2 received PBS (vehicle control) 24 hours after tumour induction. Group 3 remained untreated. One week after the initial treatment mice received treatment intraperitoneally twice a week for 4 weeks. The intraperitoneal injection was performed as previously described.

Pilot study 3 (Combination approach)

This pilot study contained 12 mice where group 1 received the treatment approach, group received the prophylactic approach, group 3 received PBS (vehicle control) and group 4 remained untreated. The concentration of the antibody was increased to 5 mg/kg of body weight where treatments were administered twice a week for 4 weeks. However, the route of administration was altered whereby the mice were injected intravenously via the tail vein.

After the four-week treatment period for all studies, all mice were euthanized with 0.5 ml Euthapent and assessed for internal and external tumour formation. The tumours were harvested, then weighed and the tumour volumes determined using the following formula: V= (Length x width x width)/ 2.

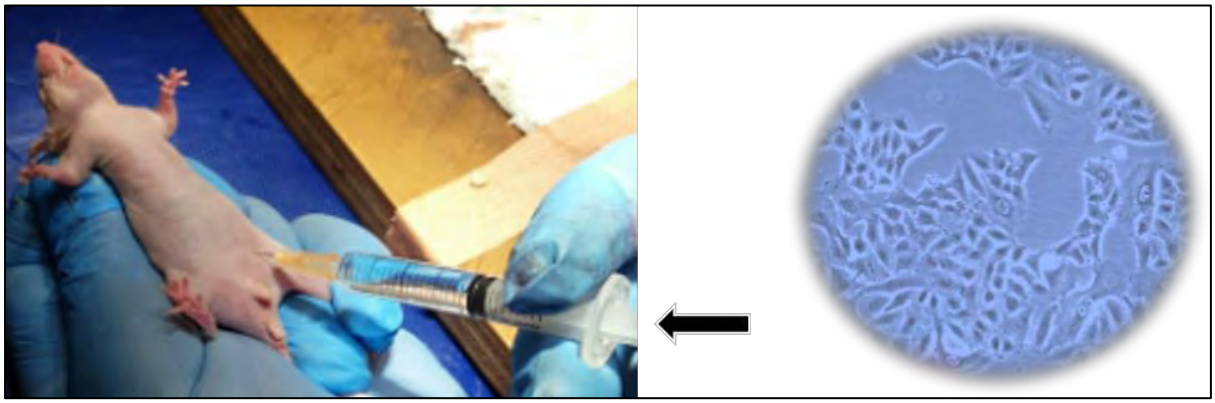


Figure 20: Injection and treatment procedure of the MF-1 nude mouse model. HT-29 cells were pelleted at a density of 5x10^{6} cells/ml and prepared in 100 µl culture medium prior to intraperitoneal injection into the abdominal region of the subject mice. This was followed by the treatment procedure using the IgG1- iS18 antibody. The IgG1-iS18 antibody was

administered twice a week for 4 weeks intraperitoneally for pilot studies 1 and 2, and tumour size was monitored.

Chapter 4: Results

4.1.*In vitro* study

4.1.1. siRNA technology successfully results in knock-down of LRP expression in early and late stage colorectal cancer cells.

To understand the effect LRP/LR expression has on early and late stage colorectal cancer cell viability, down-regulation of the receptor was performed. Once early (SW-480) and late (DLD-1) stage colorectal carcinoma cells were transfected with the Dharmacon™ ON-TARGETplus SMARTpool Human-RPSA siRNA (targets four different regions of the 37kDa LRP mRNA), evaluation of LRP levels was performed via western blotting and quantitative real-time PCR. To visualize whether the cells were undergoing morphological changes, bright field microscopy images of the DLD-1 cells were taken before and after Human-RPSA transfection of 72 hours. It was found that the upon transfection with Human-RPSA, the DLD-1 cells appeared to be reduced in size with diminished cell membranes as well as condensed nuclei – all indicative of apoptotic induction (Fig. 21). To confirm this, Western blotting was performed, and analysis showed that 37 kDa LRP was significantly knocked down in both SW-480 and DLD-1 cells when transfected with the Human-RPSA siRNA. The SW-480 and DLD-1 transfected cells exhibited a 75% and 78% decrease in 37 kDa LRP expression, respectively, when compared to cells that were not transfected, (Fig. 22). Additionally, treating SW-480 and DLD-1 cells with the negative control siRNA, MISSION® esiRNA-RLUC, indicated no notable change in 37 kDa LRP expression levels in comparison to cells that were not transfected (Fig. 22). The target sequences for the afore-mentioned siRNAs are supplied in the Appendix A section. In addition, it was found that when DLD-1 cells were transfected with Human-RPSA, the relative mRNA expression levels of LRP/LR were significantly decreased by 0.9-fold, further confirming that Human-RPSA siRNA is successful in down-regulating the receptor as seen in Figure 23.

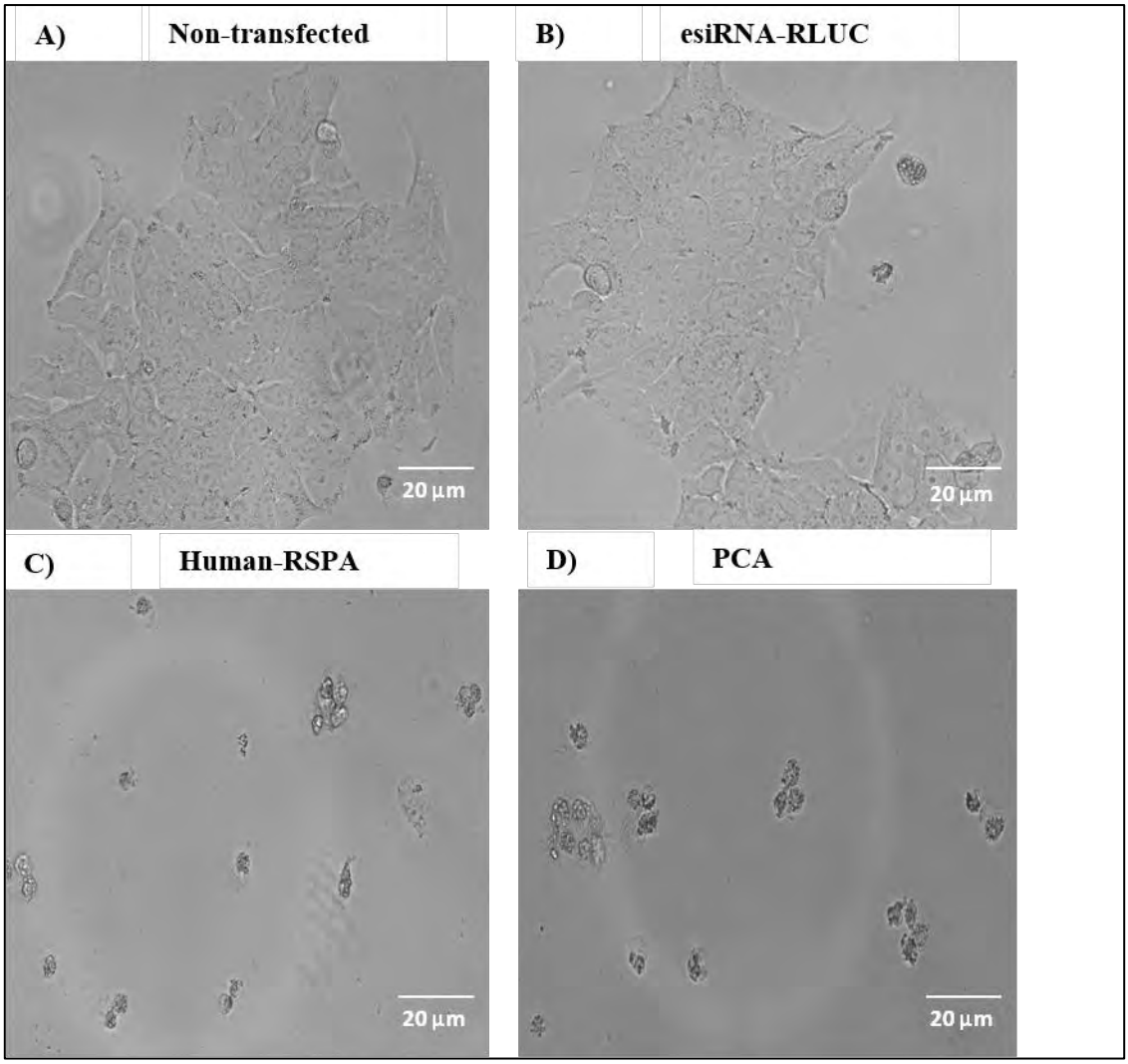


Figure 21: Late stage (DLD-1) cells observed using bright field microscopy. A) and B) Non-transfected and esiRNA-RLUC (negative control) transfected cells are found to be large with uncompromised membrane integrity. **C) and B)** Human-RPSA-transfected and PCA (positive control) treated cells are found to have a reduced size together with compromised membrane integrity i.e. membrane blebbing and condensed nuclei – indicative of apoptosis occurring. Images were obtained at 200X magnification. Scale bars are indicative of 20 µm (Adopted from Vania et al., 2018 [204].

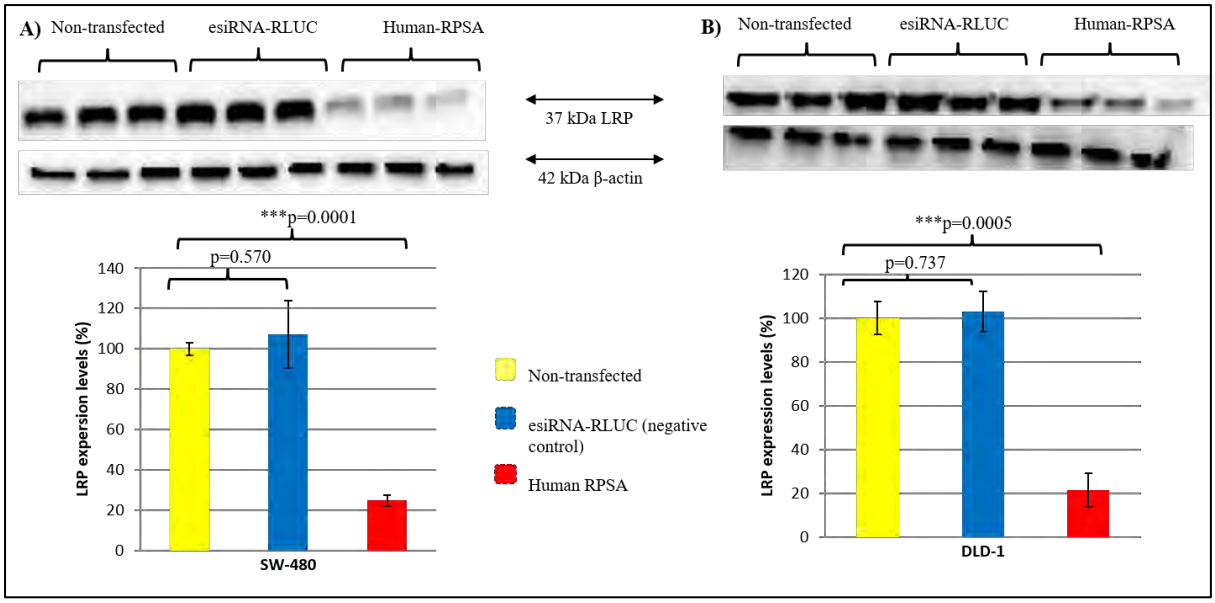


Figure 22: The effect of siRNA-mediated knock-down on LRP expression in early (SW-480) and late (DLD-1) stage colorectal cancer cells. Upon transfection of SW-480 (A) and DLD-1 (B) cells with Human-RPSA siRNA, significant 75% and 79% decreases in LRP expression levels was revealed, respectively, in contrast to cells that were not transfected. Densitometric analysis of LRP levels was performed where levels of the non-transfected cells were set to 100%. β-actin was used as the loading control and the graphs are representative of an average of experiments performed in triplicate. Error bars represent standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001, significant: The One-way ANOVA was significant and a Bonferroni corrected post-hoc *t*-test indicated that the Human-RPSA samples are significantly lower than the non-transfected and esiRNA-RLUC samples (Adapted from Vania et al., 2018 [204]).

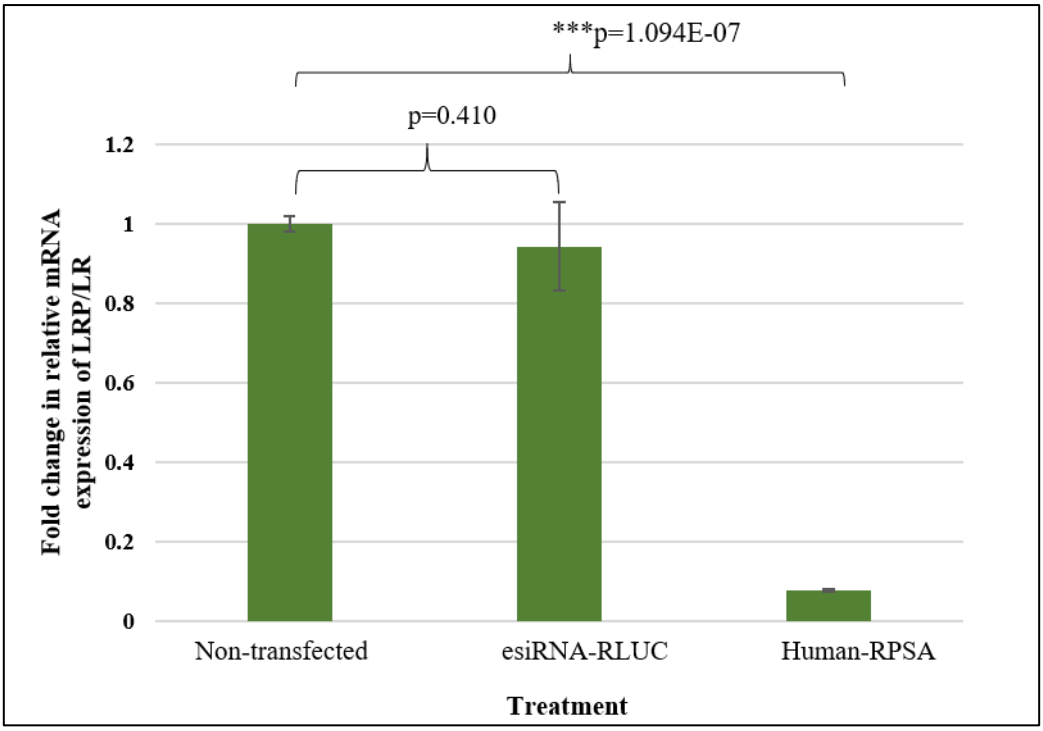


Figure 23: Relative mRNA expression levels of LRP/LR in late (DLD-1) stage colorectal cancer cells. Upon transfection with the Human-RPSA siRNA, relative LRP/LR mRNA expression levels decreased by 0.9-fold, when compared to non-transfected cells, confirming that the siRNA successfully down-regulates LRP/LR. esiRNA-RLUC served as the negative control. This data represents three biological replicates with two technical repeats with newly transfected samples each time. Error bars represent standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001, significant: The One-way ANOVA was significant, and a Bonferroni corrected post-hoc *t*-test indicated that the Human-RPSA samples are significantly lower than the non-transfected and esiRNA-RLUC samples.

4.1.2. siRNA-mediated knock-down of LRP expression results in significantly reduced viability of early and late stage colorectal cancer cells.

Once LRP expression was successfully down-regulated using siRNA technology, its effect on the viability of both cell lines were observed. MTT assays were performed which showed that when SW-480 and DLD-1 cells were transfected with the Human-RPSA siRNA, cell viability was significantly decreased in contrast to cells that were not transfected, indicating that siRNA-mediated knock-down of the receptor leads to reductions in cell viability in both early and late stage colorectal cancer cell lines. The MTT assays revealed that post-transfection with the Human-RPSA siRNA resulted in 60% and 55% decreases in SW-480 and DLD-1 cellular viability, respectively, in comparison to the cells that were not transfected (Fig. 24). Additionally, transfecting SW-480 and DLD-1 cells with the negative control siRNA, esiRNA-RLUC, caused no notable change in cell viability in comparison to cells that were not

transfected (Fig. 24). Protocatechuic Acid (PCA), a well-known apoptotic inducer was used as a positive control.

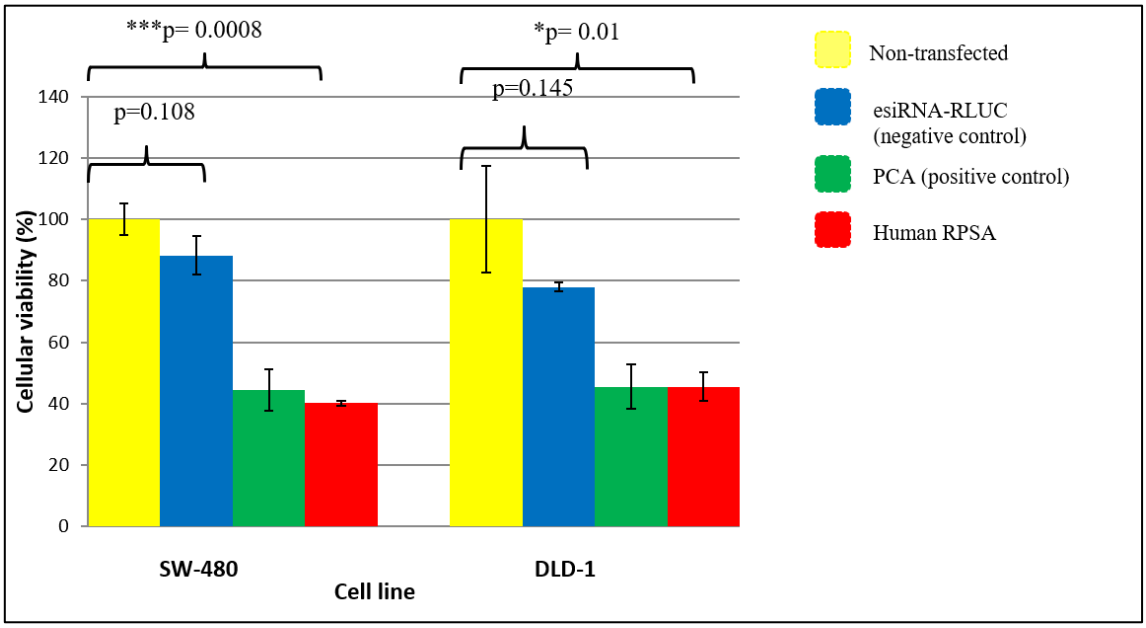


Figure 24: The effect of siRNA-mediated knock-down of LRP on the cellular viability of early (SW-480) and late (DLD-1) stage colorectal cancer cells. MTT assays were performed to evaluate the effect on cellular viability post transfection with the Human-RPSA siRNA. It was found that when SW-480 and DLD-1 cells were transfected with the Human-RPSA siRNA, cell viability was significantly decreased by 60% and 55%, respectively, in contrast to cells that were not transfected. Cells transfected with the negative control siRNA-RLUC showed no significant difference in cellular viability when compared to the non-transfected cells of both cell lines. PCA was used as a positive control and the value for the non-transfected cells was set to 100% for both cell lines. The graph is representative of an average of experiments performed in triplicate. Error bars represent standard deviation.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significant: The One-way ANOVA was significant and a Bonferroni corrected post-hoc *t*-test indicated that the Human-RPSA samples are significantly lower than the non-transfected and esiRNA-RLUC samples (Adapted from Vania et al., 2018 [204]).

4.1.3. The use of an alternative siRNA confirms that LRP down-regulation is responsible for reductions in cellular viability in both early and late stage colorectal cancer cells.

To determine whether the reduction in LRP expression and cellular viability had resulted due to Human-siRNA-mediated LRP knock-down and not just an off-target effect, an alternative siRNA, MISSION® esiRNA-RPSA, that targets another region of LRP was utilized (see Appendix A for siRNA target sequence). When comparing the western blot analysis and densitometry to the non-transfected cells, both early (SW-480) and late (DLD-1) stage

colorectal cancer cells transfected with esiRNA-RPSA, displayed a knock-down of 72% and 61% in LRP expression, respectively (Fig. 25). SW-480 and DLD-1 cells transfected with the esiRNA-RLUC showed no significant LRP knock-down when comparing them to non-transfected cells.

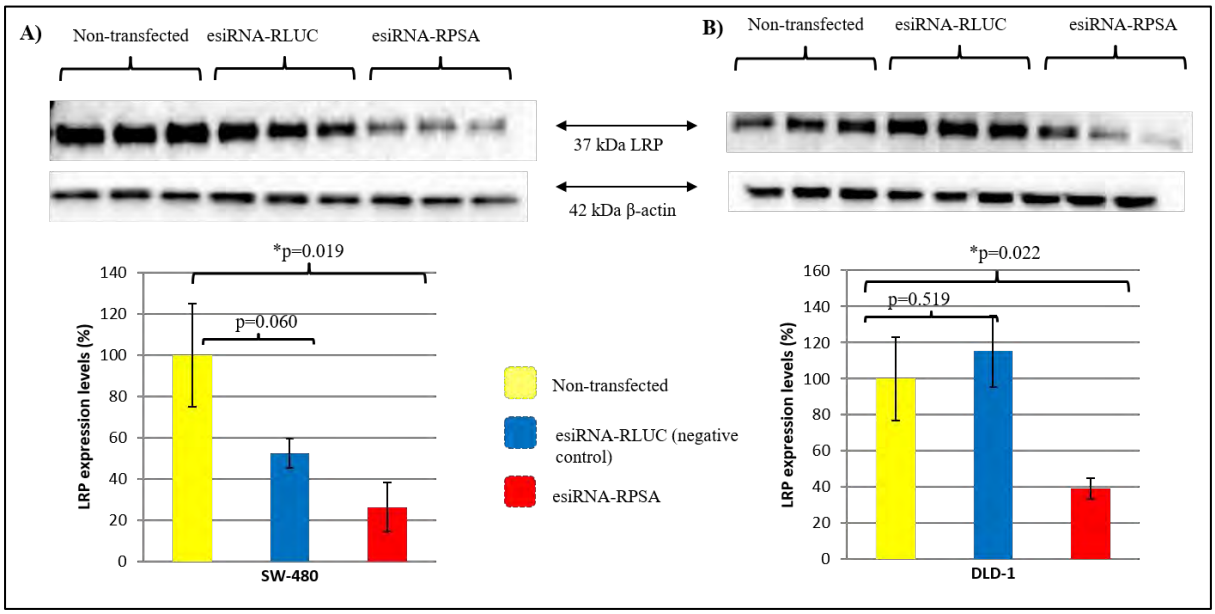


Figure 25: Detection of LRP expression in early (SW-480) and late (DLD-1) colorectal cancer cells after transfection with alternative esiRNA-RPSA siRNA. Upon transfection of SW-480 (A) and DLD-1 (B) cells with the esiRNA-RPSA siRNA, significant 72% and 61% decreases in LRP expression levels was revealed, respectively, in contrast to cells that were not transfected. Densitometric analysis of LRP levels was performed where levels of the non-transfected cells were set to 100%. β-actin was used as the loading control and the graphs are representative of an average of experiments performed in triplicate. Error bars represent standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001, significant: The One-way ANOVA was significant and a Bonferroni corrected post-hoc *t*-test indicated that the esiRNA-RPSA samples are significantly lower than the non-transfected and esiRNA-RLUC samples (Adapted from Vania et al., (2018) [204]).

To evaluate the effect of esiRNA-RPSA-mediated LRP knock-down on the cellular viability of SW-480 and DLD-1 cells, MTT assays were completed. When compared to the non-transfected cells, it was found that there were significant decreases of 44% and 89% in SW-480 and DLD-1 cells, respectively, upon transfection with the alternative esiRNA-RPSA siRNA (Fig. 26). Furthermore, both cell lines showed no significant effect on cell viability when transfected with the negative control esiRNA-RLUC siRNA, when compared to non-transfected cells (Fig. 26). From the results obtained, it can be seen that both siRNAs targeted to LRP/LR, Human-RPSA and esiRNA-RPSA, display similar effects on cellular viability in

SW-480 and DLD-1 colorectal cancer cells. Thus, this confirms that down-regulation of LRP/LR does indeed decrease cellular viability and is not an off-target effect. All subsequent experiments were performed with the Human-RPSA siRNA with negative control esiRNA-RLUC.

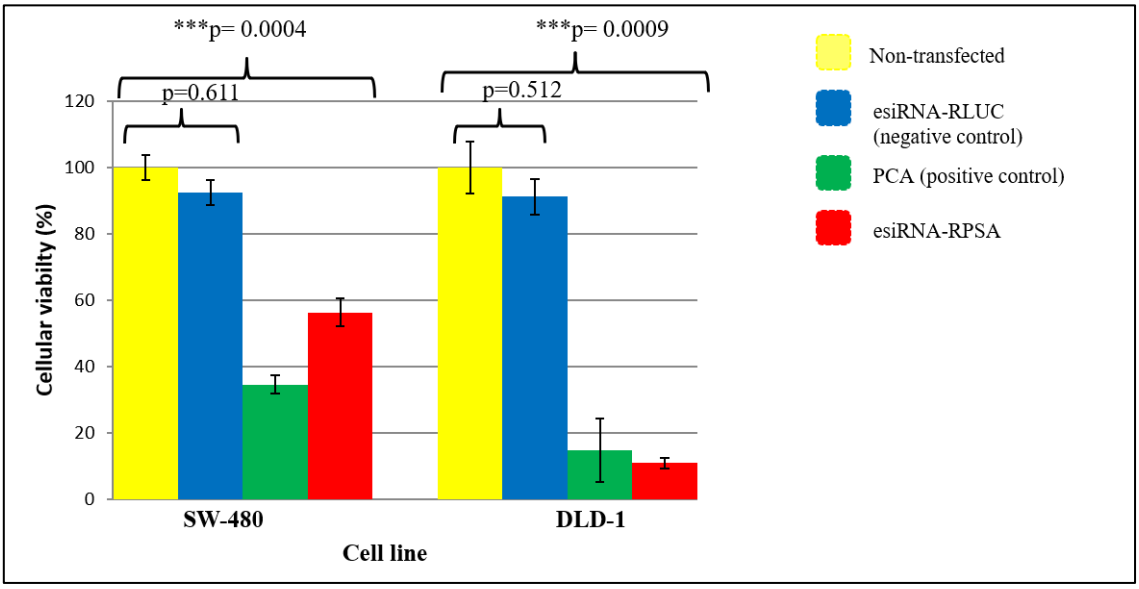


Figure 26: The effect of esiRNA-RPSA-mediated LRP knock-down on the cellular viability of early (SW-480) and late (DLD-1) stage colorectal cancer cells. Upon transfection of SW-480 and DLD-1 cells with esiRNA-RPSA, MTT assays were performed to evaluate the effect on cellular viability. When compared to non-transfected cells, SW-480 and DLD-1 cells showed significant decreases of 44% and 89%, respectively, in cellular viability, when transfected with esiRNA-RPSA. In addition, non-significant differences were observed in the viability of cells upon transfection with the negative control esiRNA-RLUC siRNA in comparison to the non-transfected cells for both cell lines (set to 100%). PCA was used as the positive control and the graph is representative of an average of three repeats. The graph is representative of an average of experiments performed in triplicate. Error bars represent standard deviation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significant: The One-way ANOVA was significant and a Bonferroni corrected post-hoc *t*-test indicated that the esiRNA-RPSA samples are significantly lower than the non-transfected and esiRNA-RLUC samples (Adapted from Vania, et al., 2018 [204]).

4.1.4. Knock-down of LRP expression via siRNA technology leads to changes in nuclear morphology signifying apoptosis in early and late stage colorectal carcinoma cells.

To determine whether the decreases in cell viability after transfection with the Human-RPSA siRNA was caused by apoptotic induction, nuclear morphological changes were studied by confocal microscopy and Airyscan™. Human-RPSA-transfected early (SW-480) stage

colorectal cancer cells exhibited nuclear morphological changes in the form of condensed nuclei and reduced nuclear size (Fig. 27D), when compared to the nuclei of cells that were not transfected (Fig. 27A). Late (DLD-1) stage colorectal cancer cells transfected with Human-RPSA presented nuclear morphological changes such as cellular fragmentation into membrane-bound bodies, weakened membrane integrity and membrane blebbing (Fig. 28D), in contrast to nuclei of cells that were not transfected (Fig.28A). Moreover, membrane blebbing confirmed bright field microscopy data obtained in Figure 21. Results obtained for Human-RPSA-transfected cells were consistent with those of the positive control, PCA (Fig. 28C). In addition, upon transfection with esiRNA-RLUC, both cell lines did not reveal any morphological changes in nuclei, when comparing them to cells that were not transfected (Fig. 28B).

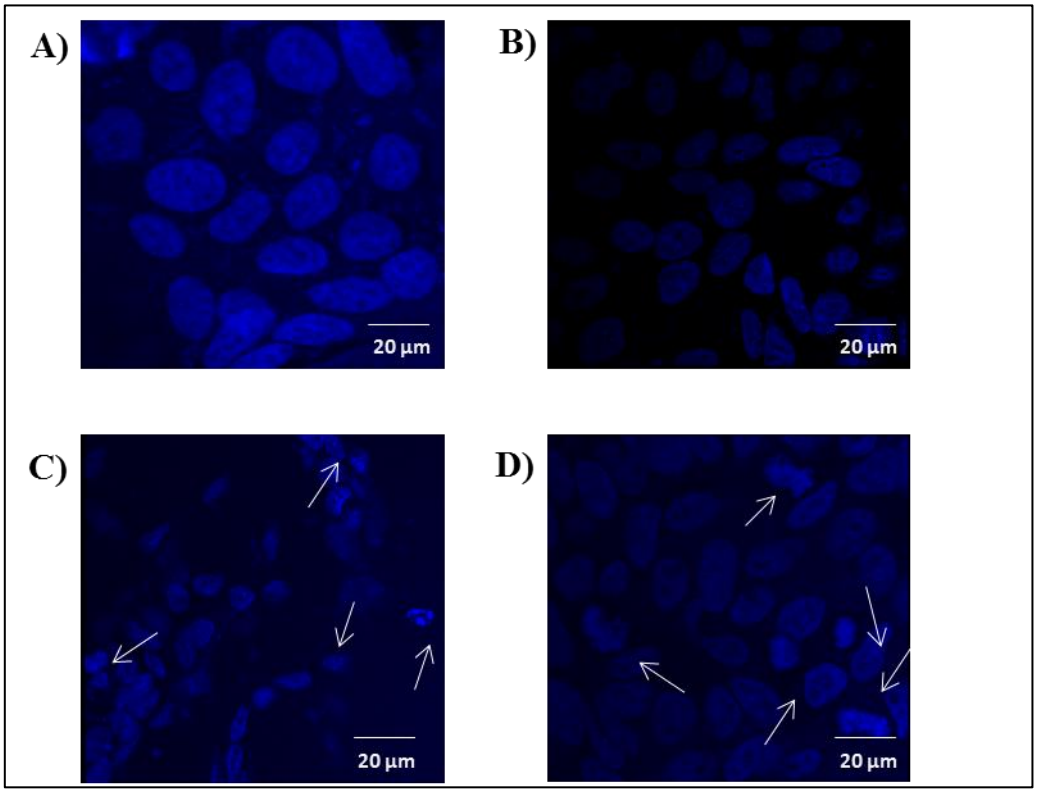


Figure 27: The effect of siRNA-mediated knock-down of LRP on nuclear morphology of early (SW-480) stage colorectal cancer cells. Confocal microscopy with Airyscan analysis was completed to investigate changes in nuclear morphology upon siRNA transfection. **A)** Non-transfected SW-480 cells displayed large nuclei with healthy membrane integrity. **B)** SW-480 cells transfected with negative control esiRNA-RLUC showed parallel characteristics to the non-transfected cells with no changes in nuclear morphology. **C)** SW-480 cells treated with positive control PCA revealed nuclei that underwent apoptotic body formation (indicated by arrows). **D)** SW-480 cells transfected with Human-RPSA siRNA exhibited nuclear shrinkage

and condensed nuclei, proposing induction of apoptosis (indicated by arrows). Images were obtained at a 630X magnification and Airyscan analysis was applied to each image. Scale bars are indicative of 20 µm. (Adapted from Vania et al., 2018 [204]).

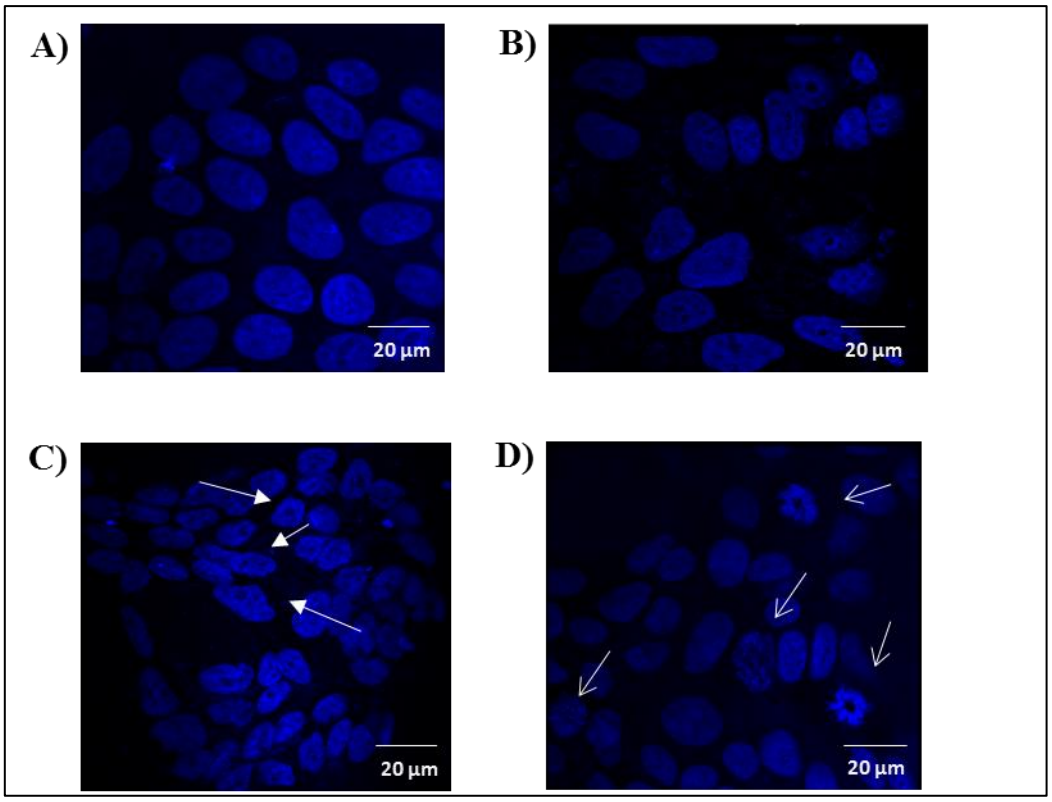


Figure 28: The effect of siRNA-mediated knock-down of LRP on nuclear morphology of late (DLD-1) stage colorectal cancer cells. Confocal microscopy with Airyscan analysis was completed to investigate changes in nuclear morphology upon siRNA transfection. **A)** Non-transfected DLD-1 cells displayed large nuclei with healthy membrane integrity. **B)** DLD-1 cells transfected with negative control esiRNA-RLUC showed similar characteristics to the non-transfected cells with no changes in nuclear morphology. **C)** DLD-1 cells treated with PCA displayed weakened membrane integrity and condensed nuclei. **D**) DLD-1 cells transfected with Human-RPSA showed formation of apoptotic bodies and weakened membrane integrity. Images were obtained at a 630X magnification and Airyscan analysis was applied to each image. Scale bars are indicative of 20 µm. (Adapted from Vania et al., 2018 [204]).

4.1.5. siRNA-mediated knockdown of LRP expression induces apoptosis in early and late stage colorectal cancer cells.

Due to confocal microscopy proposing that the knock-down of LRP expression in early (SW-480) and late (DLD-1) stage colorectal cancer cells leads to morphological changes of the nuclei (a key feature of cells undergoing apoptosis), Annexin-V/PI assays were performed to confirm this result quantitatively.

SW-480 cells transfected with Human-RPSA revealed 36.6% of cells undergoing early apoptosis, while 44.1% of cells underwent late apoptosis (Fig. 29D) thus, a total of 80.7% of cells underwent apoptosis in contrast to cells that were not transfected (Fig. 29A). Transfection of DLD-1 cells with Human-RPSA resulted in 10.0% of cells undergoing early apoptosis while 74.3% of cells underwent late apoptosis (Fig. 30D) thus, a total of 84.3% of cells underwent apoptosis, compared to cells that were not transfected (Fig. 30A) Additionally, upon transfection with esiRNA-RLUC, SW-480 and DLD-1 cell lines did not undergo apoptosis (Fig. 29B and 30B). Treatment of cells with PCA, as positive control, induced late apoptosis (Fig. 29C and 30C). Further statistical analysis confirmed a significant increase in apoptotic cells when transfected with Human-RPSA, in contrast to non-transfected cells in both cell lines (Fig.31).

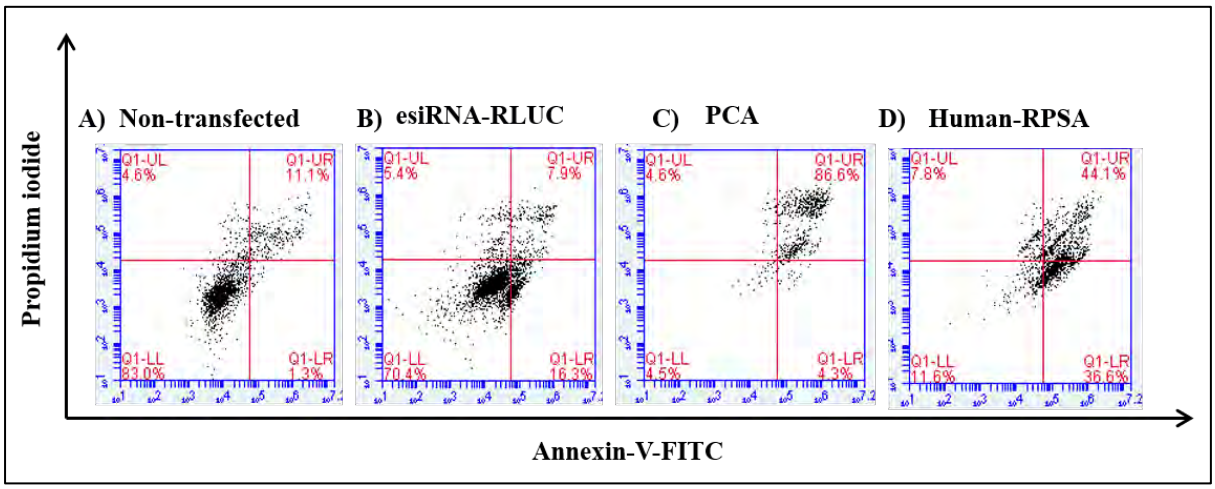


Figure 29: Apoptotic induction in early (SW-480) stage colorectal cancer cells post siRNA transfection. It was revealed that most of the non-transfected SW-480 cells fell in the lower quadrant (Q1-LL), known to represent normal live cells. SW-480 cells transfected with negative control esiRNA-RLUC mostly appeared in the lower quadrant indicating live cells. Positive control PCA, revealed that most cells were found in the upper right quadrant (Q1-UR), representing SW-480 cells undergoing late apoptosis. Cells transfected with Human-RPSA resulted in 36.6% of SW-480 cells undergoing early apoptosis, which is depicted in the lower right quadrant (Q1-LR); while 44.1% of cells underwent late apoptosis. This indicates that upon transfection with Human-RPSA, a total of 80.7% of SW-480 cells underwent apoptosis. The upper left quadrant (Q1-UL) represents cells undergoing necrosis. 20 000 cells were counted per sample. (Adapted from Vania et al., 2018 [204]).

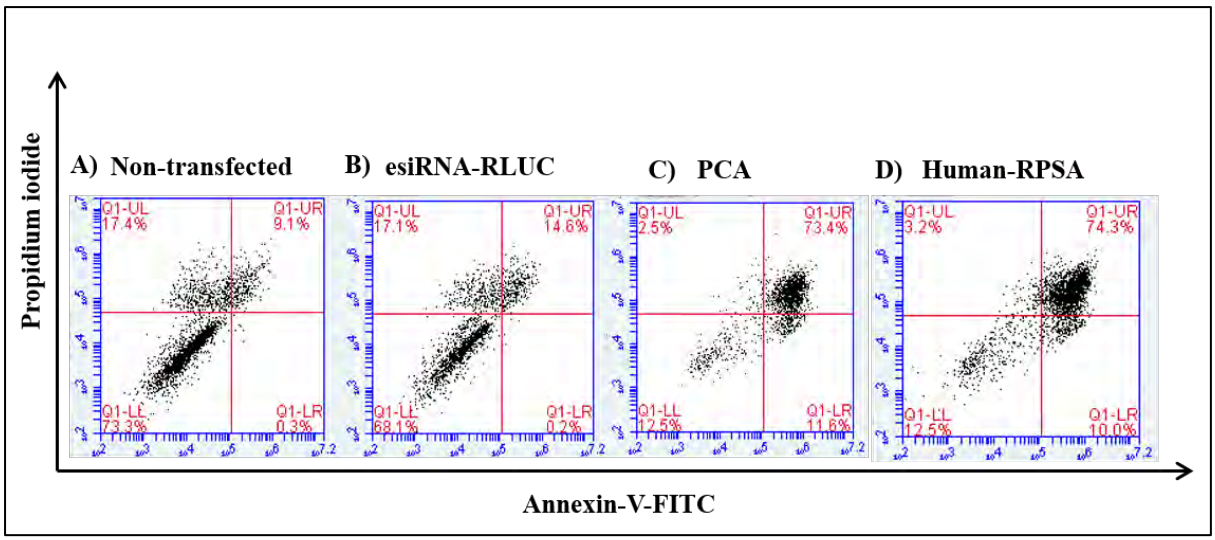


Figure 30: Apoptotic induction in late (DLD-1) stage colorectal cancer cells post siRNA transfection. It was revealed that most of the non-transfected DLD-1 cells fell in the lower quadrant (Q1-LL) known to represent normal live cells. DLD-1 cells transfected with negative control esiRNA-RLUC mostly appeared in the lower quadrant indicating live cells. Positive control PCA, revealed that most cells were found in the upper right quadrant (Q1-UR), representing DLD-1 cells undergoing late apoptosis. Cells transfected with Human-RPSA resulted in 10% of DLD-1 cells undergoing early apoptosis, which is depicted in the lower right quadrant (Q1-LR); 74.3% of DLD-1 cells underwent late apoptosis. This indicates that upon transfection with Human-RPSA, a total of 84.3% DLD-1 cells underwent apoptosis. (Adapted from Vania et al., 2018 [204]).

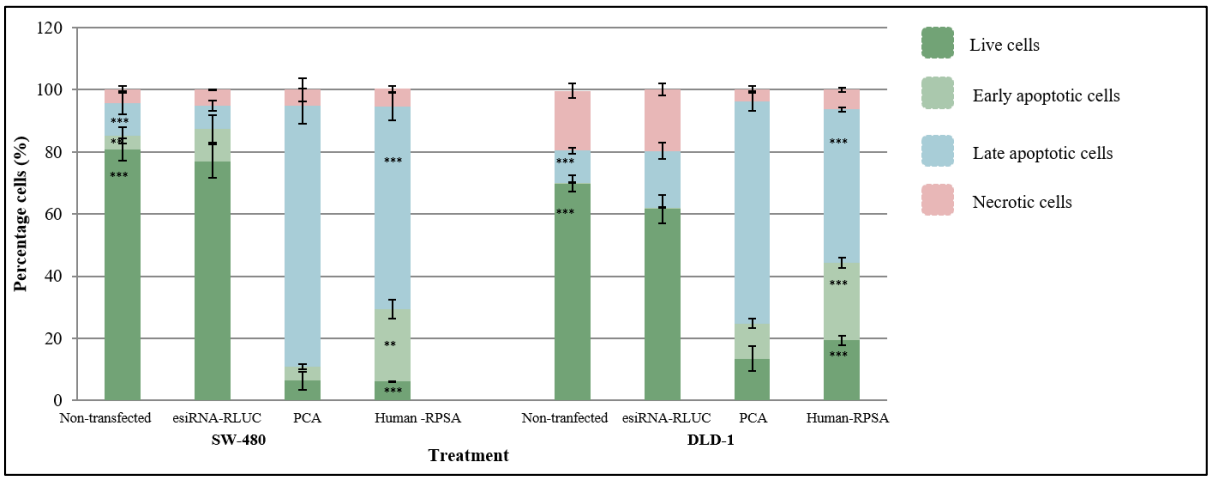


Figure 31: Bar graph illustrating apoptotic induction in early (SW-480) and late (DLD-1) colorectal cancer cells after siRNA transfection. This graph displays an average of three experiments completed in triplicate. Percentages for each quadrant were pooled together and compared to one another for both cell lines. It was found that SW-480 and DLD-1 cells had a significant increase in early and late apoptosis when transfected with Human-RPSA, in contrast to cells that were not transfected, and both cell lines were seen to undergo more late apoptosis than early apoptosis. SW-480: ***p=8.92029E-06 (live), ***p= 0.0001 (early apoptosis), **p= 0.002 (late apoptosis); DLD-1: ***p= 1.97127E-05 (live), ***p=3.72195E-05 (early apoptosis), ***p= 1.08522E-06 (late apoptosis). Error bars represent standard deviation. *p <

0.05, **p < 0.01, ***p < 0.001; One-way ANOVA and two-tailed student's *t*-test with Bonferroni post hoc analysis. (Adapted from Vania et al., 2018 [204]).

4.1.6. siRNA-mediated knock-down of LRP expression causes a notable increase in caspase-3 activity.

For additional validation that apoptosis was indeed occurring in early (SW-480) and late (DLD-1) stage colorectal cancer cells once LRP has been down-regulated, caspase-3 activity assays were completed. Post transfection with Human-RPSA, SW-480 cells were found to have a significant 4-fold increase in caspase-3 activity, in comparison to cells that were not transfected (Fig. 32). DLD-1 cells treated with Human-RPSA displayed a 5-fold increase in caspase-3 activity in contrast to non-transfected cells, which were set to 100% (Fig. 32). Furthermore, no differences in caspase-3 activity was seen when both cell lines were transfected with esiRNA-RLUC (Fig. 32). Treatment with PCA, as positive control, caused a 3-fold and 5-fold increase in caspase-3 activity in SW-480 and DLD-1 cells, respectively (Fig. 32).

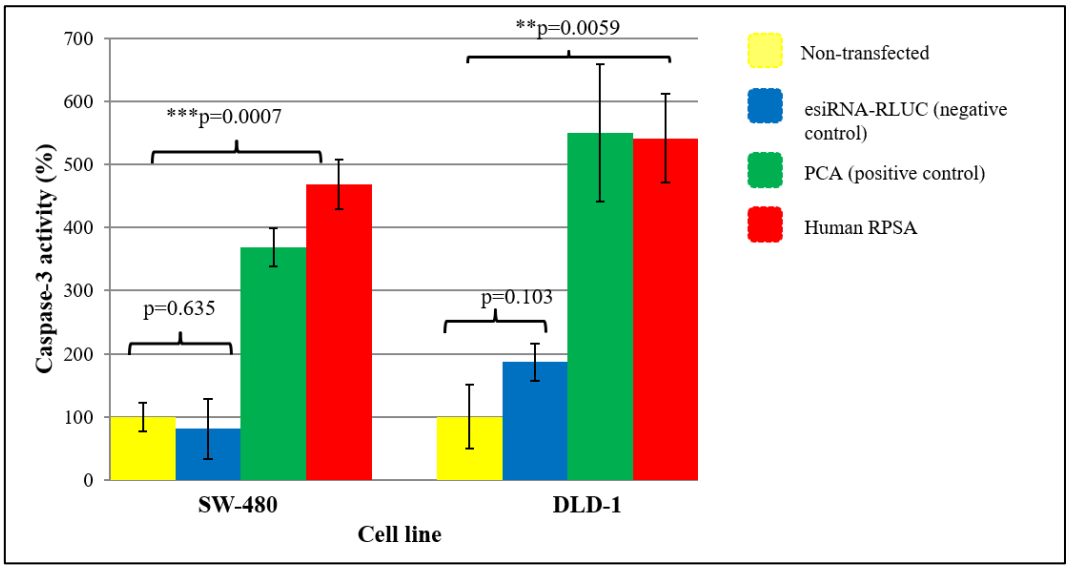


Figure 32: The effect of siRNA-mediated LRP knock-down on caspase-3 activity in early (SW-480) and late (DLD-1) stage colorectal cancer cells. Upon transfection of SW-480 and DLD-1 cells with Human-RPSA, a significant 4-fold and 5-fold increase in caspase-3 activity was revealed, respectively, in comparison to cells that were not transfected (set to 100%). Both cell lines showed no significant difference in caspase-3 activity between cells transfected with negative control esiRNA-RLUC and non-transfected cells. PCA was used as a positive control. The graph is indicative of an average of experiments performed in triplicate. Error bars represent standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001, significant: The One-way ANOVA was significant and a Bonferroni corrected post-hoc *t*-test indicated that the Human-RPSA samples are significantly higher than the non-transfected and esiRNA-RLUC samples (Adapted from Vania et al., 2018 [204]).

4.1.7. siRNA-mediated knock-down of LRP results in significant increases in caspase-8 and caspase-9 activity in early and late stage colorectal cancer cells.

Caspase-3 activation occurs through both apoptosis pathways (intrinsic and extrinsic) hence, further insight of how the receptor aids in tumourigenic cell survival was required; therefore caspase-8 and -9 activity assays were performed. These assays determined whether transfection with Human-RPSA leads to the activation of the extrinsic pathway (facilitated through caspase-8) or the intrinsic pathway (facilitated through caspase-9) in each of the cell lines. SW-480 and DLD-1 cells transfected with Human-RPSA were found to undergo a 4-fold increase in caspase-8 activity, in comparison to cells that were not transfected (which were set to 100%) (Fig. 33). Moreover, SW-480 cells and DLD-1 cells indicated a significant 7-fold and 4-fold increase in caspase-9 activity, respectively, in comparison to cells that were not transfected (Fig. 34). In addition, both cell lines transfected with esiRNA-RLUC displayed no differences in caspase-8 and -9 activity in comparison to cells that were not transfected.

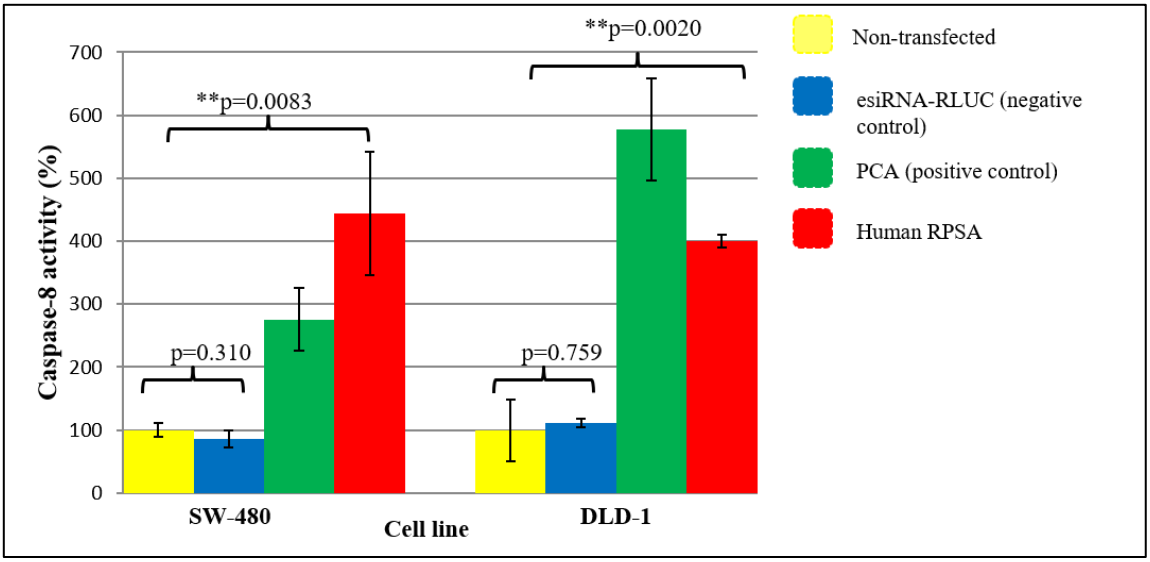


Figure 33: The effect of siRNA-mediated LRP knock-down on caspase-8 activity in early (SW-480) and late (DLD-1) stage colorectal cancer cells. Upon transfection of SW-480 and DLD-1 cells with Human-RPSA, a 7-fold significant increase and 4-fold in caspase-9 activity, respectively, compared to cells that were not transfected (set to 100%). Both cell lines showed no significant difference in caspase-9 activity between cells transfected with the esiRNA-RLUC and non-transfected cells. PCA was used as a positive control. The graph is indicative of an average of experiments performed in triplicate. Error bars represent standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001, significant: The One-way ANOVA was significant and a Bonferroni corrected post-hoc *t*-test indicated that the Human-RPSA samples are significantly higher than the non-transfected and esiRNA-RLUC samples (Adapted from Vania et al. , 2018 [204]).

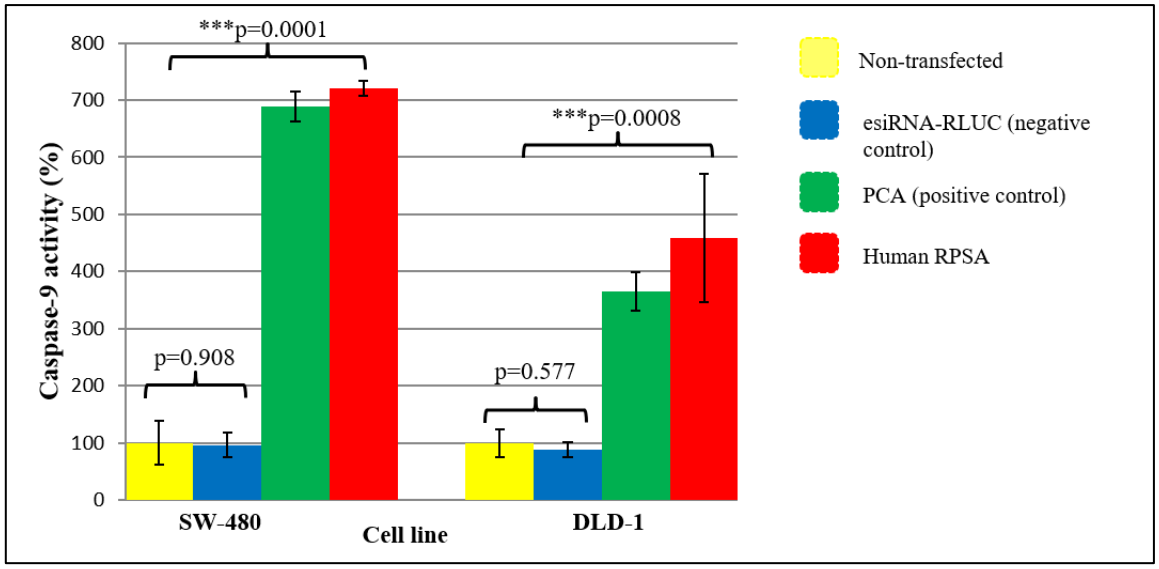


Figure 34: The effect of siRNA-mediated LRP knock-down on caspase-9 activity in early (SW-480) and late (DLD-1) stage colorectal cancer cells. Upon transfection of SW-480 and DLD-1 cells with Human-RPSA, both cell lines displayed a 4-fold significant increase in caspase-8 activity, compared to cells that were not transfected (set to 100%). Both cell lines showed no significant difference in caspase-8 activity between cells transfected with the esiRNA-RLUC and non-transfected cells. PCA was used as a positive control. The graph is indicative of an average of experiments performed in triplicate. Error bars represent standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001, significant: The One-way ANOVA was significant and a Bonferroni corrected post-hoc *t*-test indicated that the Human-RPSA samples are significantly higher than the non-transfected and esiRNA-RLUC samples (Adapted from Vania et al., 2018 [204]).

4.1.8. siRNA-mediated knock-down of LRP/LR expression causes apoptotic cell cycle arrest in late stage colorectal cancer cells.

Cell cycle analysis is made by measuring the DNA in cells stained with a DNA specific dye known as propidium iodide (PI). The fluorescence intensity of the stained cells at specific wavelengths therefore correlates to the amount of DNA the cells possess. In order to obtain insight into the cell cycle once cells were transfected with the Human-RPSA siRNA, DLD-1 cells were transfected for a duration of 3 days as well as 5 days.

Upon Human-RPSA transfection of DLD-1 cells for 3-days, there was a significant 15% increase in the number of dead cells in the sub G0/G1 stage (apoptotic stage) and 17.4% decrease in G0/G1 phase, indicating the occurrence of apoptosis (Fig. 35). Although this result was significant, it was not a substantial increase. It is known that apoptosis is a time-dependent process where the occurrence of apoptotic biochemical alterations to cells may depend on several internal cellular factors, where some alterations may take longer than others to show an effect [205]. Thus, it was decided to extend the transfection period by 48 hours, for a total of 5 days to ensure that the period of apoptotic activity was not missed. Upon transfection of DLD-

1 cells with Human-RPSA for 5 days, there was a significant 41% increase in the number of dead cells in the sub G0/G1 stage (apoptotic stage) with a 43% decrease in G0/G1 phase, indicating a higher percentage of cells undergoing apoptosis (Fig. 36). esiRNA-RLUC and PCA acted as the negative and positive controls, respectively.

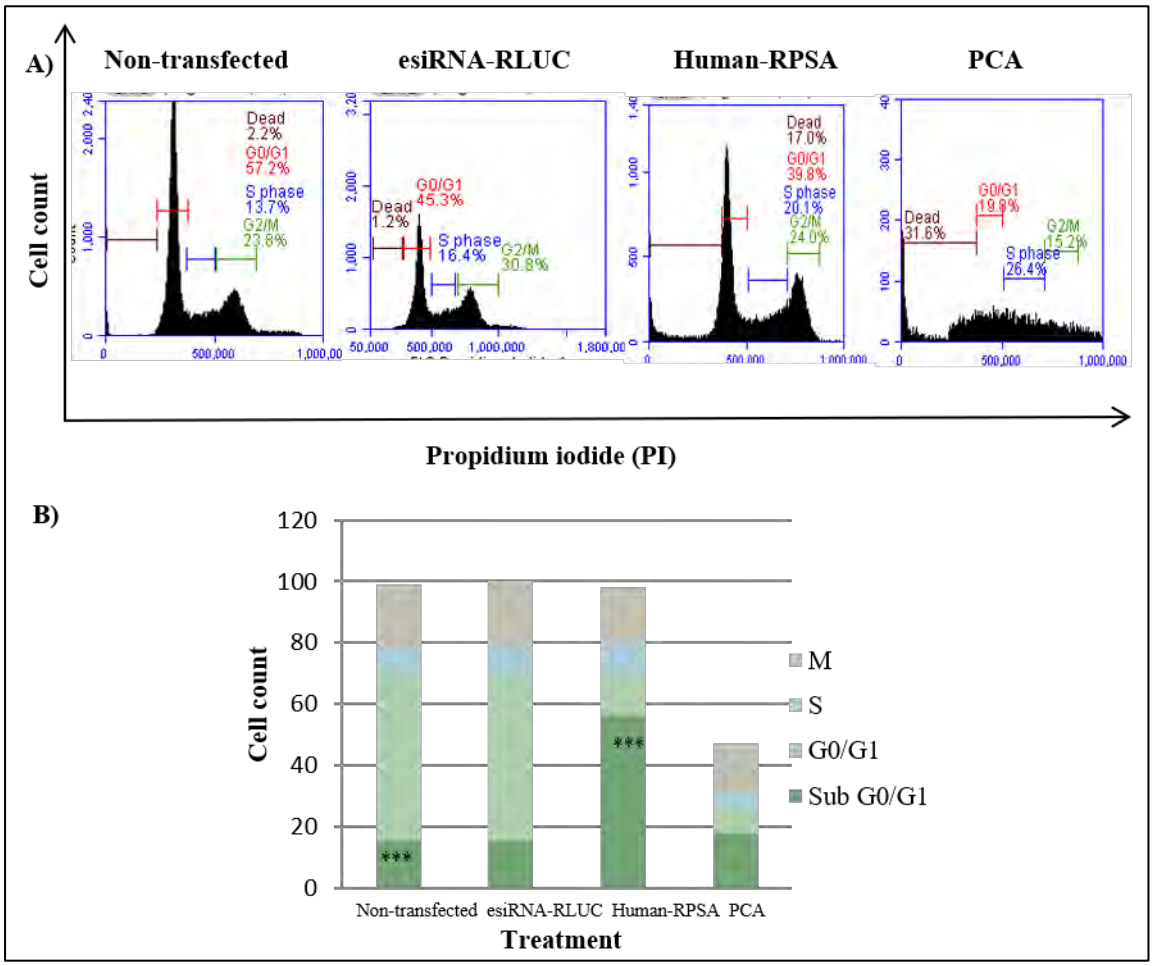


Figure 35: Cell cycle analysis on late (DLD-1) stage colorectal cancer cells after a 3-day transfection with Human-RPSA. (A) Non-transfected cells can be seen to have most cells in the G0/G1 stage of the cell cycle with only 2.2% cells being dead. Upon transfection with Human-RPSA siRNA, there is a 15% increase in the number of dead cells in the sub G0/G1 stage (apoptotic stage) and a 17.4% decrease in G0/G1 phase, indicating the occurrence of apoptosis. esiRNA-RLUC and PCA acted as the negative and positive controls, respectively. **(B)** It can be seen that there is a significant increase in the sub G0/G1 phase in cells transfected with Human-RPSA. ***p=0.0008, this data represents three biological replicates which were completed in triplicate. Error bars represent standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001; One-way ANOVA and two-tailed students *t*-test with Bonferroni post hoc analysis.

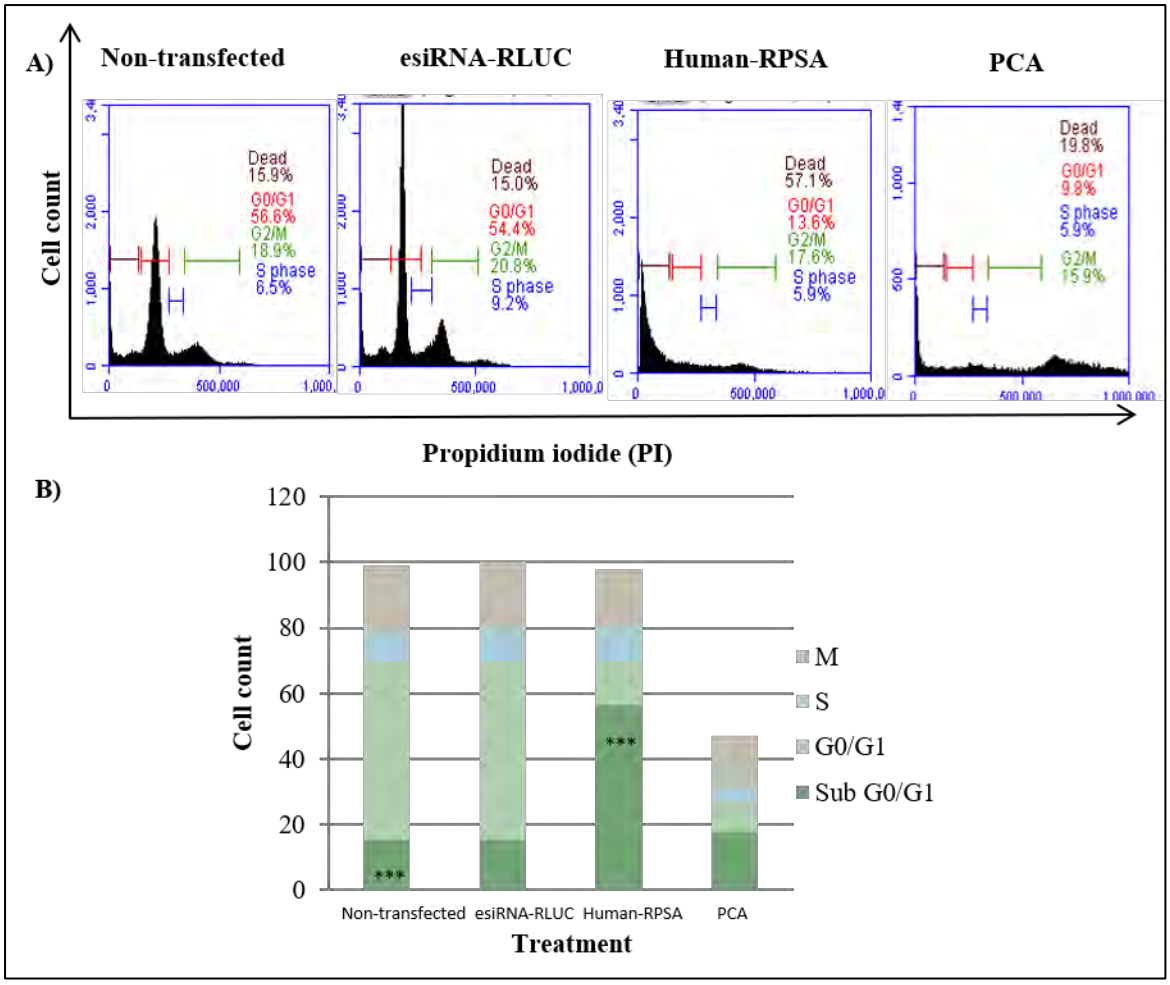


Figure 36: Cell cycle analysis on late (DLD-1) stage colorectal cancer cells after a 5-day transfection with Human-RPSA. (A) Non-transfected cells can be seen to have most cells in the G0/G1 stage of the cell cycle with only 15.9% cells being dead. Upon transfection with Human-RPSA siRNA, there is a 41% increase in the number of dead cells in the sub G0/G1 stage (apoptotic stage) while there was a 43% decrease in G0/G1 phase, indicating the occurrence of apoptosis. esiRNA-RLUC and PCA acted as the negative and positive controls, respectively. **(B)** It can be seen that there is a significant increase in the sub G0/G1 phase in cells transfected with Human-RPSA. ***p=0.0003, this data represents three biological replicates which were completed in triplicate. Error bars represent standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001; One-way ANOVA and two-tailed students *t*-test with Bonferroni post hoc analysis.

4.1.9. siRNA-mediated knock-down significantly affects telomere-related proteins in late stage colorectal cancer cells.

As mentioned above, a study showed that there is increased telomerase activity in approximately 90% of malignant human tumours [206]. Moreover, an increased level of hTERT, the catalytic subunit of telomerase, is found to increase telomerase activity and as a result, enhance tumourigenesis [122]. Additionally, it has also been shown that telomere dysfunction and elevated levels of telomerase activity are associated with cancer recurrence

and chemotherapeutic resistance [172], outlining the important role that telomerase has in cancer progression. More particularly to this study, it has been found that LRP/LR and telomerase co-localize in the perinuclear compartment of breast (MDA-MB-231) cancer cells, thereby mediating the telomerase activity in these cancer cells [122]. It has also recently been found that by down-regulating LRP/LR through siRNA technology, telomerase activity is significantly reduced in the breast cancer cells [122]. In addition, the phosphorylation of TERT, required for the activation of telomerase, has been found to correlate to increased telomerase activity levels, thereby aiding in cancer progression [207]. Taken together, these factors incited the question of whether siRNA-mediated knock-down of LRP/LR will influence hTERT and pTERT protein expression levels as well as telomerase activity in DLD-1 cells. It was found that once the DLD-1 cells were transfected with the Human-RPSA siRNA, hTERT relative mRNA and protein expression levels were significantly decreased by 0.5-fold (Fig. 37) and 0.4-fold (Fig. 38), respectively, when compared to non-transfected cells. It was also found siRNA-mediated knockdown of LRP/LR resulted in a significant 0.6-fold decrease in pTERT protein expression levels, when compared to non-transfected cells (Fig. 39). Moreover, DLD-1 cells transfected with the Human-RPSA siRNA resulted in a significant decrease of 0.8-fold in telomerase activity, when compared to non-transfected cells (Fig. 40). The esiRNA-RLUC was used as the negative control.

Furthermore, telomere-related proteins known as telomeric-repeat binding factor-1 (TRF-1) and Telomeric-repeat binding factor-2 (TRF-2) were also investigated. Specifically, TRF-1 and TRF-2 have been found to directly bind telomere repeats where they are responsible for protecting telomeres as well as regulating telomere length [166, 208]. Interestingly, it has been found that TRF-1 is over-expressed in some cancer types, however whether the increased expression contributes to cancer progression is still unclear [166]. On the other hand, TRF-2 has been found to be over-expressed in several cancer types, suggesting that TRF-2 may indeed play a role in tumour promotion [208]. Thus, this prompted investigations on how siRNA-mediated knock-down of LRP/LR would affect these telomere-related proteins. When DLD-1 cells were transfected with the Human-RPSA siRNA, it was found that the protein expression levels of TRF-2 were significantly decreased by 0.5-fold (Fig. 42), while TFR-1 was found to stay the same in comparison to non-transfected cells (Fig. 41). The esiRNA-RLUC was used as the negative control.

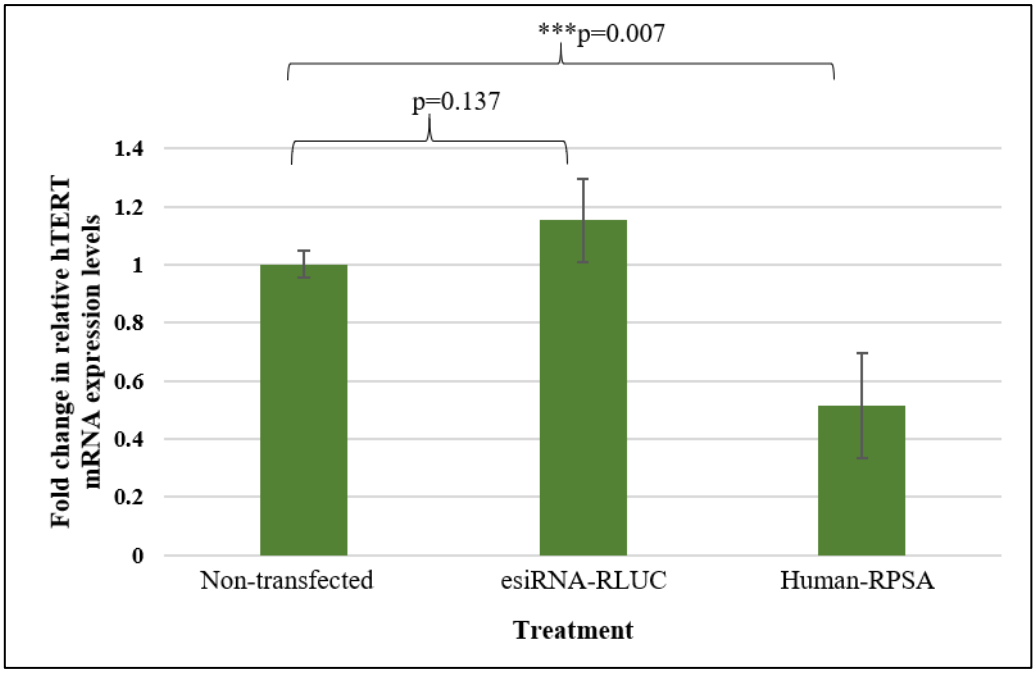


Figure 37: Relative mRNA expression levels of hTERT in late (DLD-1) colorectal cancer cells. Upon transfection with Human-RPSA, relative hTERT mRNA expression levels decreased by 0.5-fold, when compared to non-transfected cells. esiRNA-RLUC served as the negative control. This data represents three biological replicates with two technical repeats with newly transfected samples each time. Error bars represent standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001, significant: The One-way ANOVA was significant, and a Bonferroni corrected post-hoc *t*-test indicated that the Human-RPSA samples are significantly lower than the non-transfected and esiRNA-RLUC samples.

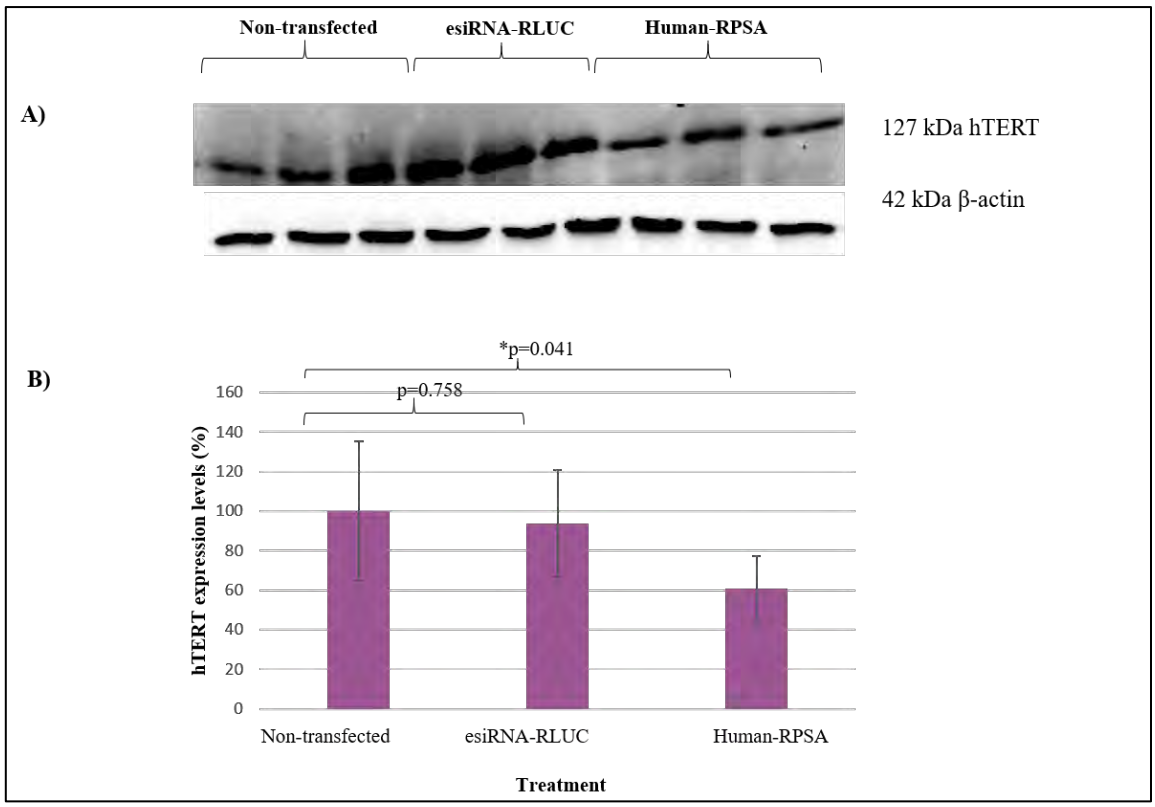


Figure 38: A) hTERT protein expression levels in late (DLD-1) stage colorectal cancer cells. Western blot showing a decrease in hTERT protein expression levels upon Human-RPSA transfection, in comparison to non-transfected cells. **B) Densitometric analysis of hTERT protein expression levels.** It can be seen that hTERT protein expression levels significantly decrease by 0.4-fold in Human-RPSA transfected cells, when compared to non-transfected cells which were set to 100%. β-actin was used as the loading control and the graphs are representative of an average of experiments performed in triplicate. Error bars represent standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001, significant: The One-way ANOVA was significant, and a Bonferroni corrected post-hoc *t*-test indicated that the Human-RPSA samples are significantly lower than the non-transfected and esiRNA-RLUC samples.

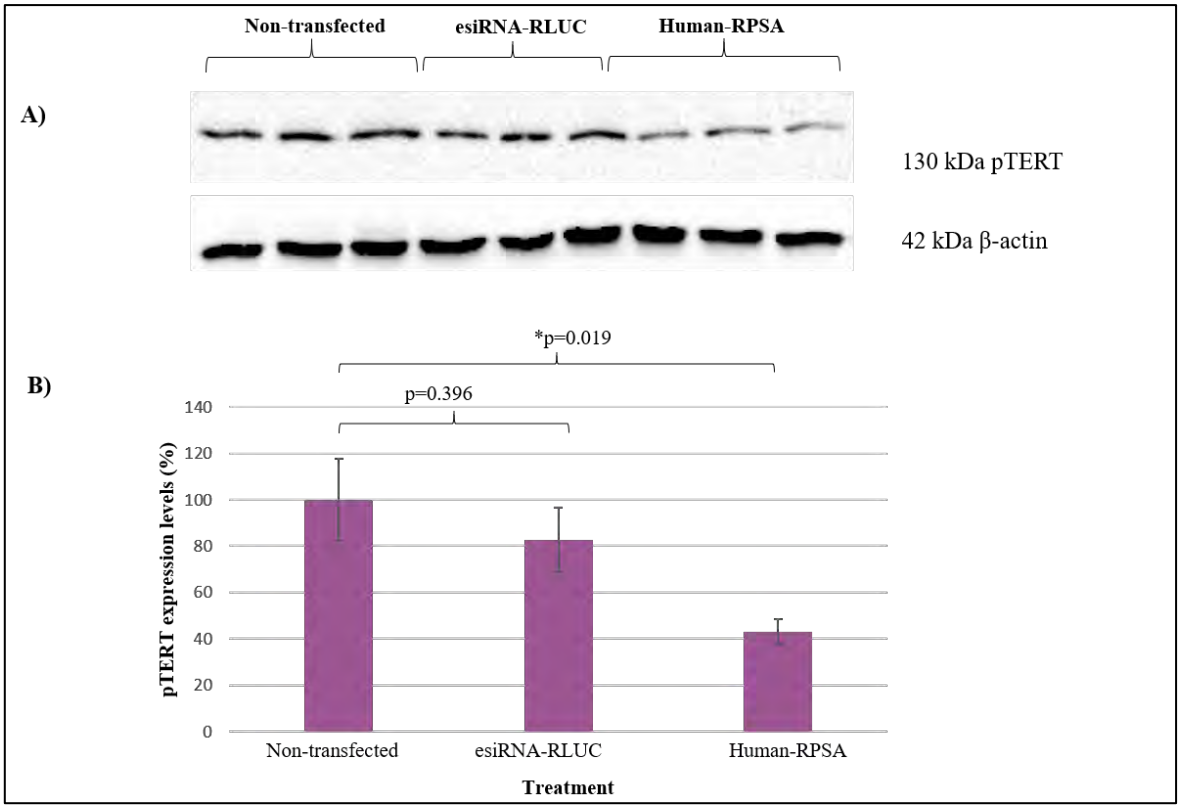


Figure 39: A) pTERT protein expression levels in late (DLD-1) stage colorectal cancer cells. Western blot showing a decrease in pTERT protein expression levels upon Human-RPSA transfection, in comparison to non-transfected cells. **B) Densitometric analysis of pTERT protein expression levels.** It can be seen that pTERT protein expression levels significantly decreases by 0.6-fold in Human-RPSA transfected cells, when compared to non-transfected cells which were set to 100%. β-actin was used as the loading control and the graphs are representative of an average of experiments performed in triplicate. Error bars represent standard deviation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significant: The One-way ANOVA was significant, and a Bonferroni corrected post-hoc *t*-test indicated that the Human-RPSA samples are significantly lower than the non-transfected and esiRNA-RLUC samples.

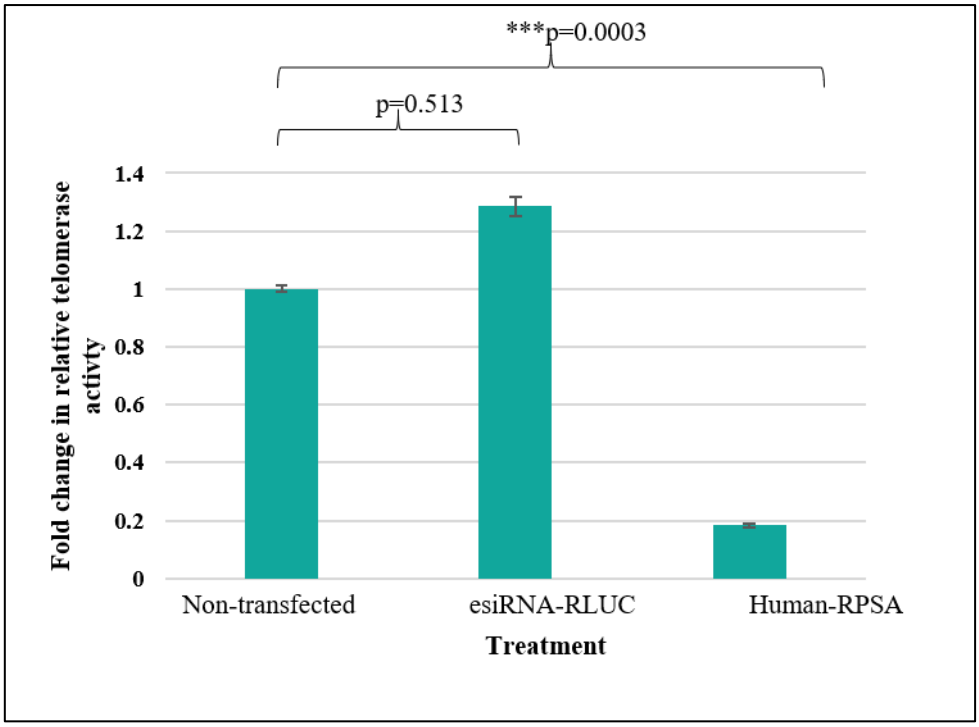


Figure 40: Relative telomerase activity in late (DLD-1) stage colorectal cancer cells. Upon Human-RPSA transfection, telomerase activity has significantly decreased by 0.8-fold. esiRNA-RLUC served as the negative control. This data represents three biological replicates with two technical repeats with newly transfected samples each time. Error bars represent standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001, significant: The One-way ANOVA was significant, and a Bonferroni corrected post-hoc *t*-test indicated that the Human-RPSA samples are significantly lower than the non-transfected and esiRNA-RLUC samples.

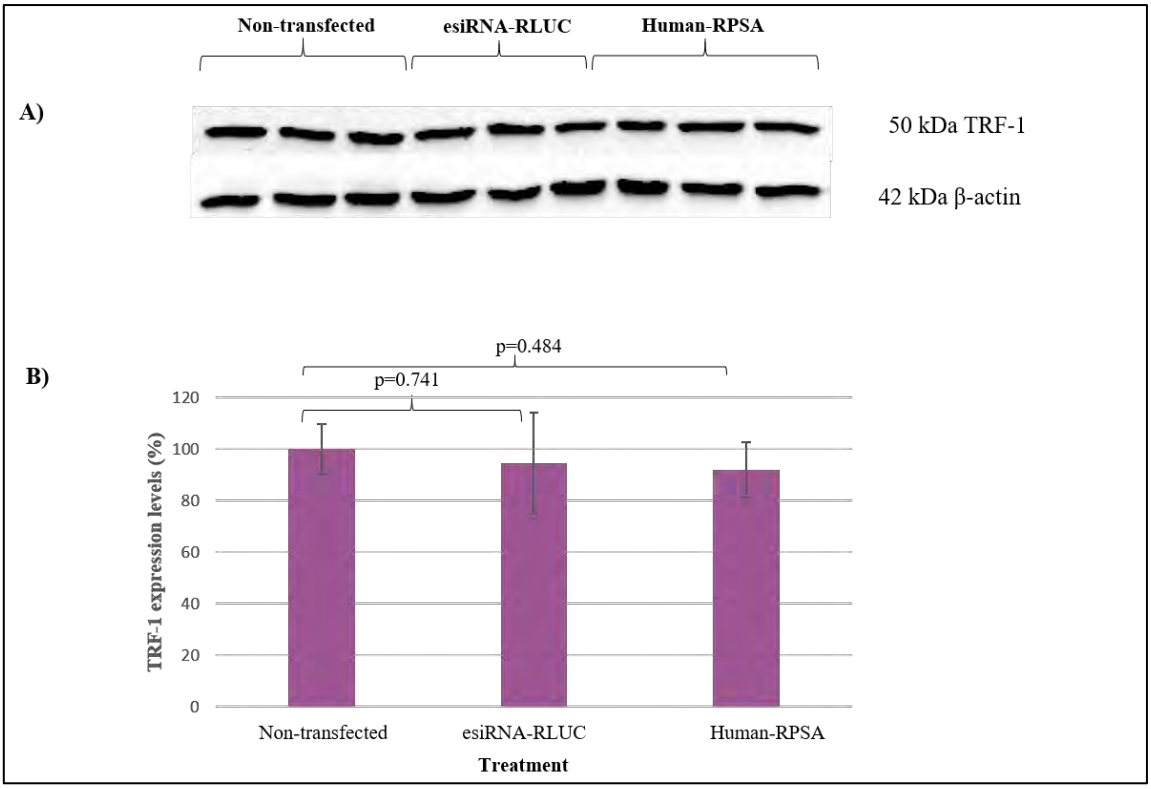


Figure 41: TRF-1 protein expression levels in late (DLD-1) stage colorectal cancer cells. Western blot shows no change in TRF-1 protein expression levels upon Human-RPSA transfection, when compared to non-transfected cells. This was confirmed through densitometric analysis where non-transfected cells were set to 100% and compared to Human-RPSA transfected cells. β-actin was used as the loading control and the graphs are representative of an average of experiments performed in triplicate. Error bars represent standard deviation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significant: The One-way ANOVA was not significant, and a Bonferroni corrected post-hoc *t*-test indicated that the Human-RPSA samples are not significantly different to the non-transfected and esiRNA-RLUC samples.

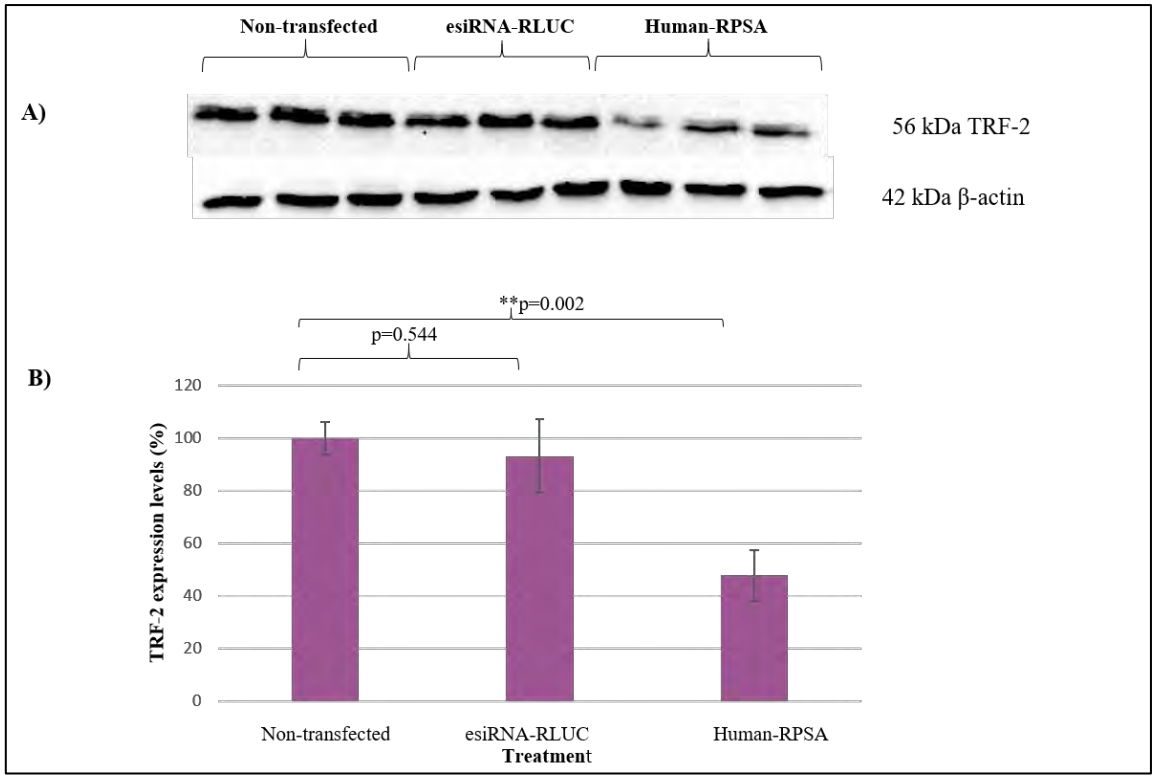


Figure 42: TRF-2 protein expression levels in late (DLD-1) stage colorectal cancer cells. (A) Western blot showing a decrease in TFR-2 protein expression levels upon Human-RPSA transfection, in comparison to non-transfected cells. **(B) Densitometric analysis of TRF-2 protein expression levels.** It can be seen that TRF-2 protein expression levels significantly decrease by 0.5-fold in Human-RPSA transfected cells, when compared to non-transfected cells which were set to 100%. β-actin was used as the loading control and the graphs are representative of an average of experiments performed in triplicate. Error bars represent standard deviation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significant: The One-way ANOVA was significant, and a Bonferroni corrected post-hoc *t*-test indicated that the Human-RPSA samples are significantly lower than the non-transfected and esiRNA-RLUC samples.

4.1.10. siRNA-mediated knock-down of LRP/LR results in the significant increase of several proteins involved the MAPK cellular pathway in late stage colorectal cancer cells.

The mitogen-activated protein kinase (MAPK) pathway consists of serine/threonine kinases which link several extracellular signals to cellular processes involved in gene expression, apoptosis, differentiation, proliferation and migration [209]. Thus, it comes as no surprise that mutations in the signalling of the MAPK pathway may play key roles in developing cancer. In addition, as mentioned above in section 1.15.2, a study performed by Sun et al. (2014) showed that the LRP/LR-laminin-1 interaction allows for LRP/LR's interaction with FAK, leading to the activation of the MAPK/ERK 1/2 cellular survival pathway as well as an increased expression of the anti-apoptotic Bcl-2 protein [180]. Thus, investigations on whether LRP/LR

influences proteins involved in the MAPK signalling pathway as well as apoptotic pathways was prompted. Proteome Profiler Antibody Arrays™ were used to analyse these pathways since these protein arrays are able to detect several proteins in a single experiment.

A Human phospho-MAPK array kit containing 43 kinase antibody capture sites was first used to determine the role of LRP/LR in the MAPK pathway. Upon siRNA-mediated knock-down of LRP/LR, densitometric analysis revealed that 9 proteins of the phospho-MAPK pathway were affected however, only 3 of these proteins such as Chk-2, β-catenin and AMPKα2 were found to be significant. The remaining 6 proteins including FAK, CREB, AMPKα1 as well as three phosphorylation sites of p53 (Fig. 43) revealed changes, however upon statistical analysis, these changes were found to be non-significant. In order to confirm the results from this profiler array, a second MAPK array kit was performed, containing 26 MAPK antibody capture sites. It displayed consistent results with the phospho-MAPK array in that proteins such as CREB, and p53 were once again found to be affected (Fig. 44). However, the change in these proteins were also found to be non-significant.

Note: A western blot was first performed to confirm that LRP/LR was successfully down-regulated (approximately 70% down-regulation) in all transfected lysates before the MAPK arrays could be performed (see Appendix A).

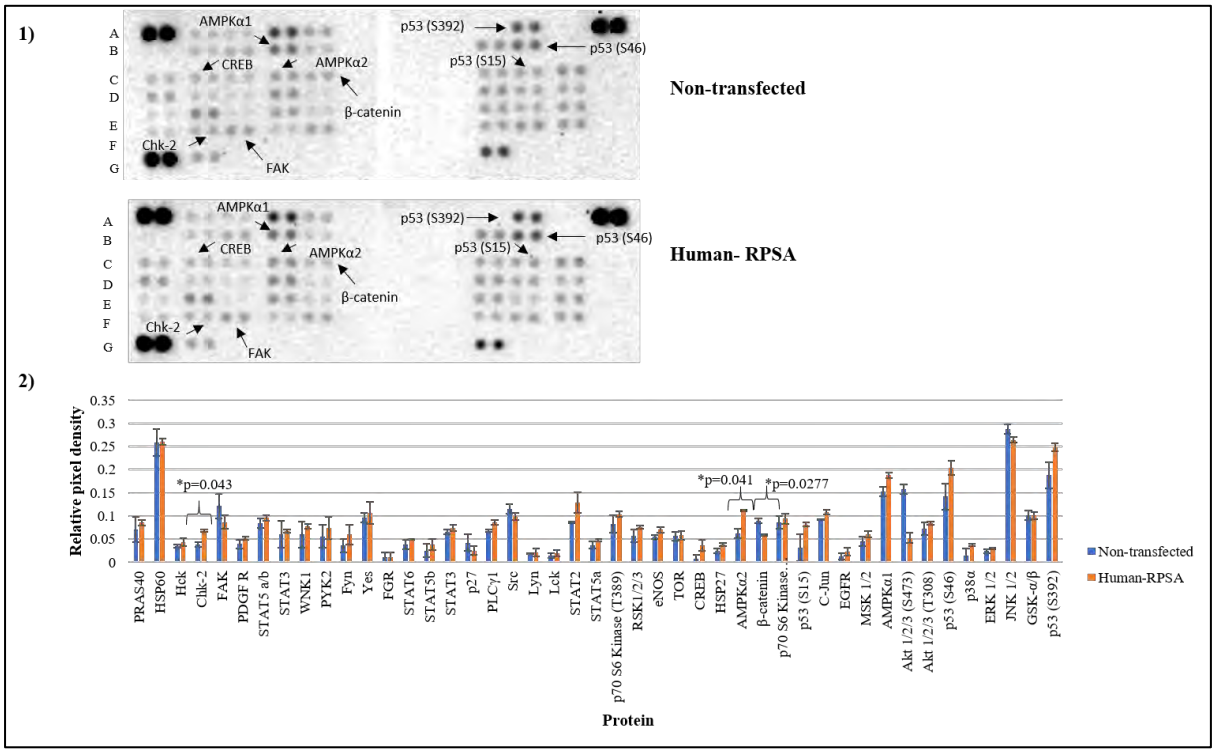


Figure 43: Proteins found to be affected upon siRNA-mediated knock-down of LRP/LR using the Human phospho-MAPK Proteome Profiler Antibody Array™ in late (DLD-1)

stage colorectal cancer cells. **43.1.** Non-transfected and Human-RPSA transfected DLD-1 cell lysates were incubated on the antibody membrane arrays. Data shown are from a 10-minute exposure. See Appendix for protein co-ordinates. **43.2. Densitometric analysis of the phospho-MAPK Proteome Profiler Antibody Array™.** Upon densitometric analysis, it was found that several proteins were affected upon siRNA-mediated knockdown of LRP/LR. Signals for each protein are displayed as a pair of spots, with three pairs of dark reference spots on the lower left, upper right and upper left corners for alignment. When compared to non-transfected cells, Chk-2, β-catenin and AMPKα2 were found to have significant differences. Proteins such as p53, CREB and FAK also displayed a difference however, this difference was seen to be non-significant. PBS spots (designated regions that had consistent background with no capture sites) were used as the negative control where each value was subtracted from PBS and divided by its corresponding reference spot. The high background may have contributed to the results being non-significant. Graph represents experiments performed in duplicate with two technical repeats. Error bars represent standard deviation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significant: The One-way ANOVA was significant, and a Bonferroni corrected post-hoc *t*-test was performed.

A second Human MAPK array kit was performed (Fig. 44) containing the main proteins involved in the signalling pathway in order to confirm the results obtained from the phospho-MAPK array kit (Fig. 43).

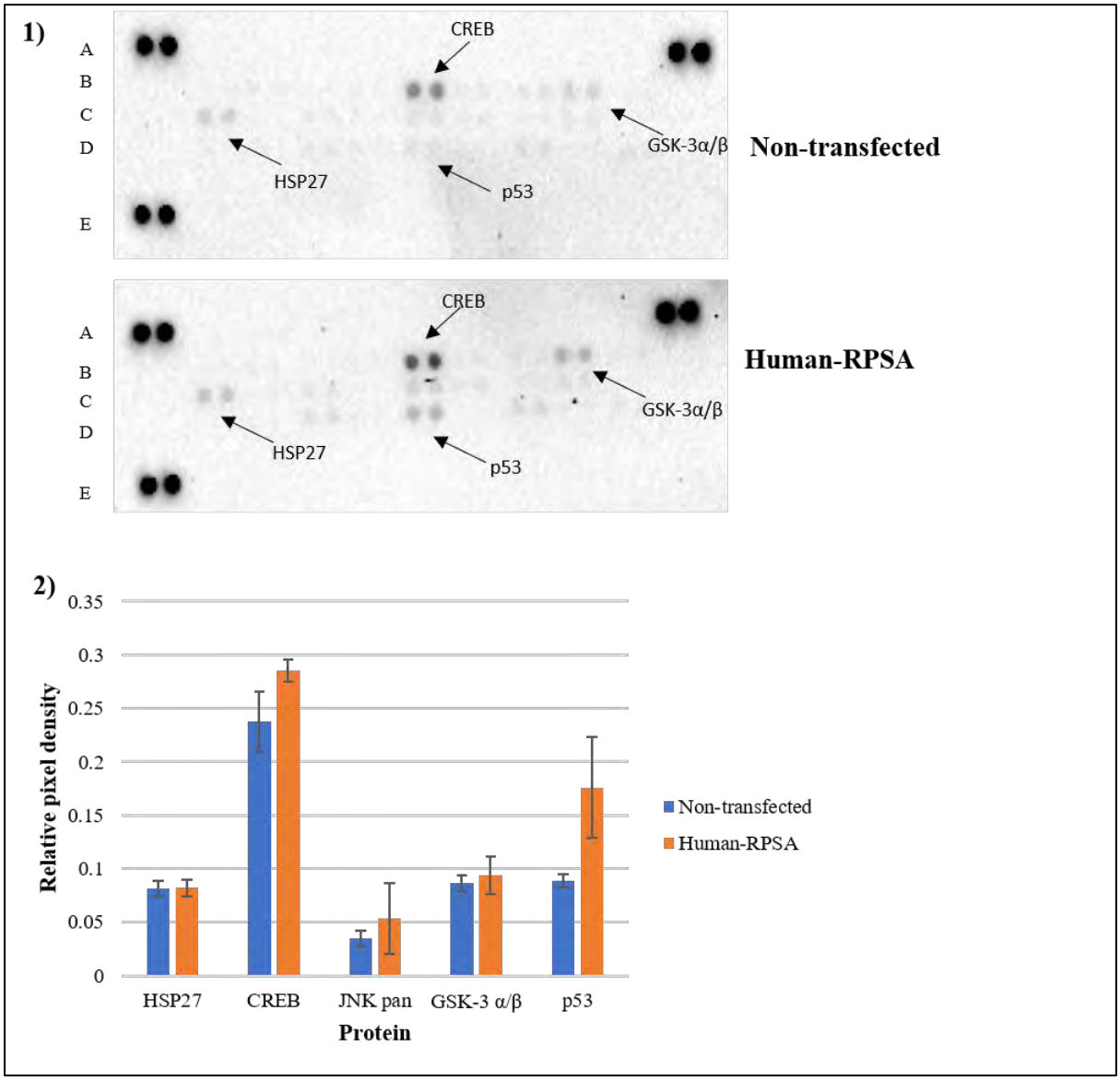


Figure 44: Proteins found to be affected upon siRNA-mediated knock-down of LRP/LR using the Human MAPK Proteome Profiler Antibody Array™ in late (DLD-1) stage colorectal cancer cells. **44.1.** Non-transfected and Human-RPSA transfected DLD-1 cell lysates were incubated on the antibody membrane arrays. When LRP/LR is down-regulated with Human RPSA siRNA, there are two proteins that seem to be affected such as CREB and p53. Data shown are from a 10-minute exposure. **44.2. Densitometric analysis of the MAPK Proteome Profiler Antibody Array ™**. Signals for each protein are displayed as a pair of spots, with three pairs of dark reference spots on the lower left, upper right and upper left corners for alignment. Upon densitometric analysis, it was found that CREB, GSK-3α/β and p53 were up-regulated once LRP/LR was silenced however, this up-regulation was found to be non-significant. PBS spots (designated regions that had consistent background with no capture sites) were used as the negative control where each value was subtracted from PBS and divided by its corresponding reference spot. Graph represents experiments performed in duplicate with two technical repeats. Error bars represent standard deviation.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significant.

4.1.11. siRNA-mediated knock-down of LRP/LR results in the significant increase of pro-apoptotic proteins as well as a decrease in anti-apoptotic proteins in late stage colorectal cancer cells.

To confirm that LRP/LR plays a role in cell survival through evasion of apoptosis, Human Apoptosis Proteome Profiler Antibody Arrays™ were performed which contained 35 capture antibody sites. It was found that upon siRNA-mediated knock-down of LRP/LR, 11 proteins were affected. Several pro-apoptotic proteins including Bax, cleaved caspase-3 and p53 phosphorylation sites were significantly up-regulated, while anti-apoptotic proteins such as claspin, cIAP1, XIAP and Survivin were significantly down-regulated (Fig. 45). Cytochrome C and HIF-1α proteins were also affected by siRNA-mediated knockdown of LRP/LR however these changes were found to be non-significant upon densitometric analysis. Several of the proteins found confirmed the results of the MAPK Proteome Profiler Arrays™ (Fig. 48). Moreover, it was found that majority of the proteins affected in the array play roles in the intrinsic mitochondrial pathway of apoptosis.

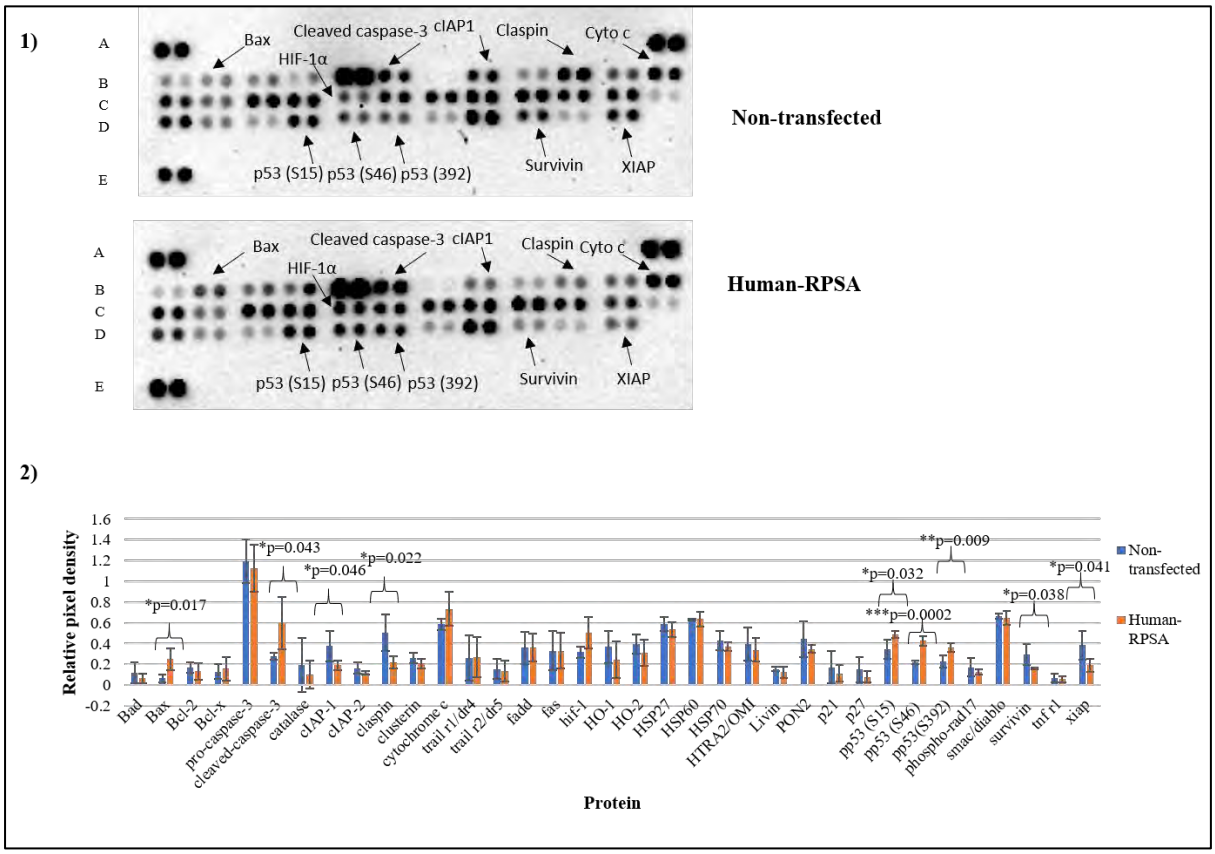


Figure 45: Proteins found to be affected upon siRNA-mediated knock-down of LRP/LR using the Human Apoptosis Proteome Profiler Antibody Array™ in late (DLD-1) stage colorectal cancer cells. **45.1.** Non-transfected and transfected late (DLD-1) stage colorectal cancer cell lysates were incubated on the antibody membrane arrays. A visible increase in

several apoptotic proteins upon Human-RPSA transfection in comparison to the non-transfected cells was observed. Data shown are from a 10-minute exposure. **45.2.** Signals for each protein are displayed as a pair of spots, with three pairs of dark reference spots on the lower left, upper right and upper left corners for alignment. PBS spots (designated regions that had consistent background with no capture sites) were used as the negative control where each value was subtracted from PBS and divided by its corresponding reference spot. Upon densitometric analysis, it was found that there several anti-apoptotic proteins were decreased while several pro-apoptotic proteins were increased, including p53 phosphorylated sites, confirming the result of the MAPK array. Furthermore, it was found that many of the proteins which were affected play a role in the intrinsic mitochondrial pathway of apoptosis. Graph represents experiments performed in duplicate with two technical repeats. Error bars represent standard deviation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significant: The One-way ANOVA was significant, and a Bonferroni corrected post-hoc *t*-test was performed.

4.1.12. siRNA-mediated LRP/LR reveals significant changes in relative mRNA expression levels of specific genes in late stage colorectal cancer cells.

The Proteome Profiler Antibody Arrays™ showed favourable results in illustrating that LRP/LR influences proteins involved in the MAPK pathway as well as the apoptotic pathways. However, similar to western blotting, Proteome Profiler Antibody Arrays™ only provide semi-quantitative data. Thus, to confirm the results obtained from the arrays, specific proteins namely: p53, Bcl-2, Bax, and CREB, were chosen for relative mRNA expression level analysis upon siRNA-mediated knock-down of LRP/LR.

It was found that upon siRNA-mediated knock-down of LRP/LR, relative mRNA expression levels of the tumour suppressor gene, p53 and the pro-apoptotic protein, Bax significantly increased by 1.7-fold and 4-fold, respectively, when compared to non-transfected cells (Fig. 46 and Fig. 47), confirming both the MAPK and apoptotic Proteome Profiler Antibody Arrays ™ (Figs. 43, 44 and 45). In addition, siRNA-mediated down-regulation resulted in a decrease in relative mRNA expression levels of the anti-apoptotic protein Bcl-2 in comparison to non-transfected cells (Fig. 48). Interestingly, the CREB protein was found to be over-expressed in cells transfected with the Human-RPSA siRNA in the Apoptotic Proteome Profiler Array™, thus investigations on whether its mRNA expression level was similarly affected were incited. Upon siRNA-mediated knock-down of LRP/LR, it was found that the relative mRNA expression level of CREB was significantly increased by 4-fold compared to non-transfected cells (Fig. 49) thus, confirming the results from the array (Fig. 44). Refer to the Appendix A for primer sequences, qPCR melt curves and peaks for each gene.

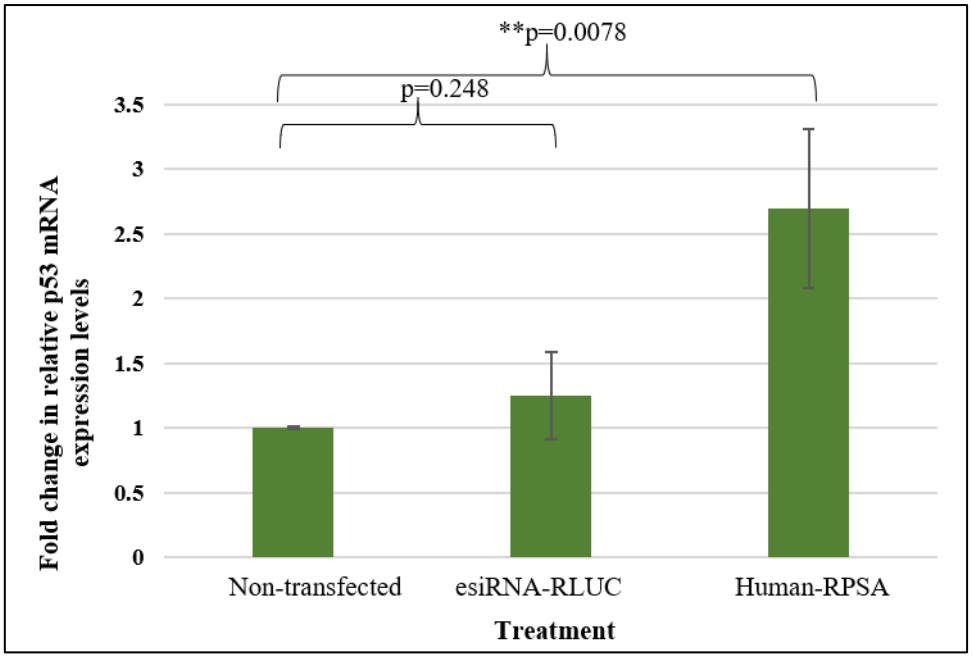


Figure 46: Relative mRNA expression levels of p53 in late (DLD-1) colorectal cancer cells. Upon transfection with Human-RPSA, relative p53 mRNA expression levels increased by 1.7-fold, when compared with non-transfected cells, confirming that p53 plays a role in DLD-1 cells once LRP/LR is down-regulated. esiRNA-RLUC served as the negative control. This data represents three biological replicates with two technical repeats with newly transfected samples each time. Error bars represent standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001, significant: The One-way ANOVA was significant, and a Bonferroni corrected post-hoc *t*-test indicated that the Human-RPSA samples are significantly higher than the non-transfected and esiRNA-RLUC samples.

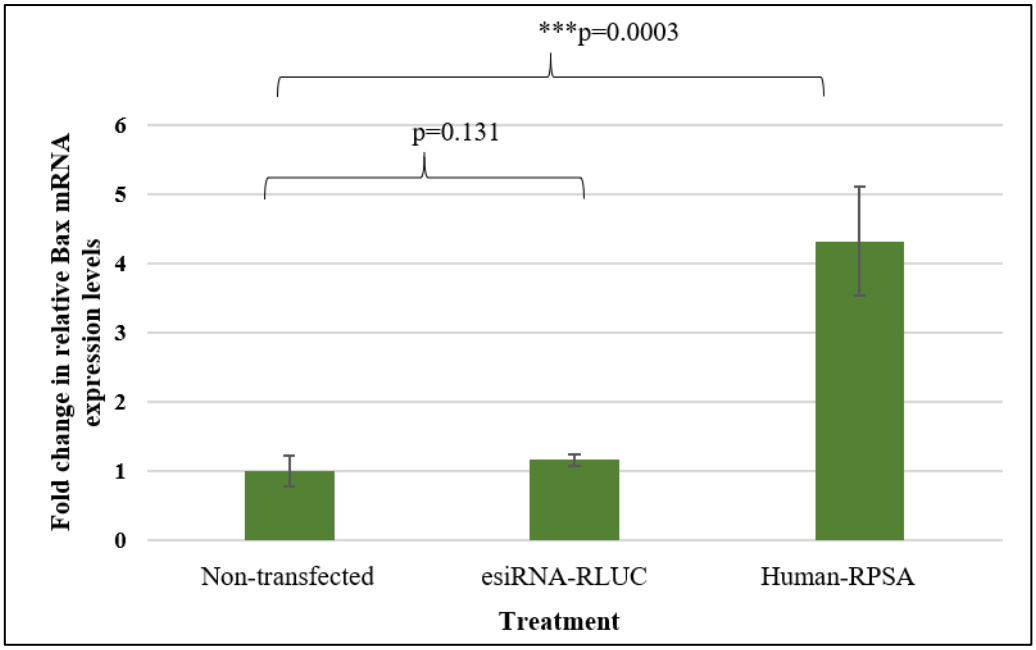


Figure 47: Relative mRNA expression levels of Bax in late (DLD-1) colorectal cancer cells. Upon transfection with Human-RPSA, relative Bax mRNA expression levels increased by 4-fold, when compared to non-transfected cells. esiRNA-RLUC served as the negative control. This data represents three biological replicates with two technical repeats with newly transfected samples each time. Error bars represent standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001, significant: The One-way ANOVA was significant, and a Bonferroni corrected post-hoc *t*-test indicated that the Human-RPSA samples are significantly higher than the non-transfected and esiRNA-RLUC samples.

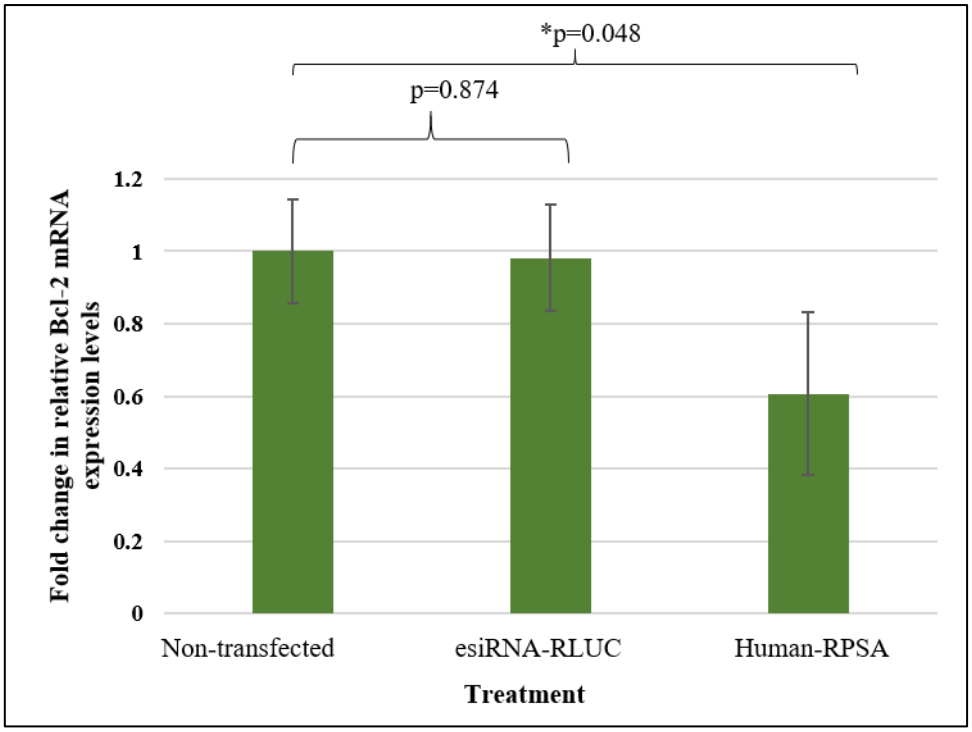


Figure 48: Relative mRNA expression levels of Bcl-2 in late (DLD-1) stage colorectal cancer cells. Upon transfection with Human-RPSA, relative Bcl-2 mRNA expression levels decreased by 0.4-fold in comparison to non-transfected cells. esiRNA-RLUC served as the negative control. This data represents three biological replicates with two technical repeats with newly transfected samples each time. Error bars represent standard deviation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significant: The One-way ANOVA was significant, and a Bonferroni corrected post-hoc *t*-test indicated that the Human-RPSA samples are significantly lower than the non-transfected and esiRNA-RLUC samples.

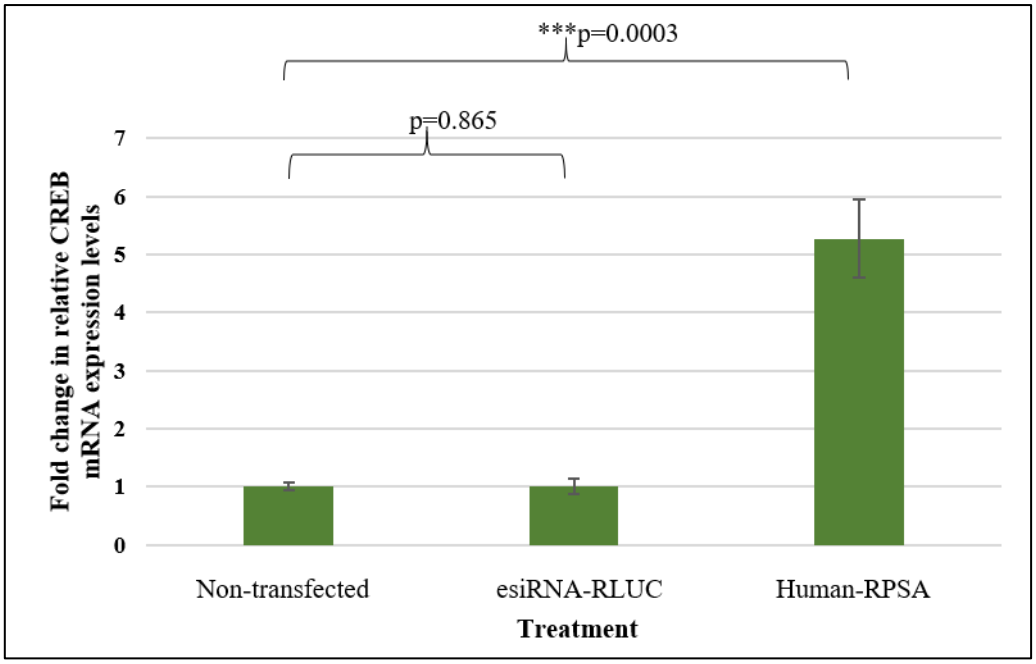


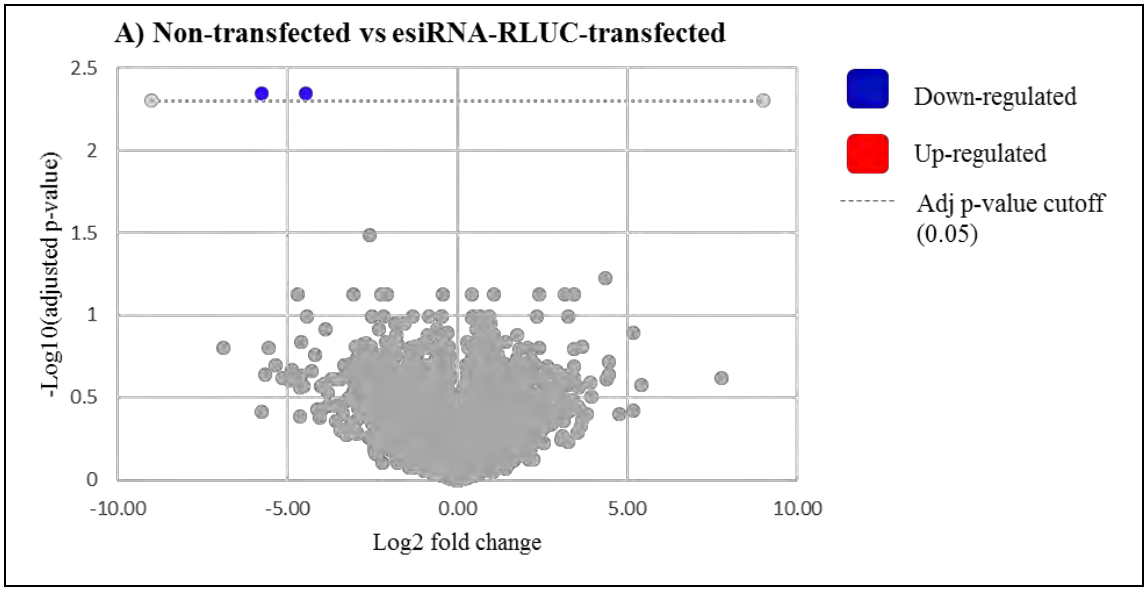
Figure 49: Relative mRNA expression levels of CREB in late (DLD-1) stage colorectal cancer cells. Upon transfection with Human-RPSA, relative CREB mRNA expression levels increased by 4-fold, in comparison to non-transfected cells, confirming its increase in the MAPK Proteome Profiler Array™. esiRNA-RLUC served as the negative control. This data represents three biological replicates with two technical repeats with newly transfected samples each time. Error bars represent standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001; *p < 0.05, **p < 0.01, ***p < 0.001, significant: The One-way ANOVA was significant and a Bonferroni corrected post-hoc *t*-test indicated that the Human-RPSA samples are significantly higher than the non-transfected and esiRNA-RLUC samples.

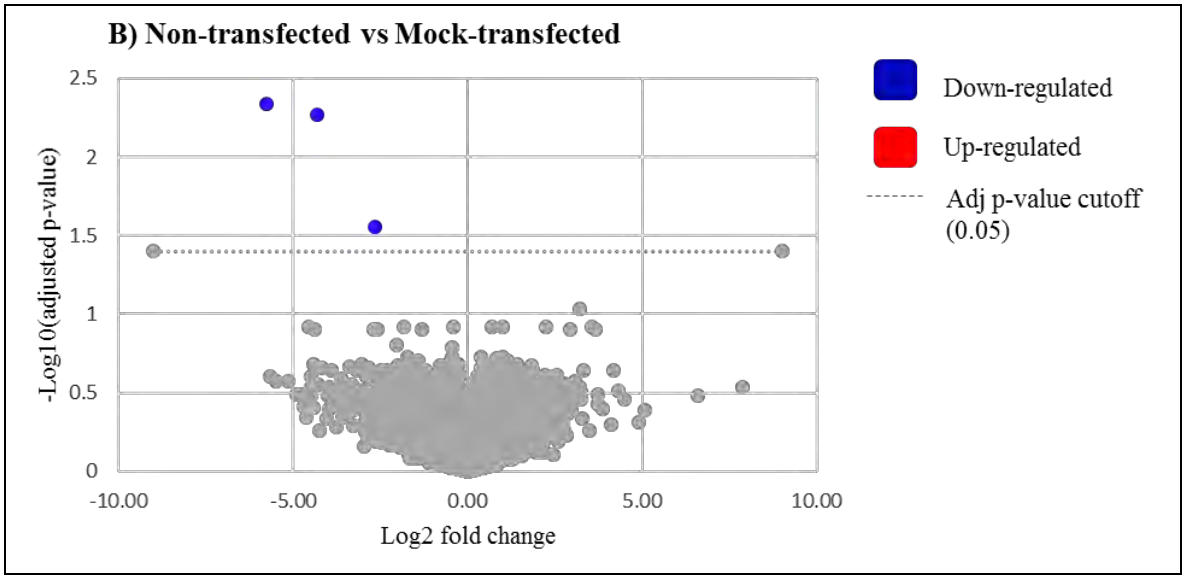
4.1.11. siRNA-mediated knock-down of LRP expression significantly changes the proteome of late stage colorectal cancer cells.

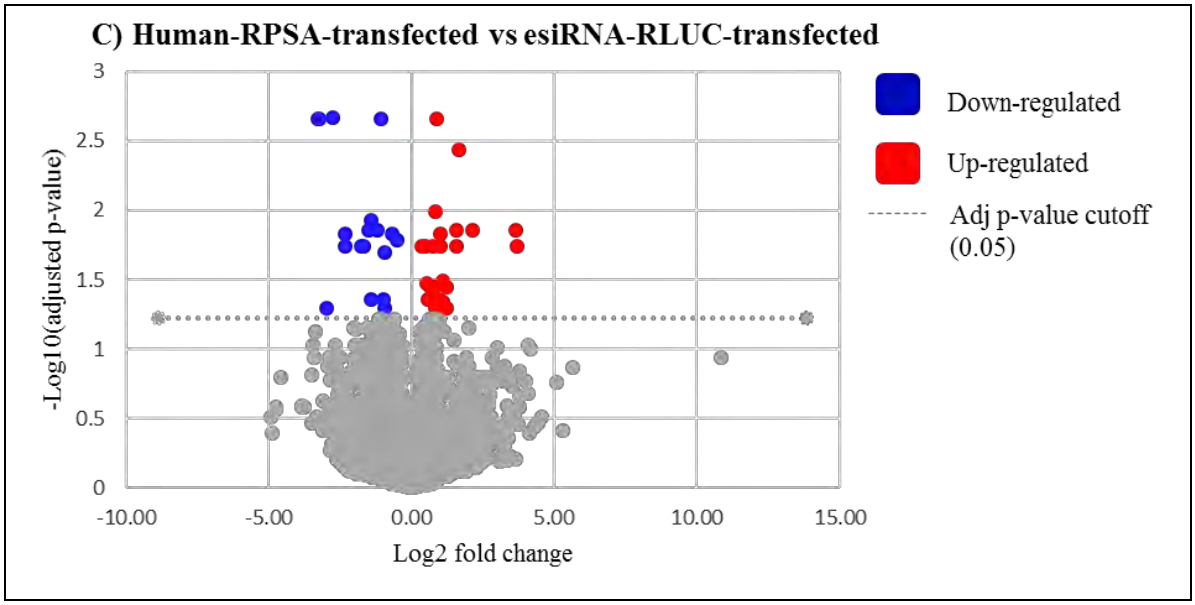
Although a significant change in various proteins was observed in the Proteome Profiler Antibody Arrays™ upon siRNA-mediated knockdown of LRP/LR, the data obtained provided only semi-quantitative results. Thus, for a more sensitive and quantitatively accurate analysis, Sequential Window Acquisition of all Theoretical Fragment Ion Spectra Mass Spectrometry (SWATH MS) was used to quantify proteins associated with LRP/LR. SWATH-MS is a modified method of data-independent analysis (DDA) MS which integrates accuracy and consistency together with the ability for deep proteome coverage on a large scale [200]. As a result, this allowed for novel screening of several proteins in a single analysis affected by siRNA-mediated down-regulation of LRP/LR in DLD-1 cells. This enabled further insight into

the mechanism by which LRP/LR exerts its effects. Samples for non-transfected cells, Human-RPSA-transfected siRNA cells as well esiRNA-RLUC-transfected cells (negative control) were prepared. An additional mock-transfected control containing only transfection reagent (with no siRNA) was also prepared in order to ensure that it was only the Human-RPSA siRNA exerting effects to down-regulate LRP/LR.

When the non-transfected and Human-RPSA siRNA-transfected samples were compared to one another, several proteins were found to be differentially expressed (Figure 50). From these proteins, only those that had a 2-fold increase or greater together with a p-value < than 0.0.5 were considered significant. From the graphs below, there is a significant change in the proteome of late stage colorectal cancer cells when transfected with Human-RPSA. Upon analysis between the Human-RPSA transfected samples and the non-transfected samples, it was found that 40 proteins were down-regulated while 44 proteins were up-regulated (Fig. 51). Moreover, when analysing the functions of each of these proteins, most were found to play roles similar to LRP/LR such as: ribosomal processing and protein synthesis [96]; nucleus and chromatin maintenance [97]; cell proliferation and differentiation [42]; cell anchorage and cytoskeletal involvement [98] and lastly, apoptotic regulation [122] (See Tables A1-7 for grouped proteins). In addition, other proteins identified were found to be involved in vesicle transportation and regulation as well as autophagy. Moreover, novel stand-alone proteins have been found to interact with LRP/LR, which could provide additional information on how the receptor works. Observations from this technique suggest that several proteins involved in suppressing tumourigenesis were up-regulated, while proteins involved promoting tumourigenesis were down-regulated proposing that the Human RPSA-siRNA-mediated knock-down of LRP/LR could be a potential anti-cancer therapeutic tool.


A) Non-transfected vs esiRNA-RLUC-transfected
-Log10(adjusted p-value)
2.5
2
1.5
1
0.5
0
-10.00
-5.00
0.00
5.00
10.00
Log2 fold change
Down-regulated
Up-regulated
Adj p-value cutoff (0.05)


B) Non-transfected vs Mock-transfected
-Log10(adjusted p-value)
2.5
2
1.5
1
0.5
0
-10.00
-5.00
0.00
5.00
10.00
Log2 fold change
Down-regulated
Up-regulated
Adj p-value cutoff (0.05)



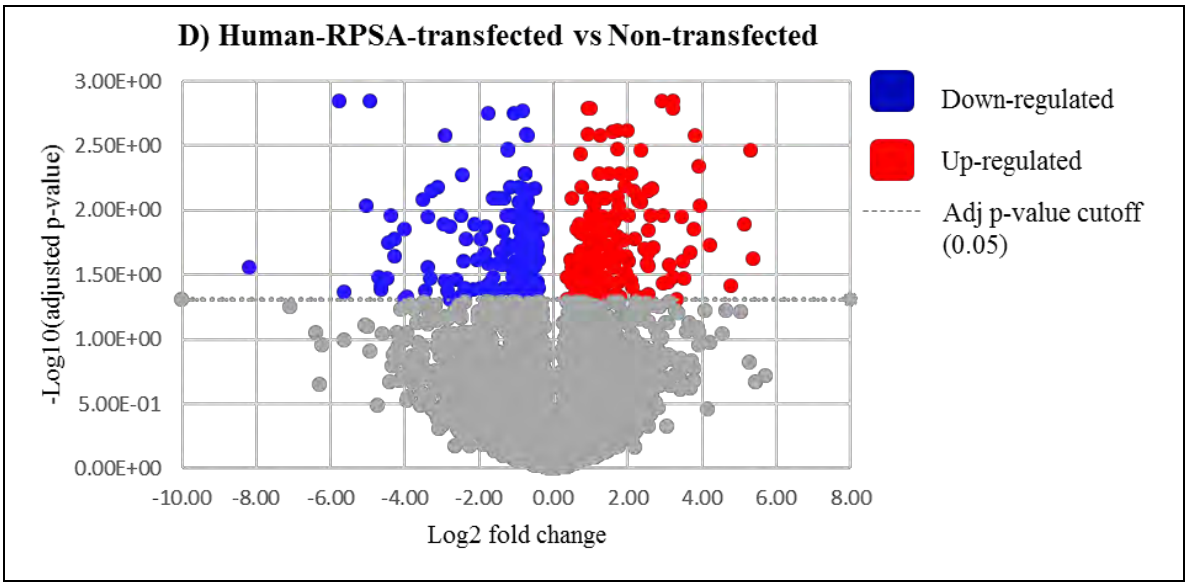


Figure 50: Proteins found to be affected upon siRNA-mediated knock-down of LRP/LR using SWATH-MS based proteomics in late (DLD-1) stage colorectal cancer cells. Only proteins with p-values smaller than 0.05 were considered significant. Red dots indicated proteins that were significantly up-regulated, while blue dots show proteins that were significantly down-regulated. (**A) Non-transfected vs esiRNA-RLUC samples.** When the non-transfected and negative control esiRNA-RLUC samples were compared to one another, only two proteins were significantly affected, which was expected. (**B) Non-transfected vs mock-transfected samples.** When the non-transfected cells were compared to the mock-transfected control samples, only three proteins were found to be significantly affected, confirming that the transfection reagent does not have an effect. (**C) Human-RPSA transfected vs esiRNA-RLUC transfected samples.** It was found that there was a significant effect on the proteome of Human-RPSA transfected samples when they were compared to esiRNA-RLUC negative control samples. **(D) Human-RPSA transfected vs non-transfected samples.** It can be seen that when Human-RPSA transfected samples were compared to non-transfected samples, it showed the largest change in the DLD-1 cells proteome with several proteins being either down- or and up-regulated. This confirms that the Human-RPSA siRNA does indeed influence LRP/LR in DLD-1 cells. Non-significant: p>0.05, non-significant.

Table 1: List of down-regulated proteins with significant fold changes of two and above observed upon siRNA-mediated knock-down of LRP/LR.

Fold change	Protein	Name	P- value	Function
-8.20	sp|P09651| HnRNPA1_HUMAN	Heterogeneous nuclear ribonucleoprotein A1	0.027	Pre-mRNA processing, mRNA transport and metabolism [210, 211]
-5.75	sp|Q14244|MAP7_HUMAN	Ensconsin	0.001	Microtubule stabilizing protein, involved in cellular differentiation and motility [212, 213]
-5.62	sp|O96028|NSD2_HUMAN	Histone-lysine N-methyltransferase	0.043	Transcription regulator [214]
-5.04	sp|Q6QEF8|CORO6_HUMAN	Coronin-like protein E	0.009	Actin binding protein, interaction with microtubules [215]
-4.95	sp|Q9UKY7|CDV3_HUMAN	Carnitine deficiency-associated gene expressed in ventricle 3	0.001	Biomarker for Hepatocellular carcinoma. Involved in cell proliferation [216]
-4.71	sp|Q9HD42|CHM1A_HUMAN	Charged Multivesicular Body	0.032	Multivesicular body sorting of proteins to the interiors of lysosomes [217]
-4.64	sp|P17152|TMM11_HUMAN	Transmembrane protein 11	0.038	Mitochondrial morphogenesis [218]
-4.63	sp|Q9NYF8|BCLF1_HUMAN	Bcl-2-associated transcription factor 1	0.040	Apoptotic induction [219]
-4.48	sp|Q9H832|UBE2Z_HUMAN	Ubiquitin conjugating enzyme E2 Z	0.033	Proteolysis [220]
-4.43	sp|Q7L9L4|MOB1B_HUMAN	Mps one binder kinase activator-like 1A	0.017	Protein kinase essential for spindle pole body duplication and mitotic checkpoint regulation/ tumour suppressor properties [221]
-4.39	sp|Q9UK76|JUPI1_HUMAN	Jupiter Microtubule Associated Homolog 1	0.011	Cell cycle and cell adhesion regulation
-4.28	sp|P41567|EIF1_HUMAN	Eukaryotic translation initiation factor 1	0.022	Initiation of mRNA translation [222]
-4.26	sp|O14530|TXND9_HUMAN	Thioredoxin Domain Containing 9	0.016	Cell differentiation/ transcription [223]
-4.02	sp|P41222|PTGDS_HUMAN	Prostaglandin-H2 D-isomerase	0.013	Cancer cell suppression [224]
-4.01	sp|Q14118|DAG1_HUMAN	Dystroglycan	0.048	Receptor for laminin; involved in basement membrane assembly and cell survival [225]
-3.94	sp|Q9NWW5|CLN6_HUMAN	Ceroid-lipofuscinosis neuronal protein 6	0.046	Regulates the transportation from the ER to lysosomes [226]
-3.50	sp|Q7Z478|DHX29_HUMAN	DExH-box helicase 29	0.008	Initiation of mRNA translation [227]
-3.45	sp|Q9UJC5|SH3L2_HUMAN	SH3 domain-binding glutamic acid-rich-like protein	0.042	Reduction of many intra-cellular protein disulphides [228]
-3.39	sp|P20042|IF2B_HUMAN	Eukaryotic translation initiation factor 2 subunit 2	0.027	Translation initiation factor activity [222]
-3.39	sp|P15529|MCP_HUMAN	Monocyte chemotactic protein	0.011	Monocyte activation [229]
-3.32	sp|Q9UKI8|TLK1_HUMAN	Serine/threonine-protein kinase tousled-like 1	0.033	chromatin assembly, DNA repair, transcription, and chromosome segregation [230]
-3.29	sp|Q8N806|UBR7_HUMAN	Putative E3 ubiquitin-protein ligase	0.006	Pathway protein ubiquitination [231]
-3.12	sp|P15151|PVR_HUMAN	Poliovirus receptor/ PVR Cell Adhesion Molecule	0.006	Mediates cell adhesion, tumour cell invasion and migration [232]
-2.95	sp|P02751|FINC_HUMAN	Fibronectin 1	0.0128	Cell adhesion, differentiation, wound healing and migration [233]
-2.92	sp|Q9Y639|NPTN_HUMAN	Neuroplastin	0.035	Cell-cell interactions or cell-substrate interactions [234]
-2.91	sp|P62875|RPAB5_HUMAN	DNA-directed RNA polymerases I, II, and III subunit	0.002	Component of RNA polymerases I, II and III which synthesize ribosomal RNA precursors, mRNA precursors /transcription and DNA binding [235]

-2.85	sp|P54105|ICLN_HUMAN	Methylosome subunit pICln	0.041	Splicing cellular pre-mRNAs [236]
-2.79	sp|Q06481|APLP2_HUMAN	Amyloid-like protein 2	0.049	Important modulator of glucose and insulin homeostasis; plays a role in cell proliferation and migration [237]
-2.79	sp|O60610|DIAP1_HUMAN	Death-associated inhibitor of apoptosis 1	0.013	Anti-apoptotic regulator [238]
-2.62	sp|Q8TAT6|NPL4_HUMAN	Nuclear protein localization protein 4	0.034	Endoplasmic reticulum-associated degradation; regulates ubiquitin-mediated mitochondria protein degradation [239]
-2.53	sp|Q99439|CNN2_HUMAN	Calponin-2	0.048	Actin filament-associated regulatory protein, involved in the regulation and modulation of smooth muscle contraction/cell proliferation, cell migration, and platelet adhesion [240]
-2.52	sp|Q15370|ELOB_HUMAN	Elongin B	0.047	Transcription elongation factor activity [241]
-2.50	sp|P61916|NPC2_HUMAN	Intracellular cholesterol transporter 2	0.011	Cholesterol regulation [242]
-2.44	sp|O15405|TOX3_HUMAN	TOX high mobility group box family member 3	0.005	Transcription factor [243]
-2.43	sp|O96005|CLPT1_HUMAN	ATP-dependent Clp protease ATP-binding subunit	0.024	Regulates the assembly of the Clp protease system [244]
-2.40	sp|Q13546|RIPK1_HUMAN	Receptor-interacting serine/threonine-protein kinase 1	0.040	Plays a role in apoptosis and necroptosis induction [245]
-2.37	sp|Q86UK7|ZN598_HUMAN	Zinc Finger Protein 598	0.043	Cell proliferation, differentiation, and apoptosis
-2.36	sp|P63220|RS21_HUMAN	Ribosomal Protein S21	0.016	Ribosomal protein that is a component of the 40S subunit [246]
-2.17	sp|Q15417|CNN3_HUMAN	Calponin-3	0.040	Regulates and modulates smooth muscle contraction/actin binding and calmodulin binding [240]
-2.12	sp|P07203|GPX1_HUMAN	Glutathione peroxidase 1	0.012	Protects cells from oxidative stress [247]
-2.06	sp|Q5JRA6|TGO1_HUMAN	Transport and Golgi organization protein 1/Melanoma Inhibitory Activity Protein 3	0.023	Transport of cargos that are too large to fit into COPII-coated vesicles from the endoplasmic reticulum / promotes the formation of metastases by inhibiting the attachment of melanoma cells to the extracellular matrix [248]

Table 2: List of Up-regulated proteins observed upon siRNA-mediated knock-down of LRP/LR.

Fold change	Protein	Name	P- value	Function
2.01	sp|P48735|IDHP_HUMAN	Isocitrate dehydrogenase NADP	0.024	Catalyses the oxidative decarboxylation of isocitrate to 2-oxoglutarate [249]
2.01	sp|Q9Y3D6|FIS1_HUMAN	Fission 1 protein	0.011	Regulation of mitochondrial morphology, the cell cycle, autophagy and apoptosis [250]
2.078	sp|Q53GQ0|DHB12_HUMAN	Estradiol 17-beta-dehydrogenase 12	0.005	Converts the potent oestrogen oestradiol into its less active metabolite estrone [251]
2.1	sp|Q12981|SEC20_HUMAN	Vesicle transport protein	0.035	Targets Golgi-derived retrograde transport vesicles with the ER/ Participates in intrinsic apoptotic pathway [252]
2.12	sp|Q5JTW2|CEP78_HUMAN	Centrosomal Protein 78	0.039	Regulates of centrosome-related events during the cell cycle, and required for ciliogenesis/Tumour suppressor properties [253]
2.16	sp|Q92597|NDRG1_HUMAN	N-myc Downstream-Regulated Gene 1	0.006	Metastasis suppressor [254]
2.18	sp|Q9P035|HACD3_HUMAN	3-Hydroxyacyl-CoA Dehydratase 3	0.016	Enzyme binding and lyase activity [255]
2.26	sp|P55795|HNRH2_HUMAN	Heterogeneous Nuclear Ribonucleoprotein H2	0.046	Pre-mRNA processing, mRNA metabolism and transport [256]

2.29	sp|Q14534|ERG1_HUMAN	Early growth response protein 1	0.008	Involved in transcription/ tumour suppressor [257]
2.3	sp|Q7Z4H8|PLGT3_HUMAN	Protein O-Glucosyltransferase 3	0.007	Regulates Notch signalling pathway [258]
2.35	sp|Q14728|MFS10_HUMAN	Major Facilitator Superfamily Domain Containing 10	0.008	Transmembrane transporter activity and tetracycline transmembrane transporter activity [259]
2.36	sp|A1L0T0|ILVBL_HUMAN	Acetolactate synthase-like protein	0.003	Catalytic enzyme involved in the biosynthesis of various amino acids [260]
2.44	sp|Q7Z3K3|POGZ_HUMAN	Pogo Transposable Element Derived with ZNF Domain/ Zinc Finger Protein 280E	0.046	Plays a role in mitotic cell cycle progression / nucleic acid binding [261]
2.46	sp|Q6NW34|NEPRO_HUMAN	Nucleolus and neural progenitor protein	0.02	Plays a role in Notch signalling pathway [262]
2.51	sp|Q86SK9|SCD5_HUMAN	Stearoyl-CoA Desaturase 5. Stearoyl-CoA desaturase	0.025	Catalyses the formation of monounsaturated fatty acids from saturated fatty acids [263]
2.54	sp|O00291|HIP1_HUMAN	Huntingtin interacting protein 1	0.021	Involved in Huntingtin's disease/ proapoptotic protein (intrinsic apoptosis pathway) [264]
2.55	sp|Q8IUX4|ABC3F_HUMAN	Apolipoprotein B MRNA Editing Enzyme Catalytic Subunit 3F	0.044	RNA processing [265]
2.55	sp|Q643R3|LPCT4_HUMAN	Lysophosphatidylcholine Acyltransferase 4	0.014	Precursor in the biosynthesis of all glycerolipids [266]
2.56	sp|Q9ULC4|MCTS1_HUMAN	Multiple Copies In T-Cell Lymphoma-1	0.026	RNA binding and translation initiation factor activity [267]
2.57	sp|P07305|H10_HUMAN	H1 Histone Family Member 0	0.006	Plays a role in apoptotic DNA fragmentation and chromatin silencing [268]
2.61	sp|Q5JU69|TOR2A_HUMAN	Torsin Family 2 Member A	0.011	Play roles in hypotension, myocardial growth and the induction of mitogenesis [269]
2.65	sp|O95994|AGR2_HUMAN	Anterior gradient protein 2 homolog	0.006	Proto-oncogene that may play a role in cell migration, cell differentiation and cell growth [270]
2.68	sp|P62854|RS26_HUMAN	Ribosomal Protein S26	0.019	Regulates the MDM2/MDMX–p53 cascade, consequently suppressing tumour cell proliferation [271]
2.92	sp|Q13228|SBP1_HUMAN	Selenium-binding protein 1	0.001	Tumour suppressor properties [272]
2.94	sp|P06733|ENOA_HUMAN	Enolase 1	0.011	May be a tumour suppressor however, ENOA's role in tumours is controversial [273]
3.09	sp|Q5U5X0|LYRM7_HUMAN	LYR Motif Containing 7	0.035	Main enzyme complex in the mitochondrial respiratory chain [274]
3.11	sp|P42858|HD_HUMAN	Huntingtin	0.026	Although the exact function of this protein is unknown, it may play a role in microtubule-mediated transport or vesicle function [275]
3.2	sp|P27144|KAD4_HUMAN	Adenylate Kinase 4	0.001	GTP binding and adenylate kinase activity/Biomarker for lung metastasis [276]
3.21	sp|P0DMM9|ST1A3_HUMAN	Sulfotransferase Family 1A Member 3	0.032	Catalyses the sulphate conjugation of many hormones, neurotransmitters, drugs [277]
3.22	sp|Q53EL6|PDCD4_HUMAN	Programmed cell death protein 4	0.001	Inhibits translation initiation and protein synthesis (tumour suppressor properties) [278]
3.32	sp|Q8TEU7|RPGF6_HUMAN	Rap Guanine Nucleotide Exchange Factor 6	0.048	Involved in Ras signalling and Tight junction pathways/involved in the activation of small GTPases [279]
3.45	sp|Q9UN37|VPS4A_HUMAN	Vacuolar protein sorting-associated protein 4A	0.011	Involved in intracellular protein trafficking/tumour suppressor properties [280]
3.46	sp|Q9P0S9|TM14C_HUMAN	Transmembrane Protein 14C	0.024	Pro-oncogene [281]
3.51	sp|O00204|ST2B1_HUMAN	Sulfotransferase family cytosolic 2B member 1	0.033	Catalyses the sulphate conjugation of numerous hormones, neurotransmitters, drugs and xenobiotic compounds[282]
3.68	sp|P22392|NDKB_HUMAN	Nucleoside diphosphate kinase B	0.021	Metastasis inhibition [283]

3.76	sp|Q9BYD2|RM09_HUMAN	Mitochondrial Ribosomal Protein L9	0.013	Structural constituent of ribosome [284]
3.8	sp|Q9Y224|RTRAF_HUMAN	RNA transcription, translation and transport factor protein	0.002	RNA transcription, translation and transport [285]
3.91	sp|Q99707|METH_HUMAN	Methionine synthase	0.004	Key enzyme involved in folate metabolism [286]
3.94	sp|Q68EM7|RHG17_HUMAN	Rho GTPase Activating Protein 17	0.009	Rho GTPase-activating protein involved in the maintenance of tight junctions [287]
4.22	sp|Q02818|NUCB1_HUMAN	Nucleobindin-1	0.018	Calcium homeostasis [288]
4.78	sp|P04632|CPNS1_HUMAN	Calpain Small Subunit 1	0.038	calcium-dependent cysteine proteinase whose proteolytic activities influence apoptosis, proliferation, migration, adhesion, and autophagy [289]
5.14	sp|Q16795|NDUA9_HUMAN	NADH dehydrogenase (Ubiquinone) 1 alpha subcomplex subunit	0.012	Involved in the mitochondrial membrane respiratory chain [290]
5.29	sp|O00442|RTCA_HUMAN	RNA 3'-terminal phosphate cyclase	0.003	RNA processing [291]
5.37	sp|P16104|H2AX_HUMAN	Histone H2AX	0.023	Key role in the double-stranded break response, DNA damage leading to apoptosis [292]

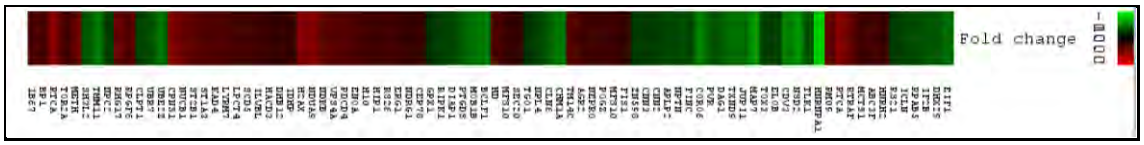


Figure 51: Heat-map illustrating up- and down-regulated proteins upon siRNA-mediated knockdown of LRP/LR. Proteins which were up-regulated upon transfection with Human-RPSA siRNA is shown in the red, while down-regulated proteins are indicated in green.

4.2.*In vivo* study

LRP/LR has been found to be over-expressed on the surface of several tumourigenic cell types, where it interacts with its primary ligand, laminin-1. This interaction is said to increase adhesion and invasion of tumour cells, ultimately resulting in an increase in the aggressiveness of tumours [33]. Furthermore, a direct correlation between LRP/LR levels and the invasive potential of several tumourigenic cells has been observed [33, 111-113, 123, 133]. However, we have previously reported that application of the anti-LRP/LR specific antibody IgG1-iS18 to early (SW-480 and HT-29) and late (DLD-1) stage colorectal cancer cells leads to a significant reduction in the adhesive and invasive potential of the cells, two main steps in metastasis [111]. Due to these promising *in vitro* results, the MF-1 nude mouse model was used to assess the effect of the anti-LRP/LR specific antibody IgG1-iS18, with regards to transformation, invasion and metastasis of colorectal carcinoma *in vivo*. Results from the pilot studies performed are shown below.

4.2.1. Successful establishment of tumours in immunocompromised MF-1 nude mouse models.

Pilot study 1 was successful in establishing tumours in all nine mice of the study. However, after the 4-week treatment regime with 2.5 mg/kg of IgG1-iS18, it was found that the treatment was ineffective in shrinking tumours or hampering metastasis. Figure 52 below represents each treatment group (consisting of three mice each).

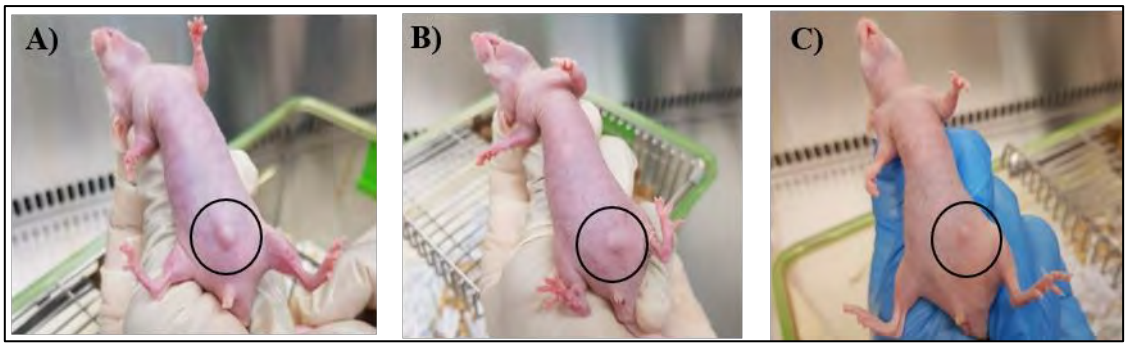


Figure 52: Injection and treatment procedure of the HT-29 cells in MF-1 nude mice for pilot study 1. The HT-29 cells were pelleted at a concentration of $5x10^6$ cells/ml and prepared in 100 µl media before intraperitoneal injection into abdomen of subject mice. The cells were injected and allowed to establish into tumours depending on treatment approach. The figure represents each treatment group (consisting of three mice each) i.e. A) Untreated, B) PBS negative control-treated and C) IgG1-iS18-treated. All nine mice established tumours however, once 2.5 mg/kg of IgG1-iS18 antibody was administered intraperitoneally for the 4-week period, it was found that there was no change in tumour size/metastasis.

4.2.2. IgG1-iS18 treatment displayed no significant change in LRP expression levels in mice samples.

Once pilot study 1 was completed, tumours were extracted for biochemical analysis. LRP expression levels were investigated through western blotting using the nine mice tumour samples. Densitometric analysis revealed that there was no change in LRP expression levels. Thus, another pilot study was performed.

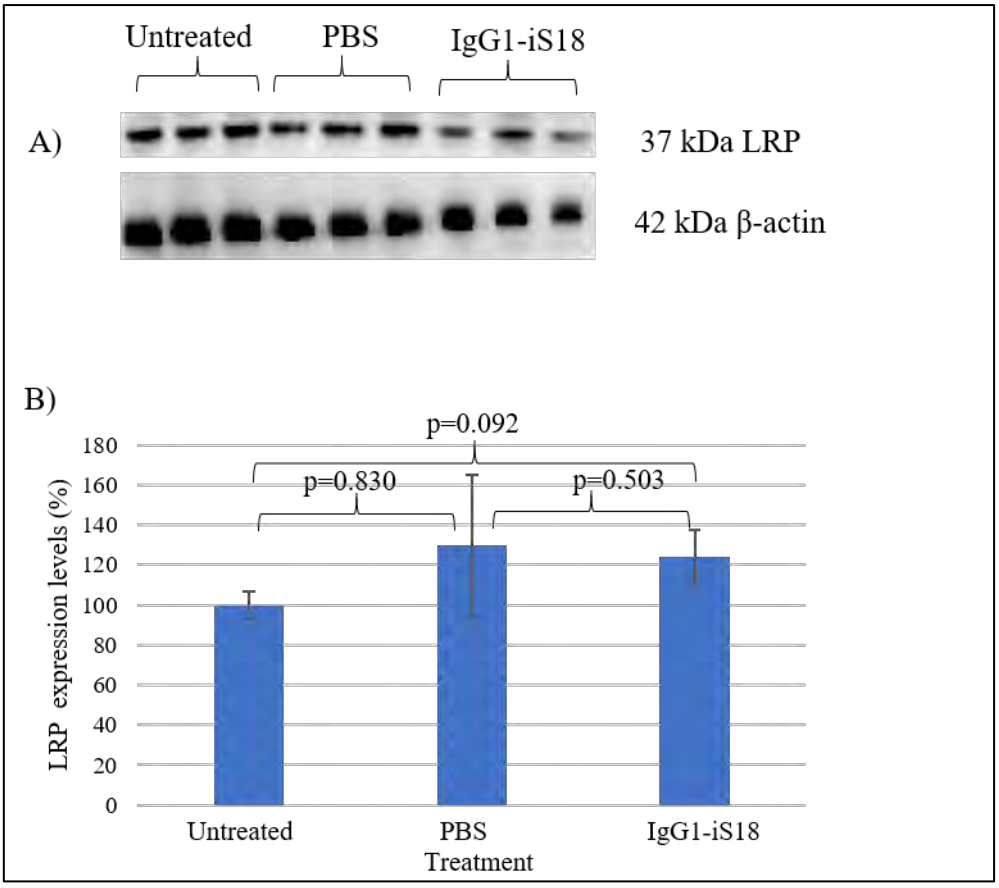


Figure 53: Expression levels of LRP in mice tissue for pilot study 1. A) Both LRP and β-actin proteins were successfully detectable in untreated, PBS-treated and IgG1-iS18-treated mice tissue samples. B) Densitometric analysis of LRP levels was performed where levels of the untreated tissue samples were set to 100%. There was no significant change in LRP expression levels between mice samples. β-actin was used as the loading control. A one-way ANOVA was performed. Error bars represent standard deviation.

Despite successful tumour induction in all nine mice, treatment with IgG1-iS18 seemed to be ineffective. This suggested that the route of administration for the antibody may not have been effective in delivering the antibody. Thus, another pilot study (pilot study 2) was performed to optimize treatment conditions. During this pilot study, the amount of antibody administered was increased to 3 mg/kg of body weight however, there was still no significant effect as seen in the table below:

Table 3: Summarized experimental parameters and outcomes observed in the pilot study 2 MF-1 nude mice model.

Mice # and treatment	Tumour weight (g)	Tumour volume (mm^3) V= (L x W x W)/2	Tumour location
#7 Untreated	1.23 1.53	1666 1800	All only metastasised to the bone next to injection site (not to other organs)
# 5 Untreated	0.71	2138	Near injection site (abdomen)
#9 Untreated	0.20 0.41	162 446	Near injection site (No metastasis)
#2 PBS	0.69	1100	Near injection site (No metastasis)
#8 PBS	0.27 0.18 0.54 0.39 0.11 0.62	792 284 480 792 75 2138	No metastasis – various tumours around injection site
#4 PBS	0.49	648	Near injection site (No metastasis)
#1 IgG1-iS18	-	-	Died (unknown date)
#3 IgG1-iS18	0.28 0.34	63 5082	Metastasised to liver and kidney
#6 IgG1-iS18	0.75 0.64	792 792	Near injection site

The results from pilot study 2 show that treatment with IgG1-iS18 was once again unsuccessful. This suggested that the concentration of the antibody may not have been enough in delivering the antibody to all sites of the body. Thus, it was decided to increase the antibody concentration to 5 mg/kg of body weight as well as to change the route of administration from intraperitoneal

to intravenous injection. The mice treated with IgG1-iS18 were split into two groups. The first group received a prophylactic approach (P) to treatment where they received one treatment with IgG1-iS18 prior to tumour induction, followed by another treatment of the antibody 24 hours later. The second group only received treatment after tumours were formed denoted as (T) (Table 4). Once treatments were completed, tumour size was assessed (Fig. 54). It was found that there was still no significant effect seen in terms of tumour growth/shrinkage and metastasis as seen in the table and figures below:

Table 4: Summarized experimental parameters and outcomes observed in the pilot study 3 MF-1 nude mice model.

Mice # and treatment	**Tumour weight (g)**	**Tumour volume (mm^3) V= (L x W x W)/2**	**Tumour location**
#3 Untreated	1.65 0.16 0.08	500 88 14	All three tumours migrated to the bottom of tail (not to other organs)
# 7 Untreated	-	-	No tumour formation (late injection)
#2 Untreated	0.12	14	Near injection site (No metastasis)
#6 PBS	3.47	1125	Tumour attached to spine (No metastasis)
#9 PBS	-	-	No tumour formation
#12 PBS	0.40	450	Near injection site (No metastasis)
#1 IgG1-iS18 (P)	1.74	2363	Multiple tumours near abdomen
#2 IgG1-iS18 (P)	-	-	Terminated due to bleeding (no tumours were found)

# 6 IgG1-iS18 (P)	-	-	Terminated due to bleeding (no tumours were found)
#4 IgG1-iS18 (T)	0.82	1125	Large tumour (no metastasis)
#5 IgG1-iS18 (T)	0.41	1350	Near injection site
#10 IgG1-iS18 (T)	0.18 0.22	125 550	Near injection site

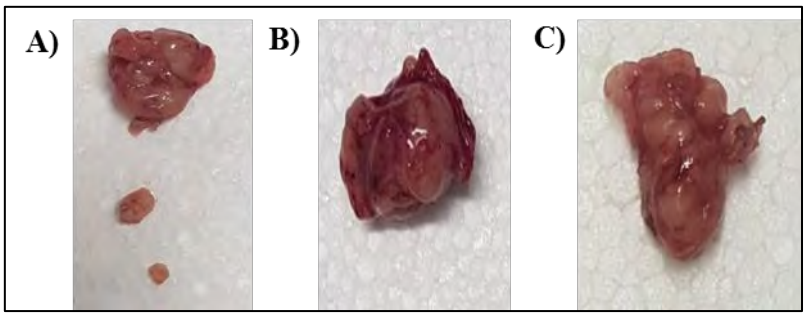


Figure 54: Representation of extracted tumours of the MF-1 nude mice from pilot study 3. A) Three tumours found in the untreated mouse 3. B) One tumour found in PBS-treated number 6 mouse. C) Multiple tumours at one site in IgG1-iS18-treated number 1 mouse and appear to be larger than untreated and PBS-treated tumours. Thus, it was concluded that the treatment IgG1-iS18 at 5mg/kg of body weight was unsuccessful in exerting an effect on tumour shrinkage and metastasis.

Due to the results from the numerous pilot studies and time constraints, it was decided to not continue with the full study.

Chapter 5: Discussion

LRP/LR has gained a large amount of interest due to the many roles it plays in the cell. Particularly, the receptor's over-expression in several cancer cell types as well as its contribution in tumourigenesis has become a target area for research. LRP/LR has been seen to assist with several tumourigenic processes including tumour adhesion and invasion (metastasis), angiogenic enhancement, tumour cell viability maintenance and apoptotic evasion [34]. Additionally, since LRP/LR is not limited to the cell surface but also localized in the perinuclear region, cytosol and nucleus, it is able to perform many intracellular and extracellular physiological roles including maintaining cell viability [42], cell adhesion, cell growth and migration [293], cell cycle regulation, ribosomal anchorage to microtubules, pre-rRNA processing and protein synthesis [87]. Cancerous cells are therefore found to characteristically over-express LRP/LR, thereby exploiting these functions, which ultimately leads to the development of the abovementioned tumourigenic processes. However, several studies have revealed that by blocking LRP/LR with anti-LRP/LR specific antibodies, adhesion and invasion of cancer cells is significantly impeded [33, 111-113, 123, 133]. In addition, it has also been found that down-regulation of LRP/LR via siRNA technology is successful in inhibiting cancer cell viability in several cell lines including liver (Hep3B) [193], cervical (HeLa) [129], lung (A549) [129], breast (MCF-7 and MDA-MB231) [127], oesophageal (WHC01) [127], neuroblastoma (IMR-32) [128], pancreatic (AsPC-1) [128] cancer cells as well as early (A375) and late (A375SM) stage malignant melanoma cells [126]. Thus, targeting LRP/LR with specific antibodies and siRNAs may be promising therapeutic tools for the treatment of cancer. Moreover, since every form of cancer has its own characteristics, it is necessary to evaluate whether LRP-specific antibodies and siRNAs are effective in all cancer cell types hence, early (SW-480) and late (DLD-1) stage colorectal cancer cells were investigated in the current study.

5.1. *In vitro* study

5.1.1. siRNA-mediated knock-down of 37 kDa LRP

To gain insight into how LRP/LR maintains cell viability, siRNA technology was used to knock-down LRP expression (Fig. 22) and to evaluate this effect on cell viability of SW-480 and DLD-1 cells (Fig. 25). The ON-TARGETplus Human-RPSA siRNA was employed to down-regulate LRP in this study since it only targets the mRNA of the 37kDa laminin receptor

precursor (LRP) form using its ON-TARGETplus alteration which allows for the reduction of any off-target effects (Refer to Table A1). Western blotting was employed to detect knocked-down LRP expression levels by using the anti-LRP/LR specific antibody IgG1-iS18, which is known to only detect the 37 kDa LRP form. Cells transfected with the Human-RPSA siRNA resulted in significant decreases in LRP levels for SW-480 and DLD-1 cells, respectively, when compared to non-transfected cells – and as a result confirming the efficacy of the Human-RPSA siRNA. To confirm this result, quantification of LRP mRNA expression levels were investigated using qPCR. It was found that Human-RPSA-transfected DLD-1 cells showed a significant reduction in relative LRP mRNA expression levels, when compared to non-transfected cells (Fig. 23), thereby solidifying the western blot results. As mentioned above, it was found that DLD-1 cells have greater total LRP expression levels compared to SW-480 cells and when both cell lines were treated with the anti-LRP/LR specific antibody, IgG1-iS18, the DLD-1 cells had the greatest reduction in adhesive and invasive potential when compared to untreated cells [111]. This was probable since the antibody is targeted against LRP/LR and the DLD-1 cells were found to have higher amounts of LRP/LR, thus enabling the antibody to have a greater effect. Accordingly, the results obtained from this study of which the Human-RPSA siRNA showed a greater down-regulation in LRP expression in DLD-1 cells confirmed the findings of Vania et al. (2016) [111]. Furthermore, a high correlation was observed between total levels of LRP before and after Human-RPSA transfection for both cell lines (Table A12). This high and positive correlation suggests that the level of LRP expression is indeed influenced by siRNA treatment.

From the results obtained (Fig. 22), the Human-RPSA siRNA showed specificity for LRP in comparison to the negative control esiRNA-RLUC, which had a negligible effect on LRP expression. To validate that the observed knock-down was not due to off target effects, SW-480 and DLD-1 cells were both transfected with an alternative siRNA, known as esiRNA-RPSA. Unlike the Human-RPSA siRNA that targets four different regions of the 37 kDa LRP mRNA sequence, this siRNA only targets a specific region i.e. nucleotides 521-929 (See Table A1). Upon transfection with esiRNA-RPSA, both cell lines showed significant decreases in LRP expression in contrast to cells that were not transfected (Fig. 26). In addition, the correlation between total LRP levels before and after esiRNA-RPSA treatment was found to be high (Table A13). Thus, with both siRNAs yielding similar effects, it can be said that the data obtained upon transfection with Human-RPSA was a direct consequence of LRP knock-down and was not an off-target effect. As mentioned above, esiRNA-RLUC was also used as

the negative control and was found to exert no significant change in LRP expression in SW-480 and DLD-1 cells. This therefore also proposes that the esiRNA-RPSA siRNA has high specificity for LRP.

5.1.2. Relationship between cellular viability maintenance and LRP expression

To further investigate the receptor's role in maintaining cell viability, MTT assays were employed to investigate the effect of transfection with both Human-RPSA and esiRNA-RPSA siRNAs on cell viability. Upon Human-RPSA siRNA transfection, a significant decrease in viability was observed for both SW-480 and DLD-1 cells after LRP down-regulation, when compared to non-transfected cells (Fig. 25). These reductions in cellular viability correlate with the decreased levels of LRP observed after siRNA-mediated down-regulation, signifying the receptor's vital role in the maintenance and survival of SW-480 and DLD-1 cancer cells (Table A12). Likewise, treatment with the alternate esiRNA-RPSA siRNA resulted in significant reductions in both SW-480 and DLD-1 cell viability, when compared to non-transfected cells (Fig. 26). In addition, a high correlation was also found between esiRNA-RPSA-mediated LRP knock-down and decreases in cell viability induced by esiRNA-RPSA transfection in SW-480 and DLD-1 cells (Table A13). This result coincides with that of cells transfected with the Human-RPSA siRNA thus, reiterating the receptor's key role in maintaining cellular viability.

Moreover, upon transfection with the negative control esiRNA-RLUC, no significant effect was seen in SW-480 and DLD-1 cells thereby proving the high efficiency of this negative control as well as confirming the specificity of the target siRNAs used in this study. In addition, the cells were treated with a known apoptotic inducer called protocatechuic acid (PCA) which thereby served as the positive control. Post treatment of SW-480 and DLD-1 cells with 8 mM PCA, decreases in cellular viability were found which paralleled that of the cells treated with Human-RPSA and esiRNA-RPSA, as expected.

Studies have shown that tumourigenic development and progression is aided through apoptotic evasion occurring from the over-expression of LRP/LR, known to play a critical role in maintaining cellular viability [126-128]. As mentioned earlier, it has been discovered that LRP/LR localised in the nucleus allows for chromosome stability and as a result cellular viability maintenance via interactions with the Midkine heparin-binding growth factor; well-known for enhancing cell proliferation, migration and survival [179]. In addition, several cancer types are showed to have an up-regulated expression of Midkine, which results in the promotion of cell survival factors as well as obstruction of apoptosis through caspase-3

inhibition [294]. However, recent studies have shown that the knock-down of LRP/LR in breast [127], oesophageal [127], pancreatic [128], neuroblastoma [128] as well as early and late stage malignant melanoma cells [126] leads to significant decreases in cellular viability through the induction of apoptosis. Thus, it can be said that by targeting LRP expression through siRNA technology, LRP/LR-Midkine interactions may decrease, and as a result decrease cell viability. Additionally, a study performed by Lu et al. (2016) demonstrated that siRNA-mediated knock-down of LRP/LR led to the knock-down of the anti-apoptotic protein Bcl-2 while an up-regulation of the pro-apoptotic Bax protein [191]. This is indeed a favourable result for anti-cancer treatment since the capacity for cancer cells to survive is reduced and increases avenues of investigations regarding knock-down of LRP/LR via siRNA technology.

5.1.3. The effect of LRP knock-down on apoptotic induction

5.1.3.1. Alterations in nuclear morphology

To establish whether the observed decrease in SW-480 and DLD-1 cell viability post LRP knock-down was due to cell death caused by apoptosis, confocal microscopy with the addition of Airyscan™ was used to evaluate nuclear morphology. Upon treatment with Human-RPSA, both cell lines revealed several changes in nuclear morphology which were all characteristic of apoptosis including: nuclear condensation, reduced nuclear size and the formation of membrane-bound bodies (Fig. 27 and Fig. 28). These alterations were further confirmed by the PCA positive control-treated cells thereby proposing that siRNA-mediated knock-down of LRP results in apoptotic induction. It is known that nuclear structures are maintained via the binding of histones to perinuclear and nuclear LRP/LR, thus when the receptor is down-regulated, loss of membrane integrity and distorted nuclear morphology is evident [97]. Moreover, disruptions within the structure of DNA is a distinctive characteristic of cells that are undergoing apoptosis [82]. Hence, the observed alterations to the nuclear morphology of SW-480 and DLD-1 cells induced by downregulation of LRP are indeed suggestive of apoptotic induction in these colorectal cancer cell lines. These findings are consistent with various other studies which have found that reduced LRP expression via siRNA technology causes morphological alterations in several cancer cell lines including breast [127], oesophageal [127], lung [129], cervical [129], pancreatic [128], neuroblastoma [128] as well as early and late stage malignant melanoma cells [126]. It must be noted that upon transfection of SW-480 and DLD-1 cells with the negative control esiRNA-RLUC, no visible alterations in

nuclear morphology were observed, confirmed by several similarities to the non-transfected cells.

5.1.3.2. Apoptotic induction confirmation through Annexin V/PI assays

Although confocal microscopy provided a visual indication that apoptosis was occurring, additional quantification and affirmation of apoptotic induction in SW-480 and DLD-1 was needed. This was made possible by means of Annexin V/PI assays.

As previously described, apoptotic induction leads to reduced cell membrane integrity together with a membrane-flip reaction that influences the phospholipid asymmetry of the cell membrane [295]. It was found that non-transfected and negative control esiRNA-RLUC-transfected SW-480 and DLD-1 cells showed negative staining for Annexin-V, thus appearing in the lower left quadrant, indicative of live cells (Figs. 29and 30). On the other hand, Human-RPSA-treated and PCA-treated SW-480 and DLD-1 cells exhibited positive staining for Annexin-V or Annexin-V and PI, therefore appearing in the lower and upper right quadrants, indicating early and late stage apoptosis, respectively (Figs. 29 and 30). This shift of Annexin-V staining from negative to positive shows that Human-RPSA siRNA-mediated down-regulation in SW-480 and DLD-1 cells activates membrane asymmetry loss, specifically membrane blebbing, as well as a membrane-flip reaction involving the externalization of phosphatidylserine (PS) on the outer leaflet of the plasma membrane – allowing these cells to be taken up by phagocytes [296]. Thus, these findings together with the nuclear morphological changes observed, confirm that siRNA-mediated down-regulation of LRP/LR leads to the induction of apoptosis in SW-480 and DLD-1 cells. More specifically, both SW-480 and DLD-1 colorectal cancer cells treated with Human-RPSA revealed that most of the cells underwent late apoptosis (Fig. 31). In addition, the correlation (Table A12) between total levels of LRP after Human-RPSA treatment and total levels of apoptosis for both cell lines were found to be high, further reiterating that LRP expression affects cell viability and apoptosis. These results corroborate previous studies performed which show that several cancer cell lines undergo early and late apoptosis through siRNA-mediated knock-down of LRP/LR [126-129]. Both cell lines experienced small amounts of necrosis appearing in the upper left quadrant, post Human-RPSA siRNA treatment. A possible reason for this result is that the 72-hour transfection period may be causing some of the non-transfected cells to undergo necrosis. However, confirmation of this theory would require time-dependent studies to determine if a similar outcome is observed when the transfection period is reduced.

5.1.4. The effect of down-regulated LRP on apoptotic pathways

Studies on nuclear morphology through confocal microscopy together with Annexin-V-FITC/PI assays confirmed that siRNA-mediated knock-down of LRP results in the induction of apoptosis in SW-480 and DLD-1 cells. However, additional caspase-3 assays were completed in order to further prove the apoptotic inducing effects of Human-RPSA-mediated LRP knock-down. This is due to Sustantad et al. (2008) observing that when LRP/LR is down-regulated via siRNA technology, not only did the cell viability of cervical cancer (HeLa) cells and lung (A549) cancer cells decrease but caspase-3 activity was increased in both cell lines [193]. Caspase-3, an effector caspase, is responsible for executing the hallmarks of apoptosis which includes the afore-mentioned nuclear morphological changes by cleaving substrates [297]. Therefore, upon apoptotic induction, caspase-3 activity is expected to be significantly increased. Upon down-regulation of LRP with Human-RPSA, a distinct increase in caspase-3 activity was observed in SW-480 and DLD-1 cells when compared to cells that were not transfected (Fig. 32). Furthermore, the correlation between the total levels of LRP after Human-RPSA-mediated knock-down (Fig. 22) and increases in caspase-3 activity (Fig. 32) was high in both cell lines (Table A12). These results noticeably point to the induction of apoptosis in SW-480 and DLD-1 cells due to the silencing of LRP through siRNA technology, which could perhaps be due to the reduction of the previously mentioned interaction between LRP/LR and Midkine.

However, it has also previously been shown that interactions between LRP/LR and focal adhesion kinase (FAK) are made possible via the binding of the receptor to laminin-1. These LRP/LR-FAK interactions were seen to be involved in activating cell signalling cascades such as MEK/ERK 1/2 and PI3-kinase/AKT as well as up-regulating the anti-apoptotic protein, Bcl-2 [180]. This ultimately led to the inhibition of apoptosis of cancerous cells. Hence, we suggest that silencing LRP/LR using siRNA technology as performed in the current study, could be impeding the LRP/LR-FAK interaction, and in this way, inducing apoptosis. Furthermore, LRP/LR has been shown to have a direct relationship with the MAPK signalling pathway – where decreased levels of LRP causes a response in the pathway – resulting in cell stress and ultimately, cell death [298]. Another reason which could have led to apoptotic induction through siRNA-mediated knock-down of LRP is the receptor's role in ribosomal processing. Research has shown that LRP/LR is involved in processing 21S pre-rRNA into mature 18S rRNA, also known as the biogenesis of ribosomes [96]. Moreover, LRP/LR has also been

shown to associate with pre-40S ribosomal subunits, providing nucleolar exits for the subunits, thereby facilitating protein synthesis [299]. This suggests that the LRP down-regulation performed in this study may have hampered formation of the ribosome as well as the resultant translation of proteins required for correct cellular functioning and survival, ultimately leading to apoptosis. Furthermore, it has been found that the translation of cytoskeletal proteins such as tubulin is carried out by ribosomes [98] and LRP/LR is known to tether tubulin to the ribosome [98, 300]. Thus, down-regulation of LRP/LR in this study could also possibly be impeding this interaction, leading to reduced translation and apoptosis.

In addition, DLD-1 cells were found to have a larger increase in caspase-3 activity in comparison to SW-480 post Human-RPSA transfection. This finding once again shows that LRP/LR plays a vital role in maintaining cellular viability and confirms that higher levels of LRP expression results in increased levels of apoptotic induction. Moreover, a high correlation was found between total levels of siRNA-mediated knock-down of LRP and increases in caspase-3 activity for both SW-480 and DLD-1, further supporting this suggestion (Table A12).

Both the MEK/ERK 1/2 and PI3-kinase/AKT cell signalling cascades are found to inhibit the caspase-8 and -9 apoptotic pathways. Caspase-8 plays a vital role in the extrinsic apoptotic signalling pathway known to initiate apoptosis through transmembrane receptor-mediated interactions which involve death receptors of the tumour necrosis factor (TNF) receptor gene superfamily [56]. On the other hand, caspase-9 is critical in the intrinsic apoptotic signalling pathway which initiates apoptosis through a diverse collection of non-receptor-mediated stimuli – which include toxins, free radicals and hypoxia – that all produce intracellular signals that act directly on cellular targets [56]. These events are mitochondrial-dependent. Thus, investigations on both apoptotic pathways were needed to determine whether caspase-8 and -9 become activated once LRP/LR is down-regulated. Upon Human-RPSA-mediated knock-down of LRP/LR, both SW-480 and DLD-1 colorectal cancer cell lines were found to have higher caspase-8 activity, in contrast to cells that were not transfected (Fig. 33). Moreover, this study revealed that Human-RPSA transfected SW-480 cells and DLD-1 also have increased in caspase-9 activity after LRP down-regulation, in contrast to cells that were not transfected (Fig. 34), indicating that both colorectal cancer cell lines undergo caspase-8 and -9 activation [125]. This is highly likely as the extrinsic and intrinsic pathways can interconnect at numerous levels and as a result, become influenced by similar factors. In fact, a study showed that activated extrinsic caspase-8 stimulates the release of cytochrome *c* and apoptosome formation,

respectively, and ultimately activation of the intrinsic pathway [301, 302]. Another possible reason as to why both apoptotic pathways are activated may be that these colorectal cancer cells undergo a mechanism known as retaliatory caspase activation where the two apoptotic pathways are found to use a feedback amplification loop in order to activate one another [303]. Specifically, activated caspase-9 initiates and proteolytically cleaves caspase-3, also leading to caspase-8 activation. Moreover, due to SW-480 and DLD-1 cells undergoing both apoptotic pathways, it can be said that down-regulated LRP/LR possibly hampers both anti-apoptotic signalling pathways on account of the reduced interaction of phosphorylated FAK and LRP/LR.

5.1.5. The effect of down-regulated LRP on the cell cycle

The cell cycle is a sequence of events involving cell replication followed by cell division. In particular, the eukaryotic cell cycle is divided into four main phases including: Gap phase 1 (G1); DNA synthesis (S); Gap phase 2 (G2) cell division preparation; and mitosis (M) where chromosomes are able to separate and the cell divides. However, the events of the cell cycle are observed at specific checkpoints known as the G1/S point, the S-phase point and the G2/M point [304]. This system allows for cells to proliferate only when stimulatory signals like growth factors are present. However, it has been found that DNA damage is able to activate this system by causing cell cycle arrest to repair the damage made to the cell. Once DNA repair has occurred, cell cycle progression continues however, if the damage could not be repaired, the cell is removed via the process of apoptosis [305]. Thus, cell cycle arrest at any stage of cell division occurs before apoptotic induction [306]. If this cell cycle system is disrupted, this leads to the development of cancer.

The four phases can be analysed through flow cytometry in order to gain insight into cell progression. In addition to the four phases, another phase known as the sub G0/G1 apoptotic phase can also be analysed. Cells undergoing apoptosis are known to experience DNA fragmentation, thereby losing some of their DNA content, in late stage apoptosis following endonuclease activity [307]. Thus, by using the propidium iodide (PI) to stain the DNA, the number of hypodiploid cells undergoing the apoptotic process can be quantified in the sub G0/G1 phase of a PI histogram [307, 308]. In this study, the DLD-1 colorectal cancer cells were transfected with Human-RPSA for 3-days and were found to undergo cell cycle arrest in the sub G0/G1 phase, when compared to non-transfected cells (Fig. 35). However, it must be noted that apoptosis is a dynamic, time-dependent process during which apoptotic cells show

their characteristic features [307]. Thus, to determine whether the number of cells undergoing apoptosis would increase, the DLD-1 cells were subjected to a 5-day transfection with Human-RPSA and it was found that there was a significantly larger number of cells in the sub G0/G1 phase, when compared to the non-transfected cells (Fig. 36). Moreover, another research group also used siRNAs to target LRP/LR and they found that by down-regulating the receptor for a 4-day period, fibrosarcoma (HT1080) cells were predominantly arrested in the G1 phase of the cell cycle and only 9% of cells appeared in the sub G0/G1 phase [181, 309]. From this, it can be seen that siRNA-mediated knock-down of LRP/LR is largely dependent on the cell line of interest, the degree of knock-down as well as the transfection period. Thus, further time-dependent and confirmation studies in various cancer cell lines must be performed to gain insight into LRP/LR's function in the cell cycle. In saying that, the increase in the sub G0/G1 population from this study together with increased levels of apoptosis (Figs. 35 and 36) and caspase-3 activity (Fig. 32) as well as compromised membrane integrity (Figs. 27 and 28), confirm that the down-regulation of LRP/LR with the Human-RPSA siRNA leads to the induction of apoptosis in DLD-1 colorectal cancer cells.

5.1.6. The effect of LRP knock-down on telomerase and telomere-related proteins

As mentioned previously, LRP/LR has been found to interact with the ribonucleoprotein, telomerase, in both tumorigenic and non-tumorigenic cells [108, 122]. Telomerase is made up of two essential components, namely: the reverse transcriptase catalytic component, hTERT, which is responsible for the addition of telomeric repeats; and the functional RNA component, hTERC, which serves as the telomeric template [139]. Telomerase mainly functions to elongate telomeres which are found on the ends of chromosomes to help prevent chromosomal degradation [139].

There is an increasing amount of evidence that suggests telomerase is also implicated in cancer by actions beyond its function in maintaining telomeres [44]. Furthermore, it has also been shown that telomere dysfunction and elevated levels of telomerase activity are associated with cancer recurrence and chemotherapeutic resistance [172], reiterating the important role that telomerase has in cancer progression. Thus, there have been several studies performed to inhibit or down-regulate telomerase for the treatment of cancer. Moreover, since most somatic cells do not possess detectable telomerase activity, inhibiting telomerase should have little off-target effects. In order to measure the catalytic subunit TERT protein expression levels, western

blotting was employed. Upon down-regulation of LRP/LR with the Human-RPSA siRNA in DLD-1 cells, it was revealed that there was a significant decrease in hTERT mRNA expression levels, in comparison to non-transfected cells (Fig. 37). To confirm this result, SDS-PAGE and western blotting was employed to analyse protein expression. It was revealed that DLD-1 cells transfected with Human-RPSA siRNA had a significant reduction in in hTERT protein expression levels, when compared to non-transfected cells (Fig. 38), suggesting that LRP/LR may indeed influence hTERT expression.

As mentioned previously, telomere capping is a highly important process in protecting the ends of chromosomes from being recognised as a DNA break by DNA damage response enzymes since this could lead to undesired responses such as unnecessary apoptosis or senescence of functional genetic material [310]. However, a study showed that by inhibiting telomerase, telomere maintenance and telomere dysfunction was decreased, ultimately leading to the induction of apoptosis [150]. Specifically, this loss of telomerase activity caused progressive telomere shortening and led to the inactivation of the telomere capping function of the shelterin complex. As a result, the ends of chromosomes fused together, leading to genetic instability and the activation of the p53-mediated DNA damage response pathway, ultimately resulting in apoptosis [310]. In addition, the catalytic subunit of telomerase, hTERT, has been found to play a role in regulating apoptosis, independent of telomere maintenance and telomerase activity. It has been demonstrated both *in vitro* and *in vivo*, that there is a direct association between TERT and an increased resistance to apoptosis [311]. Moreover, hTERT overexpression has been found to make cells more resistant to apoptosis [312]. The study therefore also showed that telomerase expression did not prevent stress-induced senescence in normal human fibroblasts, but rather protected the cells from apoptosis [312]. Another recent study showed that the knock-down of hTERT through siRNA technology in cervical (HeLa) and colon (HCT116) cells, resulted in the activation of pro-apoptotic protein Bax, which triggered the mitochondrial cell death pathway [151]. Furthermore, this did not occur as a result of telomerase inhibition, as there was no telomere erosion, ultimately suggesting a role of TERT in preventing apoptosis [151]. In addition, it has also been found that the down-regulation of LRP/LR via siRNA technology does not only lead to the induction of apoptosis [126-128] but also to a reduction in telomerase activity [122]. This suggests that hTERT may be a promising target as a double-hit anticancer therapeutic strategy since its inhibition not only limits tumour cell proliferation but also induces an acute apoptotic effect, potentially resulting in a rapid chemosensitising effect [151].

Another protein that was investigated was pTERT, the phosphorylated/activated form of the TERT telomeric subunit. Evidence shows that the phosphorylation of TERT at Serine residue 824 via Akt kinase is a pre-requisite for telomerase activity to occur [313] and if TERT is dephosphorylated, telomerase activity is halted [314]. Upon Human-RPSA-mediated down-regulation of LRP/LR in DLD-1 cells, pTERT protein expression levels were found to be significantly reduced, when compared to non-transfected cells (Fig. 39). Moreover, a telomerase activity assay was conducted to evaluate LRP/LR's effect on telomerase activity, and it was found that the DLD-1 cells transfected with Human-RPSA resulted in a significant decrease in telomerase activity (Fig. 40). In addition, a high and positive correlation of R=0.91 was found between pTERT protein expression levels and telomerase activity post knock-down with the Human-RPSA siRNA. These findings suggest that LRP/LR does indeed influence both hTERT and pTERT expression levels, resulting in the receptor influencing telomerase activity.

The knock-down of LRP/LR with Human-RPSA significantly reduced telomerase activity in DLD-1 colorectal cancer cells, and should this state be maintained it could effectively prevent the preservation of the shorter telomeres characteristic of tumorigenic cells. This would therefore lead to widespread telomere erosion, which could not only increase the susceptibility for the occurrence of crisis in the cells but also further enhance chemosensitivity [172]. This enhanced chemosensitivity would arise from the loss in genomic protection that the telomeres convey, thereby allowing chemotherapeutic drugs to induce widespread genomic instability and damage more effectively. In addition to the knockdown of LRP/LR influencing telomerase's function of maintaining telomere length, it may also influence the extra-telomeric functions of TERT. TERT also plays critical roles in angiogenesis, inflammation, cell viability as well as the EMT process, which are all found to contribute in tumourigenesis if left unregulated. Thus, by down-regulating TERT through siRNA-mediated knockdown of LRP/LR in cancer cells, it is proposed that these processes may also be impeded. This ultimately suggests that the down-regulation of LRP/LR may be a potential therapeutic intervention for cancer through the resultant down-regulation of telomerase activity, due to the relationship between LRP/LR and TERT.

Two other proteins which play a role in telomere biology and cancer are the telomere repeat-binding factors 1 (TRF-1) and 2 (TRF-2). These proteins, together with four other proteins, make up the complex known as "shelterin" which has a high specificity for telomeres [155].

The shelterin proteins' functions include the maintenance of telomere length, development of t-loop formation, recruitment of telomerase to the ends of telomeres and lastly, protection of chromosomal ends [155]. Moreover, telomere sequences are found to be directly bound to TRF-1 and TRF-2 [163]. There have been several studies suggesting that TRF-1 and TRF-2 negatively regulate telomere length since they are involved in the formation of the t-loop and limit telomere elongation [154, 315, 316]. However, despite there being extensive homology between TRF-1 and TRF-2, the proteins are not found to heterodimerize and seem to form different complexes at telomere ends, resulting in independent functions [317].

Specifically, TRF-1 is found to either limit the access of telomerase to telomeres thereby mediating the interaction of telomeres with mitotic spindles [154] or block further telomerase-mediated elongation in long telomeres, resulting in a reset of the telomere length [163]. In addition, TRF-1 is also found to play roles in DNA remodelling as well as DNA replication regulation [163]. Studies have found that the overexpression of TRF-1 leads to a steady decrease in the length of telomeres owed to its inhibition of telomerase, while down-regulation of the protein leads to the elongation of telomeres [163]. TRF-2 on the other hand has been found to have no influence on telomerase and primarily functions in stabilizing t-loop formation thereby protecting telomeric ends from DNA damage but has been found to play a role in chromatin assembly [164]. Interestingly, a study showed that telomerase ensures that the TRF-2-binding sites at chromosomal ends are constantly present by producing several telomere repeats [318]. This is due to the fact that TRF-2 is known to prevent fusion of chromosomal ends, hence telomerase preserves the protection and elongation of telomeres by continuously replacing lost TRF-2-binding sites which occurs from DNA replication. However, other studies have shown that the overexpression of TRF-2 results in progressive telomere length shortening resulting from either telomere uncapping or through a proposed mechanism that is telomerase-independent, whereby overexpressed TRF-2 stalls the replication of telomeric sequences leading to the formation of thin telomeric bridges which arise during chromosomal segregation. The formation of these telomeric bridges result in the increase of chromosomal fusion, ultimately leading to genetic instability and cancer [319]. The down-regulation of TRF-2 can also lead to chromosomal ends fusing together which can either activate DNA damage responses to induce cell cycle arrest [318] or cause genetic instability that results in cancer development [154]. Thus, by manipulating the expression of these telomere-binding proteins, the function of the telomere can be impaired resulting in senescence induction, cell cycle arrest or apoptosis [315]. A number of studies have shown that the down-

regulation of these proteins are found in gastric cancers [320], malignant hematopoietic cells [169] and breast cancers [170]. Conversely, other studies show that up-regulation of these proteins are present in lung cancer [167], lymphomas [321] and gastric carcinomas [168]. Thus, an investigation on whether these telomeric proteins have a relationship with LRP/LR, a known player in cancer development, was incited to uncover the mechanistic role of the receptor and possible novel roles of TRF-1 and -2.

Upon Human-RPSA-mediated knock-down of LRP/LR in DLD-1 cells, western blotting revealed no significant change in TRF-1 protein expression levels, when compared to non-transfected cells (Fig. 41), suggesting that there is no relationship between LRP/LR and TRF-1. A study showed that TRF-1 up-regulation together with an increase in telomerase activity, ultimately leads to the formation of gastric carcinomas [322]. While another study showed that the knock-down of TRF-1 was significantly related to cancer development and poor prognosis in oral cavity squamous cell carcinomas (OCSCC) [323]. Thus, this shows that a balance of TRF-1 expression levels is required for correct functioning of cells. Schneider et al. (2013) showed that TRF-1 plays a crucial role in maintaining pluripotency and tissue homeostasis, highlighting the need for TRF-1 not only in telomere protection but also in adult tissue regulation [324]. In addition, the result obtained from this current study is in accordance with van Steensel and de Lange (1997) which showed that TRF-1 expression does not affect the expression of telomerase and that TRF-1 binding controls telomere length by acting in cis to hamper telomerase at individual telomere ends [316].

In contrast to the findings for TRF-1, a significant reduction in TRF-2 protein expression levels was observed in DLD-1 cells upon Human-RPSA-mediated LRP/LR knock-down, in comparison to non-transfected cells (Fig. 42). A study showed that the up-regulation of TRF-2 resulted in telomerase-positive cells having progressively shortened telomeres, which thereby aided in genetic instability and the cancer development [325]. These findings also show that TRF-2 expression does not influence telomerase expression, since cancer development was not due to telomere elongation (which is normally performed by telomerase). Moreover, it has been found that the over-expression of TRF-2 results in the inhibition of the ATM (ataxia telangiectasia mutated kinase) DNA damage response, resulting in the reduction of Bax and p21 proteins, ultimately leading to the reduction of p53-mediated apoptosis [326]. As a result, Pal et al. (2015) showed that by down-regulating TRF-2 via siRNA technology, the cell viability of renal (A498) carcinoma cells decreased due to increased genomic instability, ultimately leading to apoptotic induction and cell cycle arrest [327]. Furthermore, another study

showed that TRF-2 expression levels were notably over-expressed in SW-480 colorectal cancer cells and siRNA-mediated knock-down of the protein reduced tumorigenesis [328]. Taken altogether, these findings point to TRF-2 being critical for protecting telomeric ends from uncapping as well as maintaining the structure of telomeres since disrupting TRF-2 results in dysfunctional telomeres, resulting in genomic instability and p53-mediated apoptosis. Moreover, in the current study, significant increases in caspase-9 activity in Human-RPSA transfected DLD-1 cells, a caspase known to involve p53 when inducing apoptosis was observed (Fig. 34). This suggests that down-regulated LRP/LR and the subsequent down-regulation of TRF-2 in DLD-1 cells may play a role in enhancing p53-mediated apoptosis.

Another way in which LRP/LR could be linked to TRF-2, is through protein interactions with TERT and p53. In this regard, p53 has been found to influence the expression of both TERT and LRP via transcriptional repression in order to limit proliferation [173, 174]. Additionally, p53 has also been found to affect TERT, telomerase activity as well as TRF-2 for apoptotic induction or cell cycle arrest [175]. Consequently, a decrease in LRP/LR, TERT and TRF-2 (which all share an antagonistic relationship with p53) may allow the elevation of p53 and subsequent induction of apoptosis in the cancerous cells. Furthermore, as both LRP and TERT have been intricately linked to proliferation pathways like the MAPK pathway [176, 177], knockdown of LRP and subsequently TERT and TRF-2, could severely impact the aggressive and rapid proliferation observed in cancer cells. In addition to this, LRP/LR could also be linked to TRF-2 through the association of telomerase with TRF-2. Thus, by targeting LRP/LR with siRNA technology, its consequent effects on TERT/telomerase and TRF-2, will compromise multiple key cancer hallmarks including invasion, apoptotic and growth suppressor evasion as well as replicative immortality. Presented below is a theoretical model of the effects that siRNA-mediated knockdown of LRP/LR has on telomerase, TRF-1 and TRF-2 (Fig. 55).

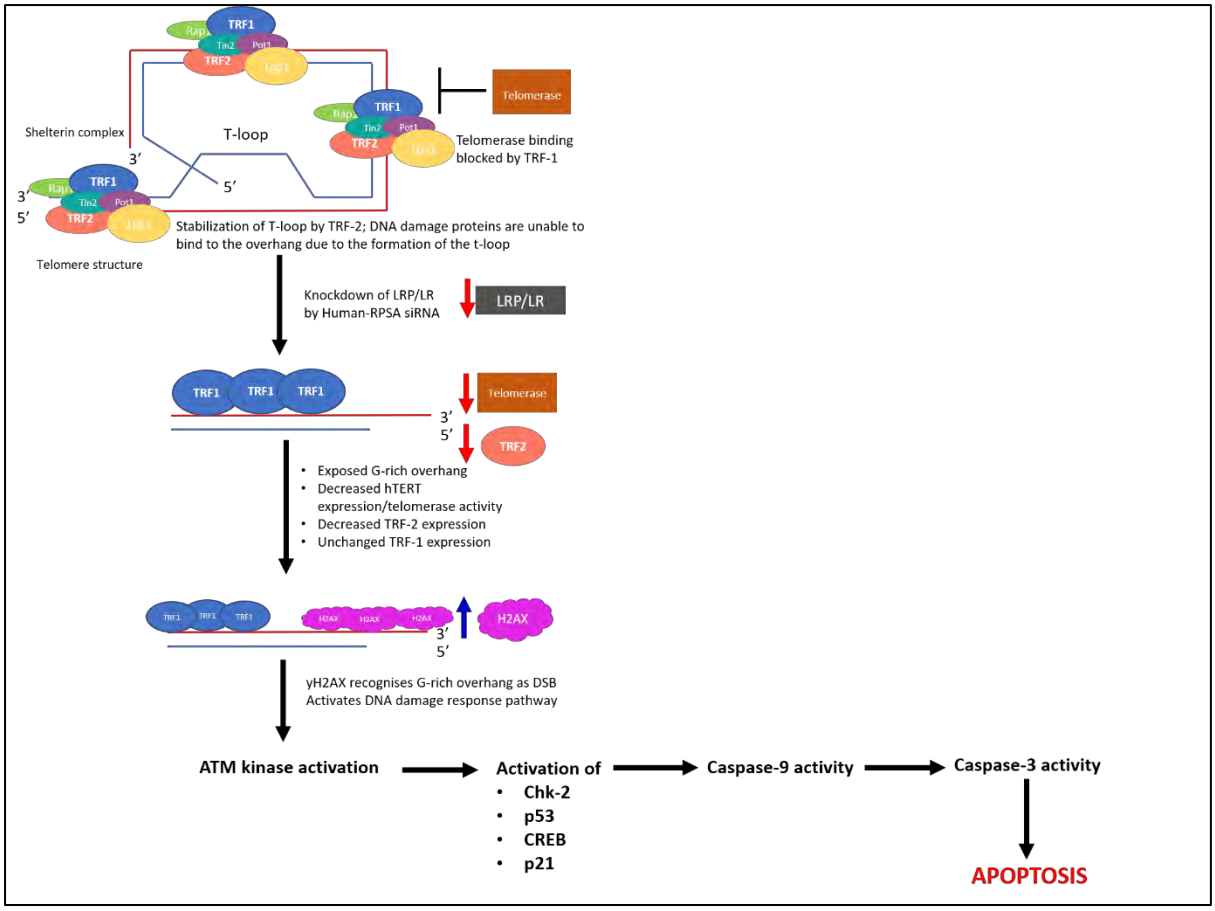


Figure 55: Schematic of possible mechanism of Human RPSA siRNA on LRP/LR, telomerase, and telomere-related proteins, TRF-1 and -2. Under normal conditions, the shelterin complex consisting of TRF-1 and TRF-2 binds to telomeres and negatively regulates telomere length by forming the t-loop structure at the ends of chromosomes to mask the G-rich overhang. TRF-1 is found to inhibit the binding of telomerase to telomere ends while TRF-2 functions to stabilize the formation of the t-loop. Upon siRNA-mediated knockdown of LRP/LR, TRF-2 protein expression levels are decreased, leaving the ends of chromosomes exposed and free to fuse with each other, which can lead to genetic instability. The knockdown of LRP/LR was also found to decrease telomerase activity and hTERT expression levels, causing progressive telomere shortening and the inactivation of the telomere capping function of the shelterin complex, further contributing to chromosomal end fusion and genetic instability. This leads to the G-rich overhang being exposed and recognised as a double stranded break by γH2AX. This results in the activation of the DNA damage response pathway whereby ATM kinase activated p53, ultimately leading to apoptotic induction.

5.1.7. The effect of knocked-down LRP in cell signalling pathways

5.1.7.1. Proteomic analysis

Proteomics is a reliable, well-known technique used to study a significantly large number of proteins in a single analysis. Proteome Antibody Profiler Arrays™ and SWATH-MS were used to analyse protein expression in the DLD-1 cells. The current study was able to identify over

4000 proteins in the DLD-1 cells using SWATH-MS. The Human-RPSA transfected cells were compared to non-transfected cells and for a more stringent analysis, only those that had a 2-fold increase or greater with a p-value < than 0.05 were considered significant (Fig. 50) based on several previous studies [329-332]. Moreover, Kammers et al. (2015) found that statistical significance is increased for proteins with larger fold changes (i.e. 2 and above) and relatively larger sample variances, thus eliminating false positives [329]. In saying that, several future experiments must be performed to investigate and confirm the proteins found to be significantly affected upon LRP/LR down-regulation in DLD-1 cells.

It was revealed that 84 proteins were affected upon siRNA-mediated knock-down of LRP/LR (Fig. 51), where 41 proteins were down-regulated (Table 1), and 44 proteins were up-regulated (Table 2). The identified up- or down-regulated proteins were grouped based on functions relating to LRP/LR such as ribosomal processing and protein synthesis [96] (Table A5); nucleus and chromatin maintenance [97] (Table A6); cell anchorage and cytoskeletal involvement [98] (Table A7) and lastly, apoptotic regulation [122] (Table A9). Groups such as: vesicle transportation and regulation (Table A8) as well as autophagy (Table A10) were added since several other proteins identified were found to play roles in these processes. Moreover, novel stand-alone proteins have been found to interact with LRP/LR, which could provide additional information on how the receptor works.

Moreover, as mentioned previously, a study performed by Sun et al. (2014) showed that LRP/LR interacts with the MAPK signalling pathway, ultimately resulting in the inhibition of apoptosis of gastric cancer cells [180]. Moreover, the current study confirms that LRP/LR does in fact play a role in apoptotic evasion and cell viability maintenance in DLD-1 cells. Thus, to gain further insight into the molecular mechanism of LRP/LR, MAPK and Apoptosis Proteome Profiler Antibody™ arrays were performed. A phospho-MAPK array kit containing 43 kinase antibody capture sites together with a second MAPK array kit, containing 26 MAPK antibody capture sites were used. Of the 43 phospho-MAPK-related proteins, 10 proteins were found to be differentially expressed upon siRNA-mediated knockdown of LRP/LR however, only 3 of these were found to be significant once densitometry was completed (Fig. 43). In addition, the second MAPK array was found to have 2 differentially expressed proteins, when compared to non-transfected cells upon siRNA-mediated knockdown of LRP/LR (Fig. 44). When compared to the non-transfected cells, it was found that there was a significant change in Chk-2, AMPKα2, β-catenin protein expression levels (Fig. 43). Several other important proteins such

as AMPKα1, FAK, CREB and the various phosphorylation sites of the tumour suppressor p53 were also found to be affected (Fig. 43). With regards to the Human Apoptosis Proteome Profiler Antibody Array™, this array contained 35 antibody capture sites in which 11 proteins were found to be affected upon siRNA-mediated knockdown of LRP/LR (Fig. 45). From the 11 proteins, 9 were found to have a significant change in expression, including pro-apoptotic proteins Bax and p53 as well as several anti-apoptotic proteins.

Although these findings provide a mechanistic insight into LRP/LR, further confirmation with several other experimental techniques should be considered. Nevertheless, observations from both techniques suggest that several proteins involved in apoptosis and cancer suppression were up-regulated, while proteins involved promoting tumourigenesis were down-regulated, proposing that the Human RPSA-siRNA-mediated knock-down of LRP/LR could be a potential anti-cancer therapeutic tool.

5.1.7.1.1. Proteins involved in ribosomal processing and translation

It is known that LRP/LR aids ribosomal processing and protein translation in the cytoplasm due to it being a crucial element of the small 40S ribosomal subunit [96]. Thus, it was expected that several proteins involved in RNA processing and protein synthesis initiation were affected upon Human RPSA-siRNA-mediated knockdown of LRP/LR. In particular, eukaryotic translation initiation factors (eiFs) EIF1 and EIF2B were found to be down-regulated (Table 1). eIFs function to stabilize the formation of the ribosomal complex and to regulate translation initiation [222]. Dysregulation in the expression of eIFs have been shown to result in tumour development. EIF1, shown to be down-regulated by 4-fold when compared to the non-transfected cells (Table 1), directly interacts with the 40S ribosome where it initiates codon selection and prepares the ribosome for mRNA loading [222]. Mutations in this protein have been related to ovarian and thyroid cancers however, it is still unclear as to how EIF1 exactly contributes to cancer formation [333]. EIF2B which was down-regulated by 3-fold when compared to non-transfected cells (Table 1), is found to activate the EIF2 protein known to bind initiator tRNA to the 40S ribosomal subunit [222]. Moreover, up-regulated levels of EIF2 have been found in colon, rectal and stomach cancers [334]. As mentioned above, LRP/LR is a vital component of the 40S ribosome where it is present during the initiation of translation [181]. Since the EIF1 and EIF2 proteins also functioning in the 40S ribosome were found to be

down-regulated upon siRNA-mediated knock-down of LRP/LR, this suggests that the receptor may be interacting with these eIFs to initiate translation of proteins required by tumours.

Moreover, another protein known as RS21, also functioning as a component of the 40S subunit was found to be down-regulated by 2.36-fold (Table 1) upon siRNA-mediated knockdown of LRP/LR, when compared to the non-transfected cells. This finding is consistent with Venticinque and Meruelo (2012) which also showed that LRP/LR associates directly with RS21 [98]. More specifically, LRP/LR directly interacts with RS21 for the processing of the 21S pre-rRNA into the 18S mature rRNA form, thereby characterizing the ribosomal biogenesis function of the receptor [96, 246]. Moreover, from the RNA integrity gels performed, a small visible decrease in both RNA species can be seen in the Human-RPSA siRNA transfected samples compared to the non-transfected samples (Refer to Appendix A). Thus, the down-regulation of RS21 suggests that LRP/LR was successfully knocked-down due to their direct interaction with each other but also the receptor's role in ribosomal biogenesis.

Although several proteins involved in protein translation initiation were down-regulated, a number of proteins were found to be up-regulated (Table 2). Although protein degradation pathways were enhanced and stress-response/apoptotic proteins were up-regulated after LRP downregulation, the cancer cells were still able to synthesise proteins and survive. This could possibly be due to the cancer cells performing a compensatory mechanism known as autophagy. Autophagy is a survival process activated in response to nutrient depletion and metabolic stress [335]. The role of autophagy is seen as a temporary survival mechanism and alternative energy source to sustain survival under stressful conditions [335]. Thus, it could be that the DLD-1 cancer cells may have been able to recycle amino acids through autophagy pathways, thereby promoting temporary protein synthesis and cell survival [336]. This is further confirmed by the large number of up-regulated enzymatic proteins found upon siRNA-mediated knockdown of LRP/LR, a key characteristic of autophagy occurring. This will however be further explained in section 5.2.7.1.6 below.

5.1.7.1.2. Proteins involved in chromatin and nuclear maintenance

As mentioned above, LRP/LR is also found to localize in the nucleus. This is most likely due to LRP/LR's ribosomal biogenesis function by which pre-40S subunits require the receptor for nucleolar exit [96]. LRP/LR performs this by associating with the nucleolar pre-40S ribosomes and nucleolar small nucleolar ribonucleoproteins, thereby allowing for the receptor to be

present in both the nucleus and cytoplasm [96]. Notably, the protein with the largest fold-change of 8.2 was the Heterogeneous nuclear ribonucleoprotein A1 (HnRNPA1) and was found to be down-regulated after LRP knockdown, when compared to the non-transfected cells from the SWATH-MS data (Table 1). HnRNPA1 is a ribonucleoprotein present in the nucleus and has been reported to play key roles in pre-mRNA processing which not only includes splicing and stabilizing the mRNA but also transporting and metabolising it, thus it is deemed a nucleocytoplasmic shuttling protein [243]. In addition, HnRNPA1 has also been interestingly found to play a role in telomerase and telomere biology whereby Ford et al. (2002) showed that both HnRNPA1 and the telomerase ribonucleoprotein co-immunoprecipitated from lung (H1299) cancer cells, confirming their direct interaction [337]. In another study, increased HnRNPA1 was found to disrupt telomeric DNA structure and enhance telomerase activity, while decreased levels of HnRNPA1 significantly decreased telomerase activity in HEK293 cells [338].

Moreover, since HnRNPA1 is present in the nucleus throughout transcription and post-transcriptional modifications of mRNA, it is suggested that the protein may be regulated by oncogenes [243]. In fact, several studies show that the silencing of HnRNPA1 leads to the inhibition of tumour cell invasion [339] and apoptosis induction in various cancer cell lines [340]. Another study showed that experimental remodelling of the ECM metalloproteinase 3 (MMP-3) in mouse mammary epithelial cells resulted in HnRNPA1 inducing the expression of the RAC1b slice variant [341]. This led to an increase in cellular ROS and stimulation of the Snail transcription factor, resulting in epithelial-to-mesenchymal transition induction [342]. Furthermore, there is also evidence that increased expression of HnRNPA1 is directly correlated with an increase in colorectal cancer progression, thereby serving as a potential biomarker for colorectal cancer [343]. Specifically, a study showed that the MAPK extracellular signalling kinase (ERK) became activated in colorectal cancer cells which led to the phosphorylation of splicing factors [344]. The change in the splicing-factor profile triggered the binding of HnRNPA1 to a silencing element, ultimately promoting the epithelial-to-mesenchymal transition [345]. Therefore, these findings together with the results from the current study confirm that LRP/LR is implicated not only in ribosomal biogenesis but also in telomere regulation. Furthermore, since HnRNPA1 showed the largest down-regulation upon siRNA-mediated knockdown of LRP/LR suggests that it significantly depends on LRP/LR to exert its functions and vice versa. Lastly, HnRNPA1 does not only link the interaction of

LRP/LR and telomerase but may also provide a further explanation as to how LRP/LR and the MAPK pathway are related in colorectal cancer cells.

It must be noted that although there was a decrease observed in proteins involved in protein synthesis, there was still an increase in several proteins involved in apoptosis. This is not surprising since in this study, an entire cell population was investigated rather than an individual cell. Apoptosis affects all cells individually and is said to be an asynchronous, time-dependent process [346] which is mainly due to the highly variable duration of the initiation phase of apoptosis in each cell [347]. It has also been shown that apoptosis may only occur in some cells once they have completed the cell cycle and pass through the mitotic phase [346]. As a result, most cells undergoing apoptosis tend to interact with larger numbers of viable cells [346]. This is evident by the Annexin-V/PI data and cell cycle analysis of the DLD-1 cells in that not all cells transfected with the Human-RPSA siRNA were found to be in the apoptotic quadrants (Figs. 29 and 30) and the sub G0/G1 apoptotic phase (Fig. 35 and 36). Thus, from this study it seems that the Human-RPSA siRNA was not only able to down-regulate proteins involved in protein synthesis in some cells, but was also able to induce a large enough response in other cells where they were able to synthesize proteins involved in apoptosis and autophagy due to stress.

Moreover, the inhibition of mRNA or protein synthesis can either enhance or prevent apoptosis, depending on the number of apoptotic stimuli or how prolonged the stress is [348]. In stressed cells and during apoptosis, several translation initiating factors including eIF2 are targeted for biological regulation [349]. In particular, once apoptosis is induced, eIF2 is phosphorylated and is found to be a target of a post-translational modification in that eIF2 itself becomes a substrate for caspases [349]. Caspase-3 synthesises a cleaved version of eIF2, which is still able to interact with other translation initiating factors, however, is not able to stimulate overall translation [350]. Thus, this further illustrates the importance of eIF2 inactivation in completing the apoptotic process.

In a similar way, although HnRNPA1 normally functions in splicing and RNA processing, it is also able to be phosphorylated and cleaved upon stress, resulting apoptosis. In particular, Hermann et al. (2001) found that apoptotic induction resulted in the cleavage of HnRNPA1 and increased expression of caspase activity [351]. Interestingly, another study showed that eIF2 and HnRNPA1 are linked whereby the phosphorylation/decativation of eIF2 is crucial for

the translocation of HnRNPA1 from the nucleus to the cytoplasm, ultimately blocking translation of mRNAs that encode inhibitors of apoptosis [352] - further illustrating why these proteins were down-regulated upon siRNA-mediated knockdown of LRP/LR. Another way that apoptosis could have been induced is through the autophagolysosomal degradation of HnRNPA1 [353]. Fijiya et al. (2014) found that apoptosis was induced in colon cancer cells via micro-RNAs binding to HnRNPA1 and inducing autophagolysosomal degradation of the protein. Since several autophagic proteins were upregulated upon siRNA-mediated knockdown of LRP/LR, perhaps they aided in degrading in HnRNPA1, ultimately leading to apoptosis. In addition, as mentioned above, HnRNPA1 also shares a relationship with telomerase and that down-regulating HnRNPA1 not only causes a decrease in invasion of tumour cells and EMT transition but also induction of apoptosis. Thus, further investigation into HnRNPA1's relationship with LRP/LR, telomerase and the MAPK pathway should be investigated.

5.1.7.1.3. Proteins involved in cytoskeletal maintenance and cell anchorage

Besides maintaining nuclear and chromatin structures, LRP/LR is also found to maintain the cytoskeleton by binding to cytoskeletal components such as actin and tubulin [99]. Upon siRNA-mediated knockdown of LRP/LR, it was found that a few proteins involved in microtubule and actin stabilization were down-regulated (Table 1). In particular, the Ensconsin (MAP7) protein known to play a vital role in microtubule reorganization and differentiation of epithelial cells, was found to be down-regulated by a significant 5-fold, when compared to the non-transfected cells (Table 3). In addition, two other proteins found to be down-regulated were Calponin-2 (CNN2) and -3 (CNN3) which bind to and stabilize actin, another vital participant of cytoskeletal rearrangement [240]. It is known that both actin and LRP/LR associate with integrins hence, signalling via focal adhesion complexes may possibly be occurring in the DLD-1 cells, since LRP/LR has been shown to colocalize with actin at lamellipodia through focal adhesion contact [99]. The findings from the current study are consistent with previous literature, therefore confirming LRP/LR's role in maintaining the cytoskeleton, where the receptor enhances protein translation as well as cell attachment and migration through tubulin and actin interactions, respectively.

Interestingly, the Dystroglycan (DAG1) protein was also found to be down-regulated upon siRNA-mediated knockdown of LRP/LR, when compared to non-transfected cells (Table 1). DAG1 is an adhesive protein required for important interactions between the ECM and the

actin cytoskeleton [225]. DAG1 binds to dystrophin, a protein that is part of the cytoskeleton, and laminin in the ECM, thereby simultaneously transducing signals and providing structural integrity [354]. Dystroglycan has also been found to be colocalize with transmembrane signalling proteins such as FAK (Focal Adhesion Kinase) [354]. Thus, the protein has been found to play important roles in proliferation, migration and polarization in epithelial cells [354]. In addition, a recent study has shown that both DAG1 and LRP/LR bind to laminin however, at different regions i.e. DAG1 binds only to the α-chains of laminin, whereas LRP/LR binds to the β-chains thereby exerting different functions. As mentioned previously, increased LRP/LR is associated with tumour development however, several studies show that increased levels of DAG1 possess tumour suppressor qualities [354]. In saying that, one study showed that by disrupting the interaction between DAG1 and laminin, apoptosis in muscle cells is induced via an impaired phosphoinositide 3-kinase (PI3K)/AKT signalling pathway [355]. This finding however must still be investigated and confirmed in cancer cells. This, however, opens a discussion since DAG1 and LRP/LR bind to laminin in different places, they are not likely to encounter each other however, the DLD-1 cells could be using both proteins association with FAK to help induce apoptosis. This is consistent with the finding of LRP/LR-FAK interactions leading to the activation of the PI3-kinase/AKT pathway as well as an increased expression of the Bcl-2 anti-apoptotic protein [180]. Thus, this would possibly explain the down-regulation of DAG1 upon siRNA-mediated knockdown of LRP/LR.

As mentioned previously, LRP/LR has been found to play important roles in cell differentiation, adhesion, growth and migration [42] This is made possible through its interaction with laminin-1 that is found in the ECM of cells. Thus, it was expected that several proteins involved in cell differentiation, proliferation and migration were affected upon siRNA-mediated knockdown of LRP/LR (Table A7). In addition, this confirms the receptor's role in promoting cell adhesion and migration, key properties of metastasis. A protein surprisingly found to be significantly down-regulated by 2-fold was Fibronectin (FINC), when compared to non-transfected cells (Table 1). FINC is a non-collagenous glycoprotein found in the ECM of cells normally involved in cell attachment, differentiation and growth [233]. It is able to bind to integrins as well as collagen and heparan sulphate proteoglycans [356]. This is however, the first report of it having any interaction with LRP/LR. There have however been studies that show tumour cell adhesion to fibronectin and phosphorylation of FAK via the α5β1 integrin are found to increase chemotherapeutic resistance [357]. Perhaps there may be a link to LRP/LR through focal adhesion contacts since as mentioned above, the receptor associates

with integrins and actin through these contacts [99]. However, further experimental procedures must be performed to confirm this theory.

5.1.7.1.4. Proteins involved in the MAPK pathway

Upon siRNA-mediated knockdown of LRP/LR, several proteins of the MAPK pathway were found to be differentially expressed when compared to non-transfected cells. As mentioned previously, a study showed that LRP/LR interacts with the MAPK signalling pathway, ultimately resulting in the inhibition of apoptosis of gastric cancer cells [180]. The MAPK pathway consists of serine/threonine kinases which regulate various downstream molecules including transcription and translation factors, kinases and cell cycle molecules [209]. Thus, the pathway is found to link several extracellular signals to cellular processes involved in gene expression, apoptosis, differentiation, senescence, proliferation and migration [209]. Depending on the time of activation and cell type, the numerous downstream targets are able to give rise to the intricacy of MAPK effects on apoptosis, where the balance of these ultimately controls the fates of cells [358].

An important protein found to play a role in both MAPK and apoptotic pathways is the tumour suppressor p53. As mentioned previously, the p53 tumour suppressor is able to respond to several stress signals including abnormal oncogene activation, ribosomal stress, DNA damage, telomere erosion and hypoxia. Once p53 is activated, it is able to regulate various processes in the cell such as DNA repair, apoptosis, cell cycle arrest or autophagy to prevent cancer progression [359]. Moreover, the extent of genotoxic stress and p53 expression are the features that impact cell fate regarding life and death i.e. low levels of genotoxic stress causes p53-induced cell cycle arrest, leading to DNA repair of the cells [305], while high levels of stress result in apoptosis. On the p53 protein itself, there are several threonine (T) and serine (S) sites which require phosphorylation for the protein to become active [360]. Furthermore, these phosphorylation sites are altered when interacting with stresses for the initiation of p53. Thus, phosphorylation is found to be a vital step in the initiation and execution of the p53 reaction [360].

Upon siRNA-mediated knockdown of LRP/LR, it was found that phosphorylated p53 was up-regulated at S15, S46 and S392, when compared to non-transfected cells in both the MAPK and apoptotic arrays (Figs. 47-49). With regards to p53 in the MAPK pathway, phosphorylation of the protein at S15 is the main target upon DNA damage stress and contributes to the protein's stability by inhibiting the binding of its transcriptional repressor, MDM2 [361]. As a result,

this can lead to ATM-mediated apoptosis. However, nutrient depletion can stimulate the activation of AMPK, which also leads to the phosphorylation of S15, resulting in either autophagy or apoptosis [362]. As a result, phosphorylated S15 promotes the interaction between p53 and the co-activator CREB-binding protein/p300, resulting in the crucial transactivation activity of p53 [363]. Hence, S15 phosphorylation is found to not only be involved in the induction of apoptosis and autophagy, but also in interactions with MAPK pathway, demonstrating that it is a key focal point of p53 activation. Phosphorylation at S46, on the other hand, has been found to preferentially activate genes that induce apoptosis rather than cell cycle arrest, in response to severe DNA damage [364]. Once phosphorylated, S46 is mainly found to selectively activate important pro-apoptotic genes such as Bax in response to the high genotoxic stress levels [364]. Moreover, the S46 phosphorylation is also found to be regulated by various kinases including ATM and AMP-activated protein kinase catalytic subunit α (AMPKα) [365], suggesting that this is a crucial modification for the correct functioning of p53. The phosphorylation of S392 on p53, on the other hand has not been studied as extensively however, it also activated due to DNA damage. Phosphorylated S392 is also able to regulate the translocation of p53 from the mitochondria as well as induce apoptosis that is transcription-independent [366]. Additionally, S392 phosphorylation has been found to aid in tetramerization of the protein and enhance the DNA binding ability of p53 [361]. Thus, it can be seen that it is vital for these residues to be phosphorylated for p53 to be activated. Moreover, as mentioned above, the relative mRNA expression levels of p53 were investigated in this study in order to confirm its presence found in each array. Upon siRNA-mediated knockdown of LRP/LR, there was a significant increase in the relative mRNA expression of p53, when compared to non-transfected cells (Fig. 46). These results, together with its consistent increase in all three arrays, confirm that p53 does indeed play a role in inducing apoptosis in DLD-1 cells.

Another protein found to be significantly up-regulated in the phospho-MAPK array was the checkpoint kinase 2 (Chk-2) protein (Fig. 43). Chk-2 is a well-known tumour suppressor that regulates the G2-checkpoint of the cell cycle with several other proteins including p53 [367]. Moreover, studies have shown that Chk-2 is activated by ATM kinase in response to DNA damage, where it is involved in phosphorylating and stabilizing p53 at additional residues Serine 20 or Threonine 18 [367]. The phosphorylation of p53 by Chk-2 is also found to activate the CREB-binding protein/p300 which facilitates functions of p53, as mentioned above. In addition, Chk-2 stabilization of p53 is found to lead to cell cycle arrest or apoptosis, depending

on the severity of the DNA damage [367]. From this study, the significant up-regulation of Chk-2 together with increases observed in p53 expression suggest that these two proteins aid in apoptotic induction, upon the down-regulation of LRP/LR in DLD-1 cells.

In addition to Chk-2, one other interesting protein linking LRP/LR to the MAPK pathway was the CREB (cyclic AMP-response element binding protein) that was found to be up-regulated (Figs. 43 and 44). To confirm this result, qPCR was performed, and it was found that upon siRNA-mediated knockdown of LRP, there was a significant 4-fold increase in mRNA expression levels when compared to non-transfected cells (Fig. 49). This protein normally functions as a transcription factor that regulates various processes including cell growth and survival by binding to cAMP response elements (CRE) sequences [368]. CREB has also been implicated in genotoxic stressed-induced transcription as a result of DNA damage and oxidative stress [368]. These stressors activate the phosphorylation of CREB which result in its further phosphorylation by ATM kinase. ATM-mediated phosphorylation of CREB leads to the recruitment of its cofactor CREB-binding protein (CBP)/p300 to initiate transcription [369]. As mentioned above, for full transcriptional activity of the p53 tumour suppressor, the coactivator CBP/p300 is required to bind to p53 [370]. The binding of these proteins is facilitated by phosphorylated CREB [369]. Moreover, Chk-2-mediated phosphorylation of p53 stabilizes the binding of CBP/p300 to p53 [367]. This results in p53 transactivating a number of proteins that are used for apoptotic induction. The findings of the current study suggest that LRP/LR may be linking CREB, CBP/p300, p53 and Chk-2, since a decrease in LRP/LR expression levels results in each of these proteins aiding in apoptotic induction.

Surprisingly, only two proteins of the MAPK pathway were found to be down-regulated upon siRNA-mediated knockdown of LRP/LR in the DLD-1 cells. One such protein was focal adhesion kinase (FAK) where upon densitometric analysis, was down-regulated compared to non-transfected cells (Fig. 43). FAK is a non-receptor tyrosine kinase that associates with several receptors including growth factor receptors, cytokine receptors, G-protein coupled receptors as well as integrins, ultimately leading to its activation [371]. FAK is found to be an important intracellular regulator of changes that occur outside of the cell including nutrient availability and ECM remodelling [371]. It also plays crucial roles in regulating cell proliferation, adhesion and survival in several cell types thus, it is said that FAK is the connection of several signalling pathways. In addition, due to its kinase-dependent regulation functions of survival and motility, FAK has been shown to contribute to the formation of cancer [372]. Moreover, a study interestingly showed that FAK associates with p53 directly in the

nucleus through a kinase-independent means, resulting in the degradation of p53 and the promotion of cell growth and survival of tumourigenic cells [372]. As mentioned previously, Sun et al. (2014) found that the LRP/LR-laminin-1 interaction allowed for FAK and LRP/LR to interact, thereby activating the MAPK signalling pathway and the anti-apoptotic Bcl-2 protein, ultimately leading to apoptotic inhibition [180]. Since FAK was down-regulated upon siRNA-mediated knockdown of LRP/LR, this shows that these results are consistent with the findings of Sun et al. (2014) and confirms the relationship between FAK and LRP/LR [180]. In addition, it was also found that the knockdown of LRP/LR decreased the mRNA expression levels of Bcl-2, further confirming the results of the previous study (Fig. 51) [180]. Lastly, since p53 was also found to be consistently up-regulated upon siRNA-mediated LRP/LR knockdown (Figs. 43-46), this might further explain the decrease in FAK levels in the DLD-1 cells since it has been shown that p53 plays a role in its degradation [372] .

Together with FAK, one other protein was down-regulated upon siRNA-mediated knockdown of LRP/LR. In fact, β-catenin was found to be significantly down-regulated, when compared to non-transfected cells (Fig. 47). β-catenin functions to regulate as well as co-ordinate cell-cell adhesion and transcription [373]. It serves as a subunit of the cadherin protein complex, thereby acting as a signal transducer of the Wnt signalling pathway[374]. Over-expressed β-catenin is linked to several cancers specifically, colorectal cancer [375]. This is because the tumour suppressor protein, adenomatous polyposis coli (APC), which is strongly linked to colorectal cancer, is normally found to regulate and destroy the β-catenin complex [376]. However, in colorectal cancer cells the APC gene is often mutated, resulting in the increase of β-catenin [373]. Once the levels of β-catenin are extremely high, it translocates to the nucleus to engage in transcription of specific pro-cancerous genes [377]. Thus, this result could only be specific to this study which investigated colorectal cancer cells, indicating that LRP/LR may also exploit mutations that occur in specific cancer types. However, as mentioned above, increased β-catenin is also found in other cancers including lung cancer, breast cancer and prostate cancer [374], suggesting that LRP/LR may also require the Wnt signalling pathway to contribute to its role in cancer development.

5.1.7.1.5. Proteins involved in apoptotic regulation

As mentioned previously, LRP/LR has been linked to a variety of cancer promoting processes, including cell viability maintenance and apoptotic evasion – focal points of this study. However, knockdown of LRP/LR via siRNA technology has shown significant decreases in

tumour cell viability through the induction of apoptosis in several cancer cell lines [126-129]. From the Apoptosis Proteome Profiler Antibody Array™, it was found that when compared to non-transfected DLD-1 cells, the pro-apoptotic Bcl-2 family member Bax was found to be significantly up-regulated (Fig. 45), while the anti-apoptotic protein Bcl-2 remained unchanged upon siRNA-mediated knockdown of LRP/LR (Fig. 45). To confirm these results, qPCR was performed to investigate the relative mRNA expression levels of both Bax and Bcl-2, key regulatory proteins of the apoptotic cascade. The results revealed that upon siRNA-mediated knockdown of LRP/LR, Bax had a significant 4-fold increase in mRNA expression levels when compared to non-transfected cells, thereby correlating with the data from the array (Fig. 47). On the other hand, Bcl-2 was found to have a significant 2.5-fold decrease in mRNA expression levels when compared to non-transfected cells (Fig. 48), showing a poor correlation between its mRNA and protein levels. There are several reasons which could account for the inconstant Bcl-2 result including the possibility of complex post-transcriptional modifications through the action of microRNAs [378]; post-translational modifications such as phosphorylation of Bcl-2 at specific serine residues [379]; as well as differential protein degradation [378] – ultimately resulting in the unchanged Bcl-2 protein expression, compared to its decreased mRNA expression. In addition, a previous study demonstrated that Bcl-2 mRNA expression was found in hepatocellular carcinomas however, the Bcl-2 protein was not expressed, further indicating that post-transcriptional control of Bcl-2 is poor [380].

It is known that Bcl-2 functions to inactivate Bax thereby inhibiting apoptosis however, in the current study Bcl-2 expression was not significantly down-regulated. This can be easily explained by the ratio between both proteins, which represents the susceptibility of a cell to undergo apoptosis [381]. Interestingly, the regulation of this Bax/Bcl-2 ratio is found to be mediated through the tumour suppressor, p53 [381]. However, tumour cells are normally found to mutate p53 and express high levels of Bcl-2, thereby blocking p53-mediated apoptosis. Thus, it comes as no surprise that high levels of the Bax/Bcl-2 ratio is associated with cancer development and poor prognosis [382]. In saying that, stress-activated p53 is known to down-regulate the expression of the Bcl-2 gene [383], which may explain the significant decrease observed in Bcl-2 relative mRNA expression levels upon siRNA-mediated knockdown of LRP/LR (Fig. 48). Taken together, these factors could have aided in inducing apoptosis in DLD-1 cells.

Moreover, it seems that Bax contributed greatly to apoptotic induction in the DLD-1 cells due to its significantly increased mRNA and protein expression levels upon siRNA-mediated

knockdown of LRP/LR, compared to non-transfected cells (Figs. 45 and 47). It is known that Bax transcription is performed directly by p53 and that there is a significantly high correlation between p53 and Bax mRNA expression levels [381] – consistent with this study (Fig. 43-47). Furthermore, several studies have shown that the expression of Bax by stress-activated p53 increases the sensitivity of the Bax/Bcl-2 ratio to apoptosis [384] and also overcomes the anti-apoptotic effects of Bcl-2 [381]. Thus, this suggests that Bax and its association with p53 were found to ultimately drive the DLD-1 cells to induce apoptosis. In addition, these findings are in line with Massard et al. (2006) which showed that the knock-down of hTERT resulted in the activation of Bax [151]. As mentioned previously in section 5.2.6, it was found that upon Human-RPSA transfection of DLD-1 cells, hTERT mRNA and protein expression levels were significantly decreased, when compared to non-transfected cells (Figs. 37 and 38). These results not only suggest that LRP/LR may influence hTERT expression but also that hTERT could be enhancing LRP/LR's role in maintaining tumour cell survival and evading apoptosis. Moreover, the significant increase found in Bax mRNA expression levels upon siRNA-mediated knockdown of LRP/LR further proposes that the inhibition of hTERT results in the induction of mitochondrial-mediated apoptosis [151].

Several other apoptotic proteins were found to be differentially expressed upon siRNA-mediated knockdown of LRP/LR, when compared to non-transfected cells. More specifically, this included anti-apoptotic members from the Inhibitor of Apoptosis (IAP) family such as the c-IAP1 (also known as BIRC2), XIAP (X-linked inhibitor of apoptosis) and Survivin (BIRC5), which were all found to be down-regulated, when compared to non-transfected cells (Fig. 45). The IAP family is suggested to be the most crucial regulators of apoptosis since they can control both apoptotic pathways, as well as the cell cycle and signal transduction [385]. These proteins are found to inhibit apoptotic induction by directly or indirectly binding to executioner caspases, thereby preventing the activation of these caspases [386]. Thus, the activation of IAP family of proteins is said to indirectly represent the active state of caspases [386]. Moreover, the overexpression of IAPs has been found to block apoptosis downstream of Bak, Bax and cytochrome c [387]. In addition, Survivin is a direct target of the Wnt pathway and is up-regulated by β-catenin (a protein that was found to be down-regulated upon the knockdown of LRP/LR in the phospho-MAPK antibody array – Fig. 43) [388]. However, it has also been found that stress-activated p53 targets Survivin and is able to induce apoptosis by repressing transcription of the protein [389]. This further shows how the Human-RPSA siRNA is able to induce apoptosis through the up-regulation of p53. In addition to this, Naseri et al. (2015)

showed that the simultaneous up-regulation of Bax and down-regulation of IAPs resulted in the release of cytochrome c as well as the activation of caspase-9 and -3 activity [390]. This therefore reiterates the findings of the current study, in that the knockdown of LRP/LR via the Human-RPA siRNA is able to induce caspase-9-mediated apoptosis (Fig. 34) through the up-regulation of Bax (Fig. 45 and 47). In addition, it also confirms that the down-regulation observed for β-catenin upon siRNA-mediated knockdown of LRP/LR in the MAPK Proteome Profiler Array, thereby providing another link between LRP/LR with the MAPK and apoptotic signalling pathways.

Another anti-apoptotic protein also found to be significantly down-regulated upon siRNA-mediated knockdown of LRP/LR, when compared to non-transfected cells was Claspin (Fig. 45). Claspin is found to interact with several proteins involved in cell cycle checkpoint activation as well as genomic stability and DNA replication [391]. Over-expressed levels of claspin have been frequently found in several cancer types [391]. Although the exact reason as to why claspin is up-regulated in cancers is not fully understood, it could simply be due to its role in DNA replication. Since tumourigenic cells are therefore known to replicate at a higher rate than their normal counterparts, much higher levels of Claspin are found in cancer cell lines [391]. However, Semple et al. (2007) found that shRNA-mediated knockdown of Claspin resulted in the promotion of apoptosis, indicating that its presence prevents apoptosis from occurring [392]. The study also found that Claspin is phosphorylated in response to DNA damage, resulting in cell cycle arrest and apoptosis. Although it is known that LRP/LR plays a role in the DNA damage response pathway through its effect on p53 and H2AX, the receptor could also be using another route through Claspin to induce apoptosis.

In addition, an interesting protein found to be differentially expressed upon siRNA-mediated knockdown of LRP/LR was the transcription factor Hypoxia-inducible factor (HIF-1α) (Fig. 45). HIF-1α is a key regulator of the mammalian response to oxygen levels [393]. The protein has also been found to regulate hypoxia, a process found in several tumours facilitating oxidative stress, which can result in increased cell proliferation and survival of the tumour [394]. Although the over-expression of HIF-1α is found to trigger hypoxia, resulting in the increase in cancer development, studies have also shown that hypoxia is also able to induce apoptosis [394]. More particularly, the lack of oxygen can lead to genomic instability and DNA damage as well as the inhibition of proton transfer in cells which leads to a decreased mitochondrial membrane potential [394]. As a result, this causes HIF-1α to increase the stabilization of p53, resulting in the activation of Bax and cytochrome c release, ultimately

resulting in caspase-9-meditated apoptosis [394]. It has been found that HIF-1α protein is also able to activate the BNIP3 pro-apoptotic protein, resulting in the stimulation of Bax and imbalance of the Bax/Bcl-2 ratio, ultimately leading apoptosis [394]. However, HIF-1α is also able to induce mitochondrial-selective autophagy by interacting directly with BNIP3, independently of p53 [395]. In addition to hypoxia, ROS production also contributes to oxidative stress formation, and it has been found that HIF-1α helps with this process [394]. This result therefore confirms the proteomic analyses from this study which showed that siRNA-mediated knockdown of LRP/LR results in the decrease of certain enzymes that normally protect the cell from oxidative stress, thereby aiding in ROS generation for temporary survival of the tumour cells through autophagy. Furthermore, this result shows that the up-regulation of HIF-1α upon siRNA-mediated knockdown of LRP/LR plays a dual role in aiding in autophagy and apoptosis of the DLD-1 cells.

Moreover, siRNA-mediated knockdown of LRP/LR resulted in the notable protein known as Histone H2AX to be up-regulated by a significant 5-fold, when compared to non-transfected cells (Table 2). H2AX is a protein marker that becomes phosphorylated to γH2AX in response to DNA damage and double stranded breaks which mostly accumulate due to the dysfunction of telomeres [292]. γH2AX aids in recruiting proteins that contain phospho-specific interaction domains which as a result, help to obtain other proteins involved in DNA damage/repair. As previously mentioned, telomere dysfunction together with elevated levels of telomerase activity have been associated with cancer recurrence and chemotherapeutic resistance [172]. However, studies have shown that dysfunctional telomeres are able to activate a DNA damage response pathway involving γH2AX, ATM and p53 proteins, ultimately leading to cell cycle arrest and apoptosis of cancer cells [396]. In the current study, it has been found that siRNA-mediated knockdown of LRP/LR not only significantly decreases hTERT expression levels and telomerase activity but also increases the expression of pro-apoptotic protein Bax and the p53 tumour suppressor. The fact that the expression of the DNA damage marker H2AX is also increased further confirms that DLD-1 cells undergo apoptosis upon LRP/LR knockdown. Moreover, this finding is also in accordance with Otgaar et al. (2017) which found that an increase in LRP/LR expression significantly decreases γH2AX expression and increases in telomere length [108].

Another interesting protein that was found to be up-regulated upon siRNA-mediated knockdown of LRP/LR was Programmed Cell Death Protein 4 (PDCD4) by 3-fold, when compared to non-transfected cells (Table 2). PDCD4 functions as a tumour suppressor involved

in transcriptional regulation [278], DNA-damage [397] and apoptotic signalling [398] as well as translation inhibition [399]. Studies have shown that a decrease in PDCD4 not only leads to the invasion and migration of tumour cells but also deregulates DNA damage responses [397, 400]. On the other hand, PDCD4 overexpression has been found to activate the pro-apoptotic Bax protein followed by the release of cytochrome C, resulting in mitochondrial-apoptotic induction [401]. Furthermore, subcellular PDCD4 is controlled by protein kinase Akt-mediated phosphorylation, thus the protein is said to be involved in the PI3K-Akt pathway [402]. Thus, the up-regulation of PDCD4 upon siRNA-mediated knockdown of LRP/LR further suggests that the down-regulation of the receptor aids in inducing mitochondrial-mediated apoptosis in tumour cells. In addition to PDCD4, another protein known as Ribosomal protein S26 (RS26) involved in the intrinsic-mitochondrial pathway of apoptosis was found to be up-regulated upon siRNA-mediated knockdown of LRP/LR by 2-fold. A study has shown that up-regulated RS26 is able to regulate the MDM2/MDMX–p53 cascade by inducing p53 activity in response to DNA damage, ultimately leading to cell cycle arrest and apoptosis [271]. These findings further suggest that siRNA-mediated knockdown of LRP/LR in DLD-1 cells may favour the intrinsic mitochondrial pathway for apoptotic induction.

Although several pro-apoptotic proteins were up-regulated upon siRNA-mediated knockdown of LRP/LR, a few of them were also down-regulated (Table 1). In particular, the Bcl-2-associated transcription factor 1 (BCLF1) was found to be down-regulated by 4-fold. BCLF1 has been found to play a role in apoptotic signalling however, the protein does not possess structural resemblances to any members from the Bcl-2 family thus, its role in apoptosis is somewhat controversial [403]. BCLF1 is rather found to possess structures that are characteristic of pre-mRNA processing/splicing and transcriptional factors [404]. In addition, Zhou et al. (2014) found that BCLF1 was able to regulate the tumourigenic potential of colorectal (HT-29 and HCT-116) cells through the serine/arginine-rich splicing factor 10 (SRSF10), its splicing regulator [219]. Moreover, it was found that upon isoform-specific down-regulation of BCLF1, colorectal cancer cell proliferation was reduced [219]. However, this finding may be specific to only colorectal cancer cells and not specific to the knockdown of LRP/LR.

5.1.7.1.6. Proteins involved in autophagic induction

Interestingly, several enzymatic proteins involved in metabolism were found to be up-regulated upon siRNA-mediated knockdown of LRP/LR in DLD-1 cells. This up-regulation could most

likely be due to the tumour cells adapting in response to stress by undergoing a process known as autophagy. Cancer cells have been found to undergo autophagy or type II programmed cell death (PCD) to degrade damaged or unessential cell constituents. This degradation serves as an alternative energy source to sustain survival [405]. The activation of autophagy is associated with stress such as low nutrient levels, DNA damage, oxidative stress and inflammation [406]. The multi-step process begins by sequestering damaged cytoplasm and organelles into isolated membranes that mature into double-membrane structures called autophagosomes [407]. These autophagosomes fuse with lysosomes thereby forming single-membrane structures called autolysosomes in which acidic lysosomal hydrolases degrade the autophagosome [407]. The products from the tumour cells undergoing autophagy include macromolecules and fatty acids that are able to be redirected to new metabolic pathways so that tumour cell viability can be sustained [408]. Studies have shown that autophagy and apoptosis can take place in the same cell in which autophagy precedes apoptosis [406].

In particular, a protein found to be up-regulated upon siRNA-mediated knockdown of LRP/LR was the AMP-activated protein kinase catalytic subunit α1 and 2 (AMPKα1 and 2), when compared to non-transfected cells (Fig. 43). AMPK is a vital energy sensor protein which is known to primarily function in regulating energy and metabolism homeostasis [409]. The protein is activated upon stressors such as ATP and nutrient depletion as well as hypoxia. As a result, AMPK is found to be one of the major regulators of autophagy, whereby it phosphorylates and activates the autophagy-initiating kinase ULK1, a key initiator of autophagic process [410]. AMPK has also been found to contribute to the maturation of the autophagosome as well as fusion of the autolysosome [411]. In addition, a study performed by Krieg et al (2018) showed that the knockdown of AMPKα1/2 revealed a key role of AMPK for the induction of autophagy [412]. Moreover, the loss of AMPK has also been shown to promote cell proliferation through the increased signalling of the MAPK pathway. Interestingly, a study performed by Choi et al. (2015) revealed that CREB binds to AMPKα in order for the transactivation of p53 in response to energetic stress [409]. Moreover, it was found that by down-regulating CREB via siRNA technology, p53 was significantly down-regulated together with a substantial decrease in AMPKα [409]. The authors therefore proposed that the interaction between AMPKα and CREB facilitates the regulation of transcriptional p53 and also contributes to the activation of p53-mediated apoptosis. Taken together with the results from the current study where CREB was found to be up-regulated (Fig. 44 and 49) upon

knockdown of LRP/LR, it is suggested that the CREB-AMPKα association additionally facilitates p53-mediated apoptosis induced by the Human-RPSA siRNA.

To further demonstrate that autophagy may have been induced, upon siRNA-mediated knockdown of LRP/LR, it was found that enzymes such as Glutathione peroxidase 1 (GPX1) and Adenylate Kinase 4 (KAD4/AK4) were found to be affected. GPX1 was down-regulated by 2-fold (Table 1), while KAD4 was up-regulated by 3-fold (Table 2), when compared to non-transfected cells. The GPX1 enzyme normally functions to protect cells from oxidative stress and damage [247]. However, since it was shown to be down-regulated, it is likely that the tumour cells were not protected from oxidative stress which triggered the cells to either activate autophagy or a DNA damage response, whereby both processes ultimately lead to apoptosis. In addition, the up-regulation of the KAD4 protein further suggests that autophagy occurred in the DLD-1 cells since it functions as a stress-responsive protein in attempt to maintain cell survival [276]. A study showed that the up-regulation of KAD4 in lung adenocarcinoma cells resulted in increased intracellular ROS levels and oxidative stress [413]. Moreover, adenylate kinase is involved in the stimulation of the previously mentioned regulator of autophagy, AMPK [414]. These findings therefore suggest that upon siRNA-mediated knockdown of LRP/LR, the DLD-1 cells may have undergone oxidative stress, due to the down-regulation of GPX1 and up-regulation of KAD4, ultimately leading to autophagic induction in an attempt to sustain survival.

In addition to the autophagic enzymes, vesicle transport proteins involved in autophagy called SEC20/BNIP1 (BCL2/adenovirus E1B 19 kDa protein-interacting protein 1) and VPS4A (Vacuolar protein sorting 4 homolog A) were found to be up-regulated by 2-fold and 3-fold, respectively, when compared to non-transfected cells (Table 2). SEC20 is normally involved in mediating membrane transport and fusion for endoplasmic reticulum (ER) organization and biogenesis but has also been found to regulate autophagy by facilitating soluble protein transport to lysosomes [252]. Moreover, the membrane delivery and vesicle trafficking qualities that SEC20 possess could possibly be facilitating the transportation of LRP/LR to the cell membrane since LRP/LR orthologs are known to associate with bound polysomes of the ER [99, 178]. In addition to its role in the ER, SEC20 has also been found to have pro-apoptotic characteristics since it belongs to the BH3 (Bcl-2 homology 3)-only family of proteins [415]. This suggests that SEC20 could be playing a dual role in the induction of mitochondrial-intrinsic apoptosis upon siRNA-mediated knockdown of LRP/LR, since it has been found that autophagy and apoptosis can occur in cells at the same time. VPS4A on the other hand, is

involved in intracellular membrane trafficking of the lysosome [280], unlike SEC20 which only transports proteins to the lysosome. As mentioned above, degrading the contents of the autophagosome is performed by the lysosome. VPS4A is involved in the formation of multivesicular bodies which are vesicle-filled endosomes that fuse with the lysosome to aid in the degradation of proteins sequestered by the acidic lysosomal hydrolases [407]. In addition, a study showed that upon VPS4A inhibition, autophagosome formation is impaired, concluding that VPS4A is vital for autophagy to occur [280].

Another protein found to be up-regulated upon siRNA-mediated knockdown of LRP/LR, was CPNS1 (Calpain Small Subunit 1) by 4-fold (Table 1). Calpains are a group of cysteine proteases located in the ER and Golgi-apparatus that are regulated by calcium (Ca^{2+}) [289]. These enzymes are found to be activated by several stimuli including starvation and nutrient depletion to cleave proteins involved in autophagy, thereby regulating the degradational system of the autophagic pathway [416]. Calpains have also been found to be involved in the formation of the autophagosome [289] as well as in cancer cell transformation and invasion. This suggests that upon nutrient depletion, cancer cells may activate calpains to induce autophagy and help in the invasion of new sites for survival [417]. Furthermore, it is known that Ca^{2+} signalling pathways are often deregulated in cancer therefore, an increase in calcium could stimulate the activation of calpains, resulting in the induction of autophagy [418]. Moreover, autophagic induction has been found to also occur through CaMKKβ (Ca^{2+}/calmodulin-dependent kinase kinase-β) activation, leading to the stimulation of AMPK activity [418]. However, the activation of calpains and CaMKKβ also induces an intrinsic pathway apoptotic response [419]. Thus, it can be seen that autophagy and apoptosis share a complex relationship involving several overlapping proteins, hence the need for cross-talk between both processes.

The current study has consistently showed that siRNA-mediated knockdown of LRP/LR results in apoptosis however, as seen from above, the DLD-1 cells may also undergo autophagy as a temporary survival mechanism in response to the cell stress. Since LRP/LR plays several roles in many signalling cascades, the levels of stress may increase due to its down-regulation. The elevated stress may lead to the formation of DNA damage, a possible downstream effect resulting from the observed decreases in telomerase activity, hTERT and TRF-2 expression levels upon siRNA-mediated knockdown of LRP/LR. This is because, as mentioned in section 5.2.6., decreased levels of all three proteins contribute to chromosomal end fusion, genetic instability and DBS formation, leading to the activation of DNA damage response pathways. In turn, this also results in the activation p53, Chk-2 and CREB of the MAPK pathway.

Moreover, due to the high stress levels and autophagic response observed in the DLD-1 cells, AMPKα may still be present in cells, allowing for the CREB-AMPKα association to occur. Ultimately, the activation of the above-mentioned proteins leads to p53-mediated apoptosis. These statements must be validated through further experimentation.

5.1.7.1.7. The role of p53 in autophagy and apoptosis

As mentioned above, autophagy and apoptosis can occur together in cells, where autophagy precedes apoptosis. This is due to autophagic induction being a strategy normally used for stress adaptation induced by external factors such as nutrient depletion, DNA damage and the inhibition of certain receptors [406]. However, if stress is found to exceed a crucial threshold, apoptosis is activated [406]. Moreover, in some cases, autophagy may promote cell death by facilitating the activation of the apoptotic process [406]. It is proposed that the p53 tumour suppressor may be a link between apoptosis and autophagy since its signal transduction is activated upon stress – a trigger for both processes. Typically, p53 is localized to the cytosol however, stress induction causes it to translocate to the nucleus where it is phosphorylated by several stress-activated kinases [406]. The translocation of p53 results in the decrease of cytosolic p53, leading to the induction of autophagy. In the nucleus, p53 is able to not only activate several genes that regulate autophagy such as DRAM (damage-regulated autophagy modulator), which promotes the formation of autophagolysosomes, but also important pro-apoptotic genes that encode for BCL-2 family members such as Bax, Bim and Puma, known to promote caspase activation [406]. While nuclear p53 promotes autophagy, cytoplasmic p53 is found to inhibit autophagy either by competitively binding to autophagic components or by activating mTOR (mammalian target of rapamycin), a negative regulator of autophagy, through the inactivation of AMPK [406]. Thus, it can be seen that p53 shares a complex relationship with both processes.

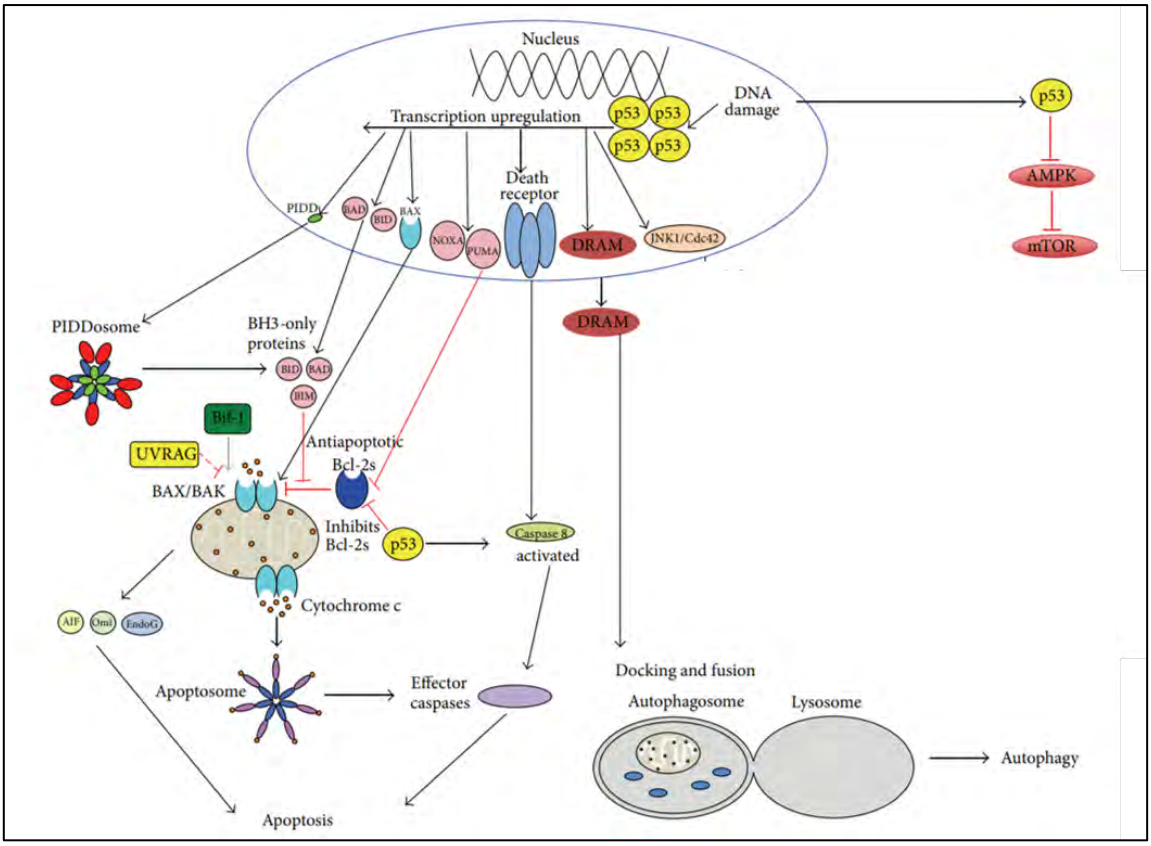


Figure 56: Summarized role of p53 in autophagy and apoptosis. Stress induces the activation of p53. Nuclear p53 is found to promote autophagy and apoptosis by activating genes required for both processes to occur. On the other hand, cytosolic p53 inhibits autophagy by activating mTOR signalling through the inactivation of AMPK. (Image adapted from Su et al., 2013 [420]).

In addition to p53, several BCL-2-homology 3 (BH3)-only proteins are also found to activate autophagy and apoptosis. For apoptotic induction, BH3-only proteins directly bind to proteins from the Bcl-2 family, thereby stimulating the pro-apoptotic proteins while causing a neutralising effect on the anti-apoptotic proteins [421]. In addition to this, BH3-only proteins including Bid (BH3-interacting domain death agonist), Bad (BCL-2 antagonist of cell death), BNIP1 and BNIP3 (BCL2 and adenovirus E1B 19 kDa protein-interacting protein-1 and -3) as well as PUMA, are also found to facilitate autophagy [421]. They perform this by blocking the inhibitory interactions between anti-apoptotic Bcl-2 proteins and Beclin-1, a main regulator of autophagy [421]. However, it still remains unclear as to how the BH3-only proteins perform sequential stimulation of both autophagic and apoptotic processes.

It has been found that stress pathways induce autophagy at early time points with low levels of stress, while apoptosis is induced at late time points with high levels of stress [406]. In the

current study, upon siRNA-mediated knockdown of LRP/LR, majority of the DLD-1 colorectal cancer cells were found to undergo late apoptosis (Fig. 30), therefore suggesting that down-regulating LRP/LR triggers initial cell stress, resulting in the induction of autophagy for cancer survival. However, due to the important role LRP/LR plays in several signalling cascades, stress levels are increased, leading to the activation of caspases to ultimately induce apoptosis. Once apoptosis is induced, autophagy becomes inactivated due to caspase-mediated cleavage of autophagic proteins [406].

The proteomics data obtained from SWATH-MS and the Proteome Profiler Antibody Arrays™ upon knock-down of LRP/LR in DLD-1 cells via siRNA technology revealed a great amount of useful and interesting information. Several new proteins were identified to have novel interactions with LRP/LR while other proteins were found to link LRP/LR to other processes thereby, exposing novel roles for the receptor. In addition, several identified proteins also confirmed the receptor's role in the various processes it has been shown to be involved in including ribosomal biogenesis and translation; cytoskeletal maintenance and cell anchorage; as well as nuclear and chromatin maintenance. The data revealed that LRP/LR does indeed play a role in the MAPK and apoptotic pathways. In addition, it also demonstrated how the DLD-1 colorectal cancer cells responded to a decrease in the levels of the receptor, in that autophagy was first induced followed by apoptosis, indicating that the cancer cells are highly affected by LRP/LR. However, there have also been proteins identified where their interaction with LRP/LR is not easily explained, highlighting the need for further experiments to confirm many of these interactions. Moreover, the identification of new proteins, when compared to previous studies, could also be due the different cell lines and treatments used as well as the different, but equally stringent, parameters used during identification and analysis of these proteins [98]. Nevertheless, several observations of the Array data are in accordance with the SWATH-MS analysis which suggest that LRP/LR may be involved in the MAPK and apoptotic pathways, further proposing that the results are still useful in elucidating the role of the receptor. Although the cellular responses observed provide an insight into LRP/LR, the exact mechanism of action of the receptor in tumourigenesis is far from being fully understood. The expression of these proteins as well as their genes should be studied at various time points post treatment to determine the exact sequence of events.

5.1.7.1.8. The proposed mechanism of cell death

It is known that p53 primarily functions in the intrinsic apoptotic pathway however, there have been several studies showing that p53 is also able to play a central role in the extrinsic pathway of apoptosis [422]. This is due to p53 being able to activate the transcription of key proteins involved in both pathways including Bax of the intrinsic pathway and the Fas death receptor of the extrinsic pathway [422]. Moreover, cytochrome c release, which is normally an important incident in the intrinsic pathway, has also been found to occur in the extrinsic pathway [422]. From this study, the apoptotic array showed that cytochrome c was increased in the DLD-1 cells upon siRNA-mediated knockdown of LRP/LR, when compared to non-transfected cells (Fig. 45). Moreover, as mentioned above, the cells were also found to have significant increases in both caspase-8 and -9 upon siRNA-mediated knockdown of LRP/LR, when compared to non-transfected cells (Figs. 33 and 34). In the intrinsic pathway, cytochrome c release is a result of increased activation of Bax by stressed-induced p53, ultimately leading to the activation of caspase-9-mediated apoptosis. On the other hand, the extrinsic pathway releases cytochrome c by cleaving the pro-apoptotic Bid protein through caspase-8 [422]. The results from this study however show that upon siRNA-mediated knockdown of LRP/LR, Bax mRNA and protein expression levels are significantly increased, correlating to the increased expression levels of p53 on account of genotoxic stress. The large increases of p53 and Bax may therefore have stimulated the increased release of cytochrome c, resulting in caspase-9 cleaving caspase-3 – also shown to be significantly up-regulated in the array (Fig. 45) – ultimately, activating mitochondrial intrinsic apoptosis. Moreover, the fact that caspase-8 was found to be increased but none of its death receptors were differentially expressed in the arrays (Fig. 33), could be due to an amplification loop as previously mentioned in section 5.2.4, where caspase-9 cleaves caspase-3, directly leading to caspase-8 activation [303]. On the other hand, activated caspase-8 is also found to cleave Bid which results in the release and activation of cytochrome c and caspase-9, respectively, ultimately leading to apoptotic induction through the intrinsic pathway [422].

Thus, from the MAPK, Apoptotic Proteome Profiler Antibody Arrays™ and SWATH-MS data, it would seem that siRNA-mediated knockdown of LRP/LR induces cell death in the DLD-1 cells in the following way: The up-regulation of p53, CREB, AMPKα, H2AX, PDCD4, RS26 and Hif-1α proteins suggests that the Human-RPSA siRNA was able to induce DNA damage in the DLD-1 cells, possibly as a result of diminished expression levels of telomerase activity as well as hTERT and TRF-2. This led to p53 stimulating the significant up-regulation

of Bax as well as BH3 pro-apoptotic protein BNIP1, resulting in an imbalance of the Bax/Bcl-2 ratio and possible dysfunction of the mitochondria. In addition, FAK and β-catenin were down-regulated as well as several anti-apoptotic proteins including Bcl-2, c-IAP, XIAP and Survivin. Consequently, Bax promoted the release of cytochrome c, resulting in caspase-9 activation and apoptosis. Complementing this pathway, was the activation of caspase-8, which may have aided the caspase-9 pathway in activating apoptosis by cleaving Bid, leading to the release of cytochrome c. Figure 57 below shows a graphical representation of the possible apoptotic pathway induced by the Human-RPSA siRNA in DLD-1 cells.

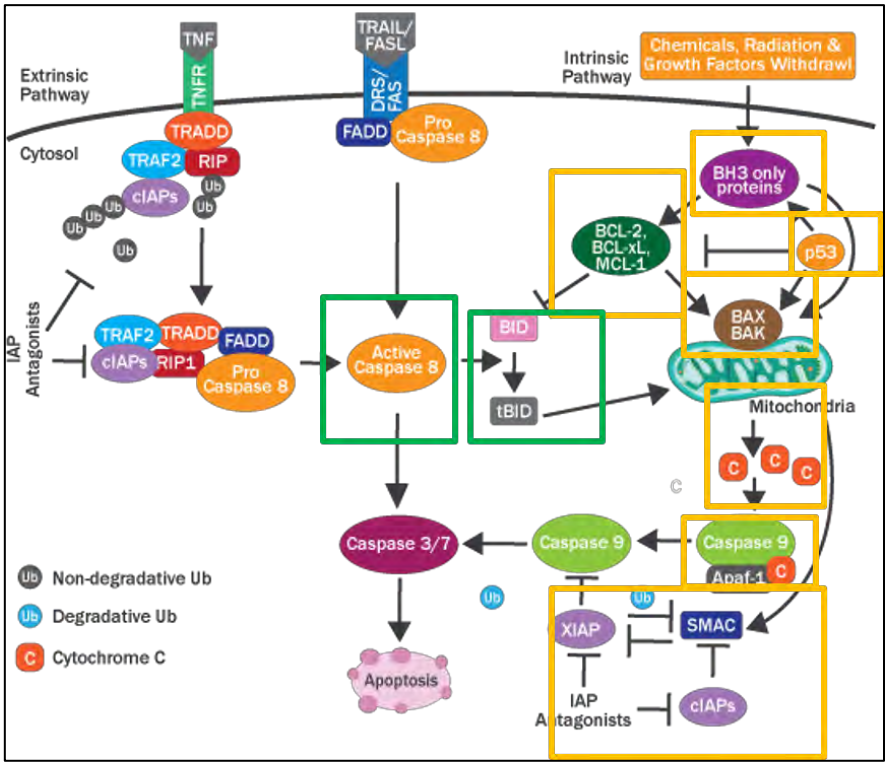


Figure 57: Graphical representation of the potential apoptotic pathway induced by the Human-RPSA siRNA in late (DLD-1) stage colorectal cancer cells. From the data obtained in this study, it is suggested that the Human-RPSA siRNA induced DNA damage, resulting in the up-regulation of p53 and several other pro-apoptotic proteins. As a result, this caused a decrease in the expression of various anti-apoptotic proteins, while pro-apoptotic protein expression such as Bax was increased. This led to the stimulation of cytochrome c release and the activation of caspase-9-mediated apoptosis. Complementing this pathway (shown in yellow) may be the activation of caspase-8, resulting in cleaved Bid and cytochrome c release, ultimately leading to the caspase-9-mediated apoptosis (shown in green) (Adapted from https://www.novusbio.com/antibody-news/antibodies/apoptosis-and-necroptosis-part-ii-inhibitors-of-apoptosis-proteins-iaps-key-regulators-of-the-balance-between-necroptosis-apoptosis-and-survival).

5.2. *In vivo* study

5.2.1. Treatment of MF-1 nude mice with IgG1-iS18

The 37kDa/ 67kDa laminin receptor (LRP/LR) has been found to be over-expressed on the surface of several cells including various cancers lines [33, 111-113, 123, 133]. However, upon treatment with the anti-LRP/LR specific antibody, IgG1-iS18, it has been found that adhesion and invasion is significantly impeded in lung (A549), prostate (LNCaP) cervical (HeLa) [33], oesophageal (WHCO1) breast (MDA-MB231) [110] liver (HUH-7) cancer cells, early (A375) and late (A375SM) stage malignant melanoma cells [113] and of particular interest, early (SW-480) and late (DLD-1) stage colorectal carcinoma cells [111]. This prompted the investigation of whether the antibody will display a similar effect *in vivo*, to ultimately provide a new therapeutic avenue for treating cancer.

For each pilot study, the induction of colorectal cancer using HT-29 cells was found to be successful in all MF-1 nude mice (Fig. 53), however, upon treatment with 2.5 mg/kg of IgG1-iS18 intraperitoneally, no significant effect on tumour growth or metastasis was observed (Fig. 54). This suggested that the antibody concentration administered may not have been potent enough to cause an effect. Thus, another pilot study was performed whereby the antibody concentration was increased to 3 mg/kg of body weight per mouse to optimize treatment conditions. However, there was still no significant effect observed on tumour growth (Table 3). This further suggested that not only the concentration but also the route of administration for the antibody may not have been effective in delivering the antibody to its target sites. Thus, a third pilot study was performed where it was decided to increase the antibody concentration to 5 mg/kg of body weight as well as to change the route of administration from intraperitoneal to intravenous injection via the tail vein –a route that has been successful in delivering antibodies directly into the bloodstream [423]. However, upon further analysis, it was found that there was still no significant effect seen in tumour growth/shrinkage and metastasis (Table 4).

There are several possibilities as to why this result may have occurred however, we believe that the main reason could be that the IgG1-iS18 became physically unstable due to temperature changes from repetitive freeze-thaw cycles. Continuous freeze-thaw cycles are known to decrease the shelf-life as well as degrade/aggregate several antibodies [424]. As a result, the supposed concentration of the administered antibody may not have been enough to exert a powerful effect on the tumours. Ferreira et al. (2017) showed that the IgG1-iS18 antibody was

able to successfully target LRP/LR in Alzheimer's Disease-transgenic mice, resulting in improved recognition and short-term memory [120]. However, in contrast to the current study, the antibody was administered intranasally and delivered directly to the target site of the brain [120]. Due to the complex microenvironmental features of tumour cells, the IgG1-iS18 antibody faces several rate-limiting steps when targeting cancers such as: distribution into the plasma, blood flow via the tumour, disorganized adhesion, extravasation as well as internalization of tumour cells [425]. Moreover, penetrating the tissue of a solid tumour is also found to severely limit the antibody treatment. This is due to tumour cells having complex matrix/stromal and vascular architectural components as well as high viscosity and interstitial fluid pressure from the cancerous blood supply [426]. As a result, monoclonal antibodies do not permeabilize through the tumour cells sufficiently as they need to diffuse against a pressure gradient [427]. Therefore, the antibody may not have only lost its effectiveness as it was distributed in the body, but also upon first encounter with its target sites on the tumour. In addition, colorectal cancer cells are able to exploit several cell signalling pathways including the Ras/Raf, MEK/ERK pathway, Wnt pathway, PI3K/Akt/ PTEN/ mTOR pathway, as well as apoptotic, autophagic and cell cycle regulatory pathways [22] to exert their tumourigenic effects. Thus, the HT-29 colorectal cancer cells could have used compensatory mechanisms by favouring one or more of these pathways – through the up- or down-regulation of certain proteins – to overcome the effects of the already weakened antibody, resulting in sustained cell survival. For a significant result to therefore be seen, a higher concentration/dosage as well as several treatments of the antibody would be required to not only elevate the plasma concentration of IgG1-iS18 but to also saturate the tumours with the antibody for faster penetration.

However, due to time constraints, optimizing parameters such as effective antibody concentrations, correct treatment intervals as well as fresh antibody production had to be halted. A proposed method to freshly express the IgG1-iS18 antibody would be to use transfected HEK293F cells that are known to be able to take up both heavy and light chain vectors of the antibody. The antibody will then get secreted from the cells into the cell media, where it must be collected and purified using a Protein G resin [428]. Thereafter, its exact concentration will have to be quantified via UV absorbance spectroscopy for dose- and time-dependent studies *in vitro,* prior to it being tested *in vivo*. Should the newly optimized parameters show favourable results in the MF-1 nude mice, this will confirm that the antibody is able to prevent the

formation or metastasis of colorectal cancer and may be a possible therapeutic tool for the treatment of colorectal cancer.

Chapter 6: Conclusion and Future Work

6.1. Conclusion

Tumourigenic cells are characteristically seen to overexpress the 37kDa/67kDa laminin receptor (LRP/LR) compared to their normal cell counterparts. This receptor is involved in promoting tumourigenic processes such as cell migration and adhesion, cellular viability maintenance as well as apoptotic evasion. The present study revealed that the knockdown of LRP/LR via siRNA technology significantly reduced the viability of early (SW-480) and late (DLD-1) stage colorectal cancer cells through the induction of apoptosis evident by decreased membrane integrity, caspase-3 activation and increased sub G0/G1 apoptotic levels. In addition, it was found that the colorectal cancer cell lines were able to undergo apoptosis through both apoptotic pathways – as the extrinsic and intrinsic pathways are able to interconnect at several levels. To understand the mechanistic role of LRP/LR in proliferation and apoptosis, pathway analysis was performed on the DLD-1 cells, which revealed that LRP/LR may alter components of the MAPK, apoptotic as well as autophagic signalling pathways to aid colorectal cancer cells in continuous growth and survival. It is also suggested that LRP/LR may play a role in enhancing telomerase activity and TRF-2 expression, thereby promoting genetic instability and further proliferation of the DLD-1 colorectal cancer cells. However, the knockdown of LRP/LR through siRNA technology was found to inhibit telomerase activity as well as decrease hTERT and TRF-2 protein expression levels while activate DNA damage responses, ultimately favouring the intrinsic pathway for apoptotic induction in DLD-1 cells.

Together, these findings show the important role that LRP/LR plays in maintaining cell viability and evading apoptosis. Moreover, the results reveal that siRNAs targeting LRP/LR as well as other proteins related to the receptor could be used as potential therapeutic tools for the treatment of early and late stage colorectal cancer cells. Although interesting responses were observed in the colorectal cancer cells, the exact mechanism of action of LRP/LR in tumourigenesis is far from being fully understood.

6.2. Future work

6.2.1. *In vitro* study

As mentioned previously, every cancer type has different behavioural characteristics and since the current study only focused on colorectal cancer cell lines (early and late stage), several other cancer types require investigation regarding siRNA-mediated knockdown of LRP/LR and cell viability maintenance. Moreover, colorectal cancer exists as five stages which are known to react differently to various stimuli, therefore it would be useful to investigate at which stage siRNA-mediated knockdown of LRP/LR would be most effective at decreasing cancer cell viability. The current study was also not able to analyse and compare the early (SW-480) stage to the late (DLD-1) stage with regards to pathway analyses therefore, it would be interesting to observe and perhaps pin point the changes that occur in the proteome of colorectal cancer cells as the stages increase – since it has been shown that LRP/LR expression levels increase as malignancy increases in colorectal cancer cells [111]. In addition, although the current study analysed the cell cycle in a time-dependent manner, several other time-dependent studies should be performed to determine whether the same proteins are affected upon siRNA-mediated knockdown of LRP/LR. The suggested mode of action of LRP/LR is mainly based on the activation and relative expression levels of specific proliferative and apoptotic proteins, in particular the p53 protein. Thus, it is essential to investigate the activation sequence and involvement of these proteins with LRP/LR more thoroughly, and to determine whether they are vital for apoptotic induction via anti-LRP siRNAs. Moreover, since proteomic analysis revealed an increase in the H2AX DNA damage marker, DNA binding studies should be performed to further confirm whether siRNA-mediated knockdown of LRP/LR does in fact cause apoptosis via DNA damage, thereby further illustrating its relationship with telomerase. Furthermore, several stimulators of autophagy (including AMPK, KAD4, SEC20 and CLPN4) were found to be up-regulated upon siRNA-mediated knockdown of LRP/LR. This novel finding therefore warrants further research to confirm that autophagy is indeed induced upon LRP/LR down-regulation by looking at specific autophagic markers through various experiments including RT-qPCR, western blotting and fluorescence microscopy. By performing these studies, a deeper understanding of LRP/LR's molecular mechanism will be achieved.

6.2.2. *In vivo* study

Due to time constraints, optimizing parameters such as effective antibody concentrations, correct treatment intervals as well as fresh antibody production were halted. Thus, future work regarding the treatment of IgG1-iS18 will require the antibody to be freshly expressed using

transfected HEK293F cells that are able to take up both heavy and light chain vectors of the antibody. Once the antibody is secreted from the cells into the media, it must be collected and purified using a Protein G resin [428]. Thereafter, its exact concentration will be quantified via UV absorbance spectroscopy for dose- and time-dependent studies to be performed *in vitro,* prior to it being tested *in vivo*. Should the newly optimized parameters show favourable results *in vitro*, this will allow for investigations in the MF-1 nude mice to commence to confirm that the antibody is able to prevent the formation or metastasis of colorectal cancer, thereby providing a possible therapeutic option for the treatment of colorectal cancer.

In addition, due to several *in vitro* studies displaying how siRNAs are successful in down-regulating LRP/LR and as a result, decreasing cancer cell viability and tumourigenesis, it is therefore necessary to assess said effect *in vivo* [126-129]. However, as mentioned in section 1.3.2., naked siRNAs at physiological conditions are highly unstable, have short half-lives and poor efficiency thus, the need for efficient packaging into vesicles for effective delivery to cancer cells. A promising delivery system is the encapsulation of siRNA in a solid polymer matrix of nanoparticles consisting of polylactic-co-glycolic acid (PLGA). These nanoparticles show high stability, low toxicity as well as sustained release of contents [429]. Thus, by producing nanoparticles that favour the positive characteristics of PLGA, this will allow for an ideal delivery system for siRNAs that not only includes a controlled siRNA release profile but also a high serum stability and encapsulation efficiency [430]. Moreover, this delivery system has been successful in efficiently down-regulating gene expression in prostate cancer [431], with low levels of cytotoxicity. Therefore, it would be interesting to evaluate this encapsulation method using anti-LRP/LR siRNAs to target colorectal cancer cells in *vivo*.

Furthermore, since siRNAs used to target LRP/LR reduce tumourigenesis through the induction of apoptosis [125-128] and inhibition of telomerase activity [122, 129] while monoclonal antibodies directed against LRP/LR inhibit angiogenesis [124] and metastasis [33, 111-113, 123, 133], combining siRNAs and antibodies targeted against LRP/LR would also be a potential avenue to test using animal studies. Should the animal studies be successful, this would be a highly efficient combination treatment. Ultimately, LRP/LR antibody-siRNA conjugates may be able to provide powerful, invaluable therapeutics for cancer therapy.

Chapter 7: References

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Appendix A

Supplementary data

Sequences for siRNA used to down-regulate LRP/LR:

Table A1: Sequence of Human-RPSA, esiRNA-RPSA and control siRNA-RLUC used for down-regulation of LRP/LR

siRNA	Organism	Sequence	Transfection reagent
ON-TARGETplus SMARTpool Human-RPSA	Homo sapiens	4 RPSA siRNA pooled together (targets 4 regions of LRP/LR): Target sequence 1: CGACAUGAGUUGUACUUCU Target sequence 2: GAUUGCAUAUCAAAGCAUA Target sequence 3: GGUCAUGCCUGAUCUGUAC Target sequence 4: UAUCAUAAAUCUCAAGAGG	DharmaFect 1
esiRNA-RPSA	Homo sapiens	CCTCTCACGGAGGCATCTTATGTTAACCTACCTACCATTGCGC TGTGTAACACAGATTCTCCTCTGCGCTATGTGGACATTGCCAT CCCATGCAACAACAAGGGAGCTCACTCAGTGGGTTTGATGTGG TGGATGCTGGCTCGGGAAGTTCTGCGCATGCGTGGCACCATTT CCCGTGAACACCCATGGGAGGTCATGCCTGATCTGTACTTCTA CAGAGATCCTGAAGAGATTGAAAAAGAAGAGCAGGCTGCTGCT GAGAAGGCAGTGACCAAGGAGGAATTTCAGGGTGAATGGACT GCTCCCGCTCCTGAGTTCACTGCTACTCAGCCTGAGGTTGCAG ACTGGTCTGAAGGTGTACAGGTGCCCTCTGTGCCTATTCAGCA ATTCCCTACTGAAGACTGGAGCG	Mission transfection reagent
esiRNA-RLUC	Homo sapiens	GATAACTGGTCCGCAGTGGTGGGCCAGATGTAAACAAATGAAT GTTCTTGATTCATTTATTAATTATTATGATTCAGAAAAACATGC AGAAAATGCTGTTATTTTTTTACATGGTAACGCGGCCTCTTCT TATTTATGGCGACATGTTGTGCCACATATTGAGCCAGTAGCGC GGTGTATTATACCAGACCTTATTGGTATGGGCAAATCAGGCAA ATCTGGTAATGGTTCTTATAGGTTACTTGATCATTACAAATAT	Mission transfection reagent

		CTTACTGCATGGTTTGAACTTCTTAATTTACCAAAGAAG ATCAT TTTTGTCGGCCATGATTGGGGTGCTTGTTTGGCATTTCA TTAT AGCTATGAGCATCAAGATAAGATCAAAGCAATAGTTCA CGCTG AAAGTGTAGTAGATGTGATTGAATCATGGGATGAATGG	

Transfection procedure for Human-RPSA:

Lyophilized Human-RPSA (5nmol/20nmol) was reconstituted in 100µl or 250µl of 1X RNase free siRNA buffer, respectively, before use in order to make a 20 µM stock. The table below shows amounts of siRNA and corresponding components used for transfections of a 6-well plate and 24-well plate.

Table A2: Transfection components and volumes for Human-RPSA

Reagent	6-well plate (Total volume of 2 ml per plate) (µl)	24-well plate (Total volume of 500 µl per plate) (µl)
Opti-MEM media – for addition of siRNA	190	47.5
Human-RPSA	10	2.5
Opti-MEM media – for addition of transfection reagent	190	47.5
DharmaFect transfection reagent	10	2.5

Transfection procedure:

Reconstituted Human-RPSA was added to the corresponding volume of Opti-MEM media in a micro centrifuge tube. In a second tube, transfection reagent was added to the corresponding volume of Opti-MEM media. Both tubes were incubated for 5 minutes at room temperature and mixed together. The resultant siRNA solution was further incubated for 20 minutes at room temperature. The suggested volumes of culture media were added into the 6-or 24-well plates containing the seeded cells and thereafter the resultant siRNA solution was added to the cells.

Transfection procedure for esiRNA-RPSA and esiRNA-RLUC:

esiRNA-RPSA (200 ng /µl) and esiRNA-RLUC (200 ng /µl) are purchased reconstituted. The table below shows volumes of esiRNA and corresponding components used for transfections of a 6-well plate and 24-well plate:

Table A3: Transfection components and volumes for esiRNA-RPSA and esiRNA-RLUC

Reagent	6-well plate (Total volume of 2 ml per plate) (µl)	24-well plate (Total volume of 500 µl per plate) (µl)
Opti-MEM media – for addition of esiRNA	250	50
esiRNA-RPSA or esiRNA-RLUC	5	1.5
Opti-MEM media – for addition of transfection reagent	250	50
Mission transfection reagent	5	1.5

Transfection procedure:

esiRNA-RPSA or esiRNA-RLUC was added to the corresponding volume of Opti-MEM media in a micro centrifuge tube. In a second tube, transfection reagent was added to the corresponding volume of Opti-MEM media. Both tubes were incubated for 5 minutes at room temperature and the contents of both tubes were mixed together. The suggested volumes of culture media were added into the 6-or 24-well plates containing the seeded cells and thereafter the resultant siRNA solution was added to the cells.

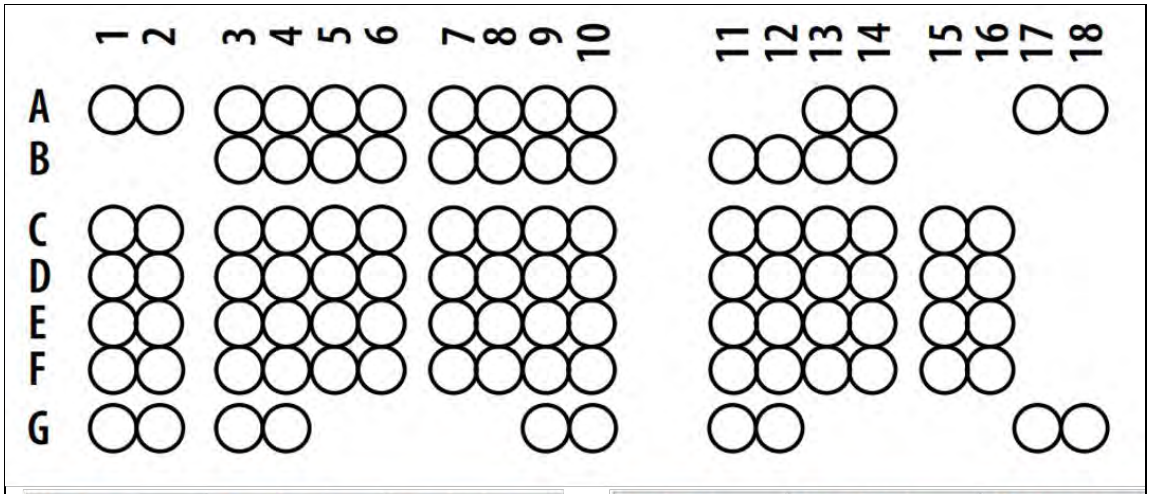
Components and volumes used for caspase assays:

The table below contains the components and volumes used in the preparation of the caspase-3, -8 and -9 assay mixtures prior to incubation in a 96-well plate for 2 hours at 37˚C:

Table A4: Caspase-3, -8 and -9 Assay mixture components

Sample	5X Assay Buffer (µl)	Caspase-3 or -8 or -9 sample (µl)	Inhibitor (µl)	dH_2O (µl)	Caspase-3 or -8 or -9 substrate (µl)	Total volume (µl)
Buffer Blank	20	0	0	80	0	100
Substrate Blank	20	0	0	70	10	100
Test sample	20	X	0	(70-X)	10	100

Visual representation of Proteome Profiler Arrays™ with corresponding coordinates:

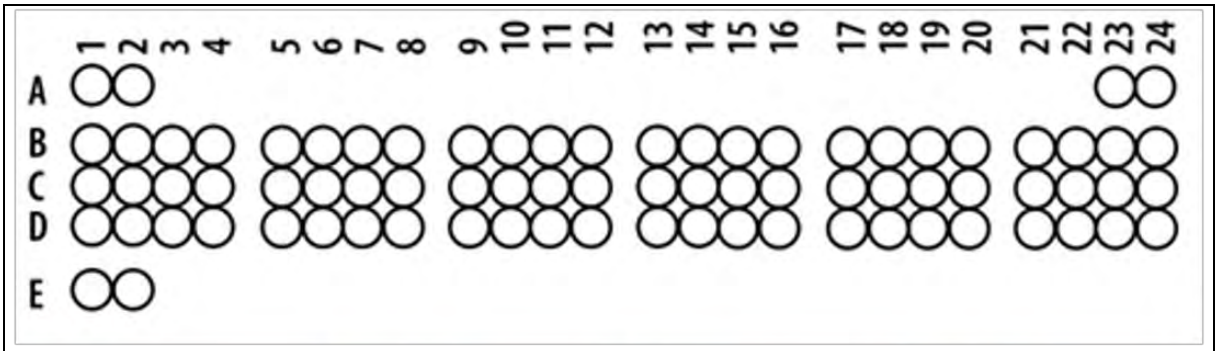


Membrane/ Coordinate	Target/Control	Phosphorylation Site
A-A1, A2	Reference Spot	—
A-A3, A4	p38α	T180/Y182
A-A5, A6	ERK1/2	T202/Y204, T185/ Y187
A-A7, A8	JNK 1/2/3	T183/Y185, T221/ Y223
A-A9, A10	GSK-3α/β	S21/S9
B-A13, A14	p53	S392
B-A17, A18	Reference Spot	—
A-B3, B4	EGF R	Y1086
A-B5, B6	MSK1/2	S376/S360
A-B7, B8	AMPKα1	T183
A-B9, B10	Akt 1/2/3	S473
B-B11, B12	Akt 1/2/3	T308
B-B13, B14	p53	S46
A-C1, C2	TOR	S2448
A-C3, C4	CREB	S133
A-C5, C6	HSP27	S78/S82
A-C7, C8	AMPKα2	T172
A-C9, C10	β-Catenin	—
B-C11, C12	p70 S6 Kinase	T389
B-C13, C14	p53	S15
B-C15, C16	c-Jun	S63
A-D1, D2	Src	Y419
A-D3, D4	Lyn	Y397
A-D5, D6	Lck	Y394
A-D7, D8	STAT2	Y689
A-D9, D10	STAT5a	Y694
B-D11, D12	p70 S6 Kinase	T421/S424
B-D13, D14	RSK1/2/3	S380/S386/S377
B-D15, D16	eNOS	S1177

Membrane/ Coordinate	Target/Control	Phosphorylation Site
A-E1, E2	Fyn	Y420
A-E3, E4	Yes	Y426
A-E5, E6	Fgr	Y412
A-E7, E8	STAT6	Y641
A-E9, E10	STAT5b	Y699
B-E11, E12	STAT3	Y705
B-E13, E14	p27	T198
B-E15, E16	PLC-γ1	Y783
A-F1, F2	Hck	Y411
A-F3, F4	Chk-2	T68
A-F5, F6	FAK	Y397
A-F7, F8	PDGF Rβ	Y751
A-F9, F10	STAT5a/b	Y694/Y699
B-F11, F12	STAT3	S727
B-F13, F14	WNK1	T60
B-F15, F16	PYK2	Y402
A-G1, G2	Reference Spot	—
A-G3, G4	PRAS40	T246
A-G9, G10	PBS (Negative Control)	—
B-G11, G12	HSP60	—
B-G17, G18	PBS (Negative Control)	—

Figure A1: Schematic of human Phospho-MAPK Proteome Profiler Antibody Array™ with corresponding coordinates. Visual representation of the membrane array consisting of

several proteins with their corresponding coordinates (shown below array). These coordinates are used to locate each protein on the array for analyte identification (Image obtained from phospho-MAPK array kit).



Coordinate	Target/Control	Coodinate	Target/Control
A1, A2	Reference Spots	C13, C14	HO-2/HMOX2
A23, A24	Reference Spots	C15, C16	HSP27
B1, B2	Bad	C17, C18	HSP60
B3, B4	Bax	C19, C20	HSP70
B5, B6	Bcl-2	C21, C22	HTRA2/Omi
B7, B8	Bcl-x	C23, C24	Livin
B9, B10	Pro-Caspase-3	D1, D2	PON2
B11, B12	Cleaved Caspase-3	D3, D4	p21/CIP1/CDKN1A
B13, B14	Catalase	D5, D6	p27/Kip1
B15, B16	cIAP-1	D7, D8	Phospho-p53 (S15)
B17, B18	cIAP-2	D9, D10	Phospho-p53 (S46)
B19, B20	Claspin	D11, D12	Phospho-p53 (S392)
B21, B22	Clusterin	D13, D14	Phospho-Rad17 (S635)
B23, B24	Cytochrome c	D15, D16	SMAC/Diablo
C1, C2	TRAIL R1/DR4	D17, D18	Survivin
C3, C4	TRAIL R2/DR5	D19, D20	TNF RI/TNFRSF1A
C5, C6	FADD	D21, D22	XIAP
C7, C8	Fas/TNFRSF6/CD95	D23, D24	PBS (Negative Control)
C9, C10	HIF-1α	E1, E2	Reference Spots
C11, C12	HO-1/HMOX1/HSP32		

Figure A2: Schematic of human Apoptosis Proteome Profiler Antibody Array™ with corresponding coordinates. Visual representation of the membrane array consisting of several proteins with corresponding coordinates (shown below array). These coordinates are used to locate each protein on the array for analyte identification (Image obtained from Apoptosis array kit).

Western blot performed prior to Proteome Profiler Antibody Arrays:

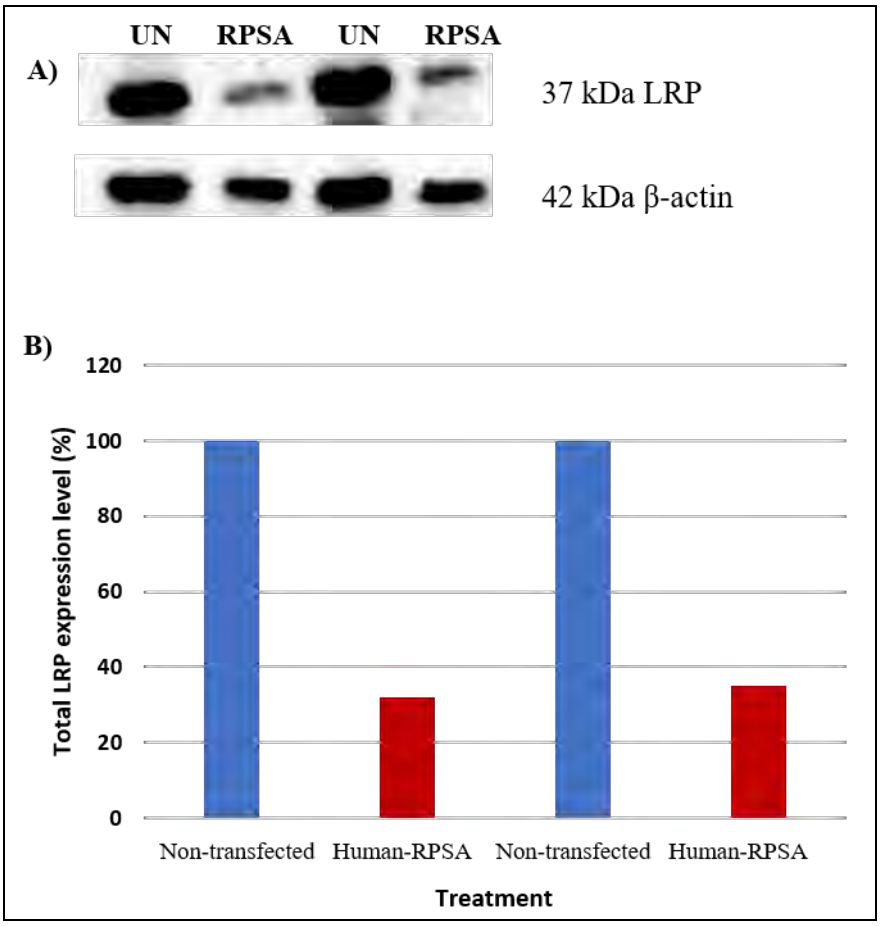


Figure A3: Representative western blot of LRP/LR levels prior to performing Proteome Profiler Antibody Arrays. Densitometric analysis revealed that upon transfection with Human-RPSA, LRP/LR was down-regulated by 70% when compared to non-transfected cells. All experiments for each array were performed using lysates that had 70% LRP/LR down-regulation for comparisons to take place.

Proteins obtained from SWATH-MS analysis:

Table A5: List of proteins involved in ribosomal processing and translation

	Fold change	Protein	Name
Down-regulated			
	-4.28	sp|P41567|EIF1_HUMAN	Eukaryotic translation initiation factor 1
	-3.5	sp|Q7Z478|DHX29_HUMAN	DExH-box helicase 29
	-3.39	sp|P20042|IF2B_HUMAN	Eukaryotic translation initiation factor 2 subunit 2
	-2.91	sp|P62875|RPAB5_HUMAN	DNA-directed RNA polymerases I, II, and III subunit
	-2.85	sp|P54105|ICLN_HUMAN	Methylosome subunit pICln
	-2.36	sp|P63220|RS21_HUMAN	Ribosomal Protein S21
Up-regulated	2.26	sp|P55795|HNRH2_HUMAN	Heterogeneous Nuclear Ribonucleoprotein H2
	2.55	sp|Q8IUX4|ABC3F_HUMAN	Apolipoprotein B MRNA Editing Enzyme Catalytic Subunit 3F
	2.56	sp|Q9ULC4|MCTS1_HUMAN	Multiple Copies In T-Cell Lymphoma-1
	3.8	sp|Q9Y224|RTRAF_HUMAN	RNA transcription, translation and transport factor protein
	5.29	sp|O00442|RTCA_HUMAN	RNA 3'-terminal phosphate cyclase
	3.76	sp|Q9BYD2|RM09_HUMAN	Mitochondrial Ribosomal Protein L9

Table A6: List of proteins involved in nuclear and chromatin maintenance

	Fold change	Protein	Name
Down-regulated	-8.2	sp|P09651|HNRNPA1_HUMAN	Heterogeneous nuclear ribonucleoprotein A1
	-3.32	sp|Q9UKI8|TLK1_HUMAN	Serine/threonine-protein kinase tousled-like 1
	-5.62	sp|O96028|NSD2_HUMAN	Histone-lysine N-methyltransferase
	-4.95	sp|Q9UKY7|CDV3_HUMAN	Protein CDV3 homolog
	-2.52	sp|Q15370|ELOB_HUMAN	Elongin B
	-2.44	sp|O15405|TOX3_HUMAN	TOX high mobility group box family member 3

Table A7: List of proteins involved in cytoskeletal maintenance and cell anchorage

	Fold change	Protein	Name
	-5.75	sp|Q14244|MAP7_HUMAN	Ensconsin
Down-regulated	-4.39	sp|Q9UK76|JUPI1_HUMAN	Jupiter Microtubule Associated Homolog 1
	-4.26	sp|O14530|TXND9_HUMAN	Thioredoxin Domain Containing 9
	-4.01	sp|Q14118|DAG1_HUMAN	Dystroglycan
	-3.12	sp|P15151|PVR_HUMAN	Poliovirus receptor/ PVR Cell Adhesion Molecule
	-3.32	sp|Q6QEF8|CORO6_HUMAN	Coronin-like protein E
	-2.95	sp|P02751|FINC_HUMAN	Fibronectin 1
	-2.92	sp|Q9Y639|NPTN_HUMAN	Neuroplastin
	-2.79	sp|Q06481|APLP2_HUMAN	Amyloid-like protein 2

	-2.53	sp|Q99439|CNN2_HUMAN	Calponin-2
	-2.17	sp|Q15417|CNN3_HUMAN	Calponin-3
	-2.37	sp|Q86UK7|ZN598_HUMAN	Zinc Finger Protein 598
Up-regulated	2.01	sp|Q9Y3D6|FIS1_HUMAN	Fission 1 protein
	2.3	sp|Q7Z4H8|PLGT3_HUMAN	Protein O-Glucosyltransferase 3
	2.44	sp|Q7Z3K3|POGZ_HUMAN	Pogo Transposable Element Derived with ZNF Domain/ Zinc Finger Protein 280E
	2.46	sp|Q6NW34|NEPRO_HUMAN	Nucleolus and neural progenitor protein
	2.65	sp|O95994|AGR2_HUMAN	Anterior gradient protein 2 homolog
	3.46	sp|Q9P0S9|TM14C_HUMAN	Transmembrane Protein 14C

Table A8: List of proteins involved in vesicle transport and membrane trafficking

Down-regulated	Fold change	Protein	Name
	-4.71	sp|Q9HD42|CHM1A_HUMAN	Charged Multivesicular Body
	-3.94	sp|Q9NWW5|CLN6_HUMAN	Ceroid-lipofuscinosis neuronal protein 6
	-2.62	sp|Q8TAT6|NPL4_HUMAN	Nuclear protein localization protein 4
Up-regulated	-2.06	sp|Q5JRA6|TGO1_HUMAN	Transport and Golgi organization protein 1/Melanoma Inhibitory Activity Protein 3
	2.1	sp|Q12981|SEC20_HUMAN	Vesicle transport protein
	2.35	sp|Q14728|MFS10_HUMAN	Major Facilitator Superfamily Domain Containing 10
	3.11	sp|P42858|HD_HUMAN	Huntingtin

Table A9: List of proteins involved in apoptotic regulation

Down-regulated	Fold change	Protein	Name
	-4.63	sp|Q9NYF8|BCLF1_HUMAN	Bcl-2-associated transcription factor 1
	-4.43	sp|Q7L9L4|MOB1B_HUMAN	Mps one binder kinase activator-like 1A
	-4.02	sp|P41222|PTGDS_HUMAN	Prostaglandin-H2 D-isomerase
	-2.79	sp|O60610|DIAP1_HUMAN	Death-associated inhibitor of apoptosis 1
	-2.4	sp|Q13546|RIPK1_HUMAN	Receptor-interacting serine/threonine-protein kinase 1
Up-regulated	-2.12	sp|P07203|GPX1_HUMAN	Glutathione peroxidase 1
	2.12	sp|Q5JTW2|CEP78_HUMAN	Centrosomal Protein 78
	2.16	sp|Q92597|NDRG1_HUMAN	N-myc Downstream-Regulated Gene 1
	2.29	sp|Q14534|ERG1_HUMAN	Early growth response protein 1
	2.54	sp|O00291|HIP1_HUMAN	Huntingtin interacting protein 1
	2.57	sp|P07305|H10_HUMAN	H1 Histone Family Member 0

	2.68	sp|P62854|RS26_HUMAN	Ribosomal Protein S26
	2.92	sp|Q13228|SBP1_HUMAN	Selenium-binding protein 1
	2.94	sp|P06733|ENOA_HUMAN	Enolase 1
	3.22	sp|Q53EL6|PDCD4_HUMAN	Programmed cell death protein 4
	3.45	sp|Q9UN37|VPS4A_HUMAN	Vacuolar protein sorting-associated protein 4A
	3.68	sp|P22392|NDKB_HUMAN	Nucleoside diphosphate kinase B
	5.14	sp|Q16795|NDUA9_HUMAN	NADH dehydrogenase (Ubiquinone) 1 alpha subcomplex subunit
	5.37	sp|P16104|H2AX_HUMAN	Histone H2AX

Table A10: List of proteins involved in autophagic regulation

Up-regulated	Fold change	Protein	Name
	2.01	sp|P48735|IDHP_HUMAN	Isocitrate dehydrogenase NADP
	2.078	sp|Q53GQ0|DHB12_HUMAN	Estradiol 17-beta-dehydrogenase 12
	2.18	sp|Q9P035|HACD3_HUMAN	3-Hydroxyacyl-CoA Dehydratase 3
	2.36	sp|A1L0T0|ILVBL_HUMAN	Acetolactate synthase-like protein
	2.51	sp|Q86SK9|SCD5_HUMAN	Stearoyl-CoA Desaturase 5
	2.55	sp|Q643R3|LPCT4_HUMAN	Lysophosphatidylcholine Acyltransferase 4
	3.09	sp|Q5U5X0|LYRM7_HUMAN	LYR Motif Containing 7
	3.2	sp|P27144|KAD4_HUMAN	Adenylate Kinase 4
	3.21	sp|P0DMM9|ST1A3_HUMAN	Sulfotransferase Family 1A Member 3
	3.51	sp|O00204|ST2B1_HUMAN	Sulfotransferase family cytosolic 2B member 1
	4.22	sp|Q02818|NUCB1_HUMAN	Nucleobindin-1
	4.78	sp|P04632|CPNS1_HUMAN	Calpain Small Subunit 1

Table A11: List of remaining proteins identified (no direct link to LRP/LR)

	Fold change	Protein	Name
Down-regulated	-2.5	sp|P61916|NPC2_HUMAN	Intracellular cholesterol transporter 2
	-4.48	sp|Q9H832|UBE2Z_HUMAN	Ubiquitin conjugating enzyme E2 Z
	-3.29	sp|Q8N806|UBR7_HUMAN	Putative E3 ubiquitin-protein ligase
	-2.43	sp|O96005|CLPT1_HUMAN	ATP-dependent Clp protease ATP-binding subunit
	-4.64	sp|P17152|TMM11_HUMAN	Transmembrane protein 11
	-3.45	sp|Q9UJC5|SH3L2_HUMAN	SH3 domain-binding glutamic acid-rich-like protein
Up-regulated	3.91	sp|Q99707|METH_HUMAN	Methionine synthase
	2.61	sp|Q5JU69|TOR2A_HUMAN	Torsin Family 2 Member A
	3.32	sp|Q8TEU7|RPGF6_HUMAN	Rap Guanine Nucleotide Exchange Factor 6
	3.94	sp|Q68EM7|RHG17_HUMAN	Rho GTPase Activating Protein 17

Primers used for mRNA quantification in qPCR reactions:

Table A11: List of primers used for mRNA quantification through qPCR

Gene	Forward Primer (5'-3')	Reverse Primer (3'-5')
GAPDH (Inqaba Biotech™) (Deng et al. , 2014 [432]	GTGGACCTGACCTGCCGTCT	GGAGGAGTGGGTGTCGCTGT
ACTB (Integrated DNA Technologies®) - original synthesis	AGTTGCGTTACACCCTTTC	CCTTCACCGTTCCAGTTT
LRP/LR (Integrated DNA Technologies®) - original synthesis	GCCATTGAAAACCCTGCTGA	AGCGCAATGGTAGGTAGGTT
p53 (Inqaba Biotech™) (Chew et al. , 2012 [433])	TAACAGTTCCTGCATGGGCGGC	AGGACAGGCACAAACACGCACC
Bax (Integrated DNA Technologies®	AAGAAGCTGAGCGAGTGTCT	GTTCTGATCAGTTCCGGCAC

) - original synthesis)		
Bcl-2 (Inqaba Biotech™) (Jackson et al. , 2006 [434])	CCTGATTCATTGGGAAGTTTCAA	AAACAAATGCATAAGGCAACGA
TERT (Integrated DNA Technologies®) – original synthesis	CTTCCTACGCTTCATGTGCC	AATCATCCACCAAACGCAGG
CREB (Inqaba Biotech™) (Shankar et al. , 2005 [435])	AAGCTGAAAGTCAACAAATGACAGTT	TGGACTGTCTGCCCATTGG

RNA integrity gels:

In order to confirm that there was no DNA contamination in the extracted RNA samples, RNA integrity gels were run each time new samples were made.

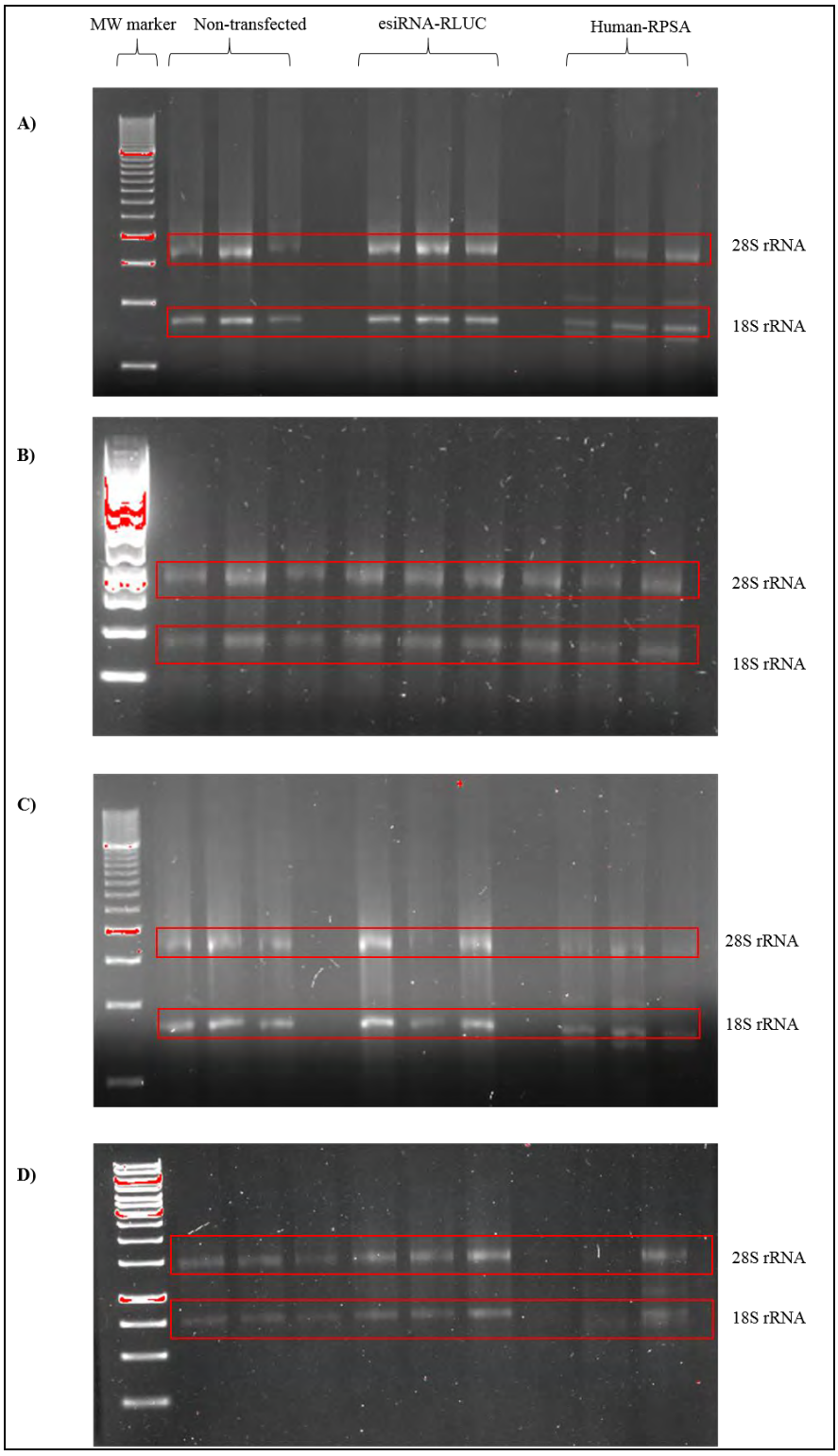


Figure A4: Agarose gel electrophoresis displaying total RNA. Agarose 1% gels were utilized to evaluate the purity and integrity of the extracted RNA from DLD-1 cells. Two

distinct bands can be seen for each sample in each gel which are indicative of ribosomal 28S rRNA and 18S rRNA. Moreover, there are no distinct bands at the top of each well, confirming no DNA contamination. One microliter of RNA was loaded for each sample. A 50 base pair molecular weight "MW" marker was used.

Melt peak and amplification curve analysis of primers used:

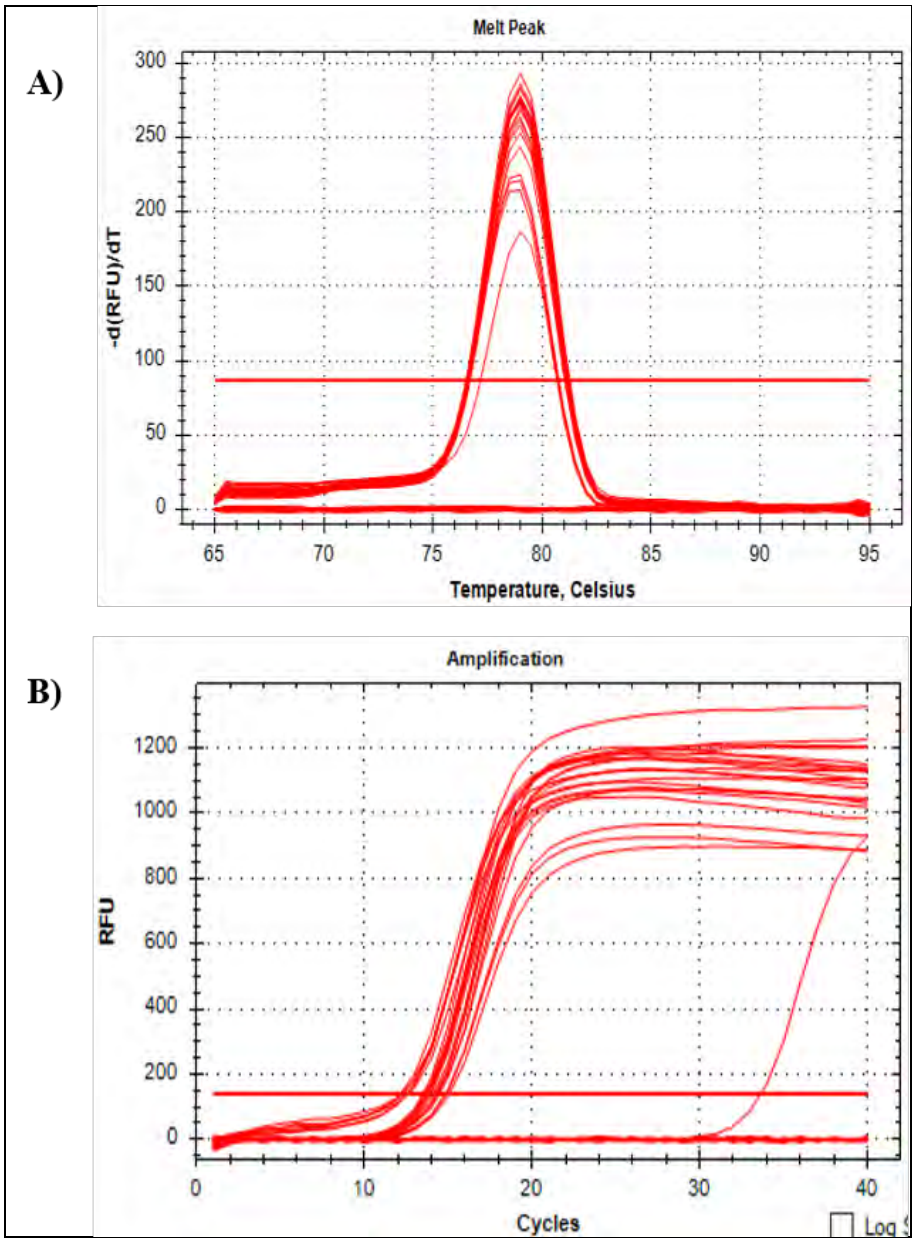


Figure A5: Melt peak and amplification curve analysis of β-actin reference gene. A) Representative qPCR melt peak analysis of the β-actin gene in non-transfected, esiRNA-RLUC control-transfected and Human-RPSA-transfected DLD-1 cell samples. B) Amplification curve following real-time PCR amplification of the β-actin gene in non-transfected, esiRNA-RLUC control-transfected and Human-RPSA-transfected DLD-1 cell samples. The No template controls (NTC's) and reverse transcriptase controls (RTC's) did not amplify only displaying noise/background.

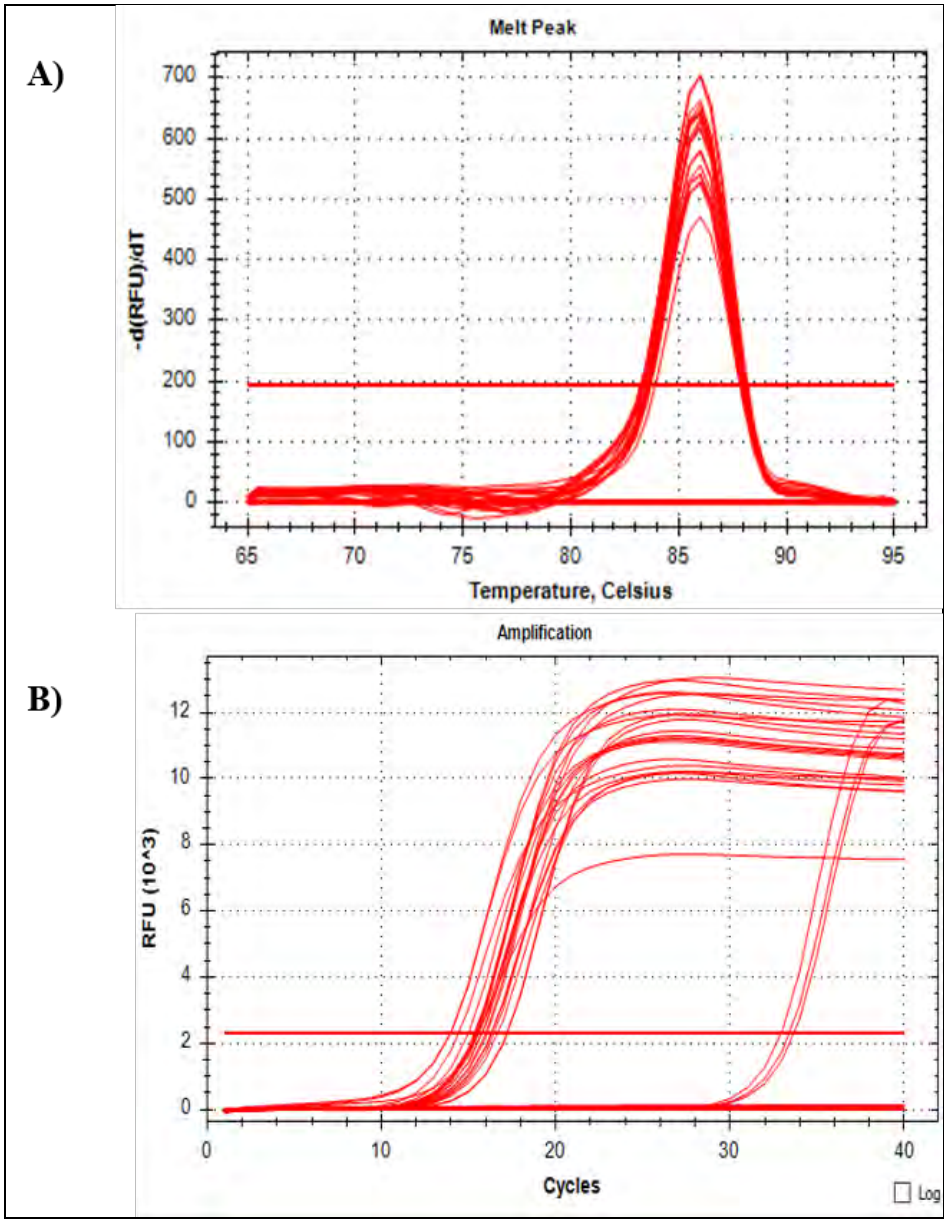


Figure A6: Melt peak and amplification curve analysis of GAPDH reference gene. A) Representative qPCR melt peak analysis of the β-actin gene in non-transfected, esiRNA-RLUC control-transfected and Human-RPSA-transfected DLD-1 cell samples. B) Amplification curve following real-time PCR amplification of the GAPDH gene in non-transfected, esiRNA-RLUC control-transfected and Human-RPSA-transfected DLD-1 cell samples. The No template controls (NTC's) and reverse transcriptase controls (RTC's) did not amplify only displaying noise/background.

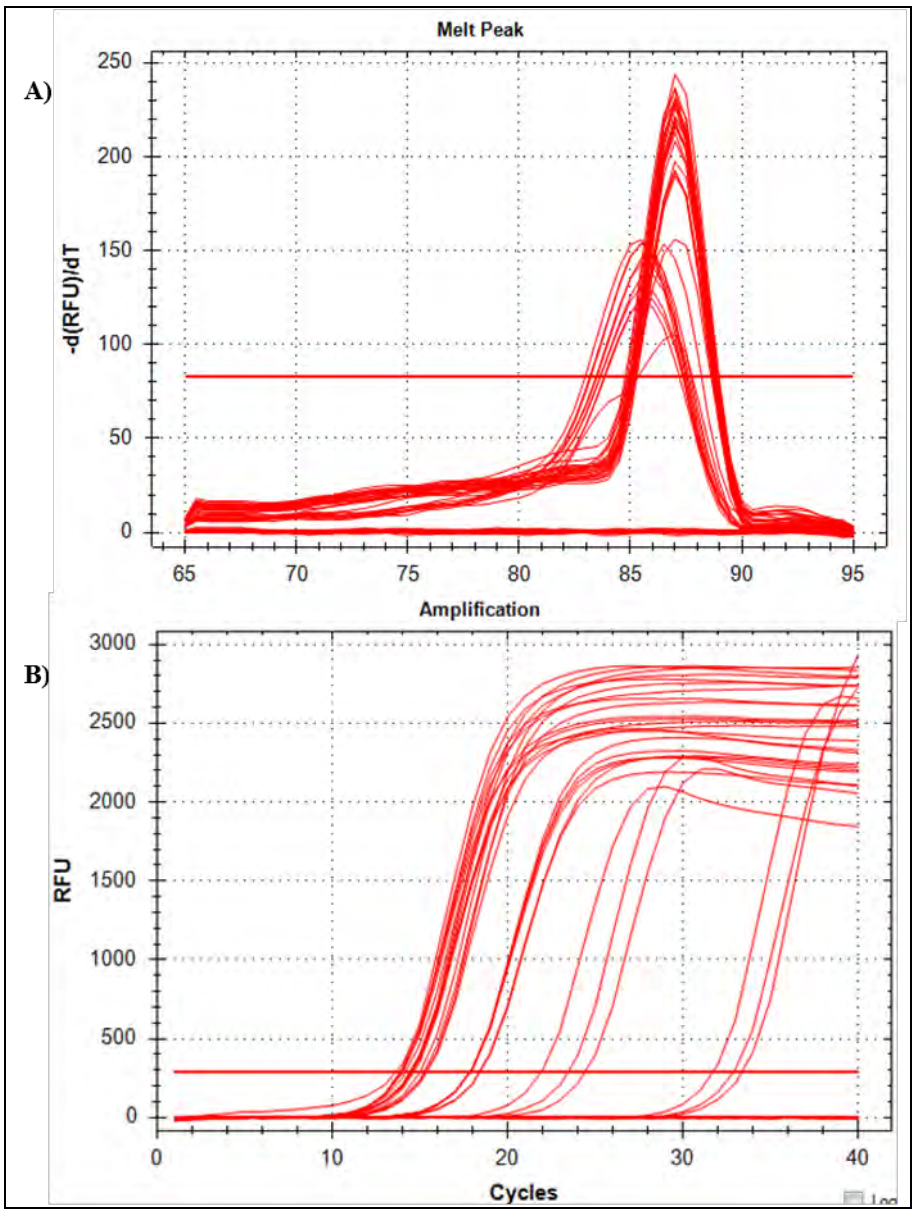


Figure A7: Melt peak and amplification curve analysis of the LRP/LR gene. A) Representative qPCR melt peak analysis of the LRP/LR gene in non-transfected, esiRNA-RLUC control-transfected and Human-RPSA-transfected DLD-1 cell samples. B) Amplification curve following real-time PCR amplification of the LRP/LR gene in non-transfected, esiRNA-RLUC control-transfected and Human-RPSA-transfected DLD-1 cell samples. The No template controls (NTC's) and reverse transcriptase controls (RTC's) did not amplify only displaying noise/background

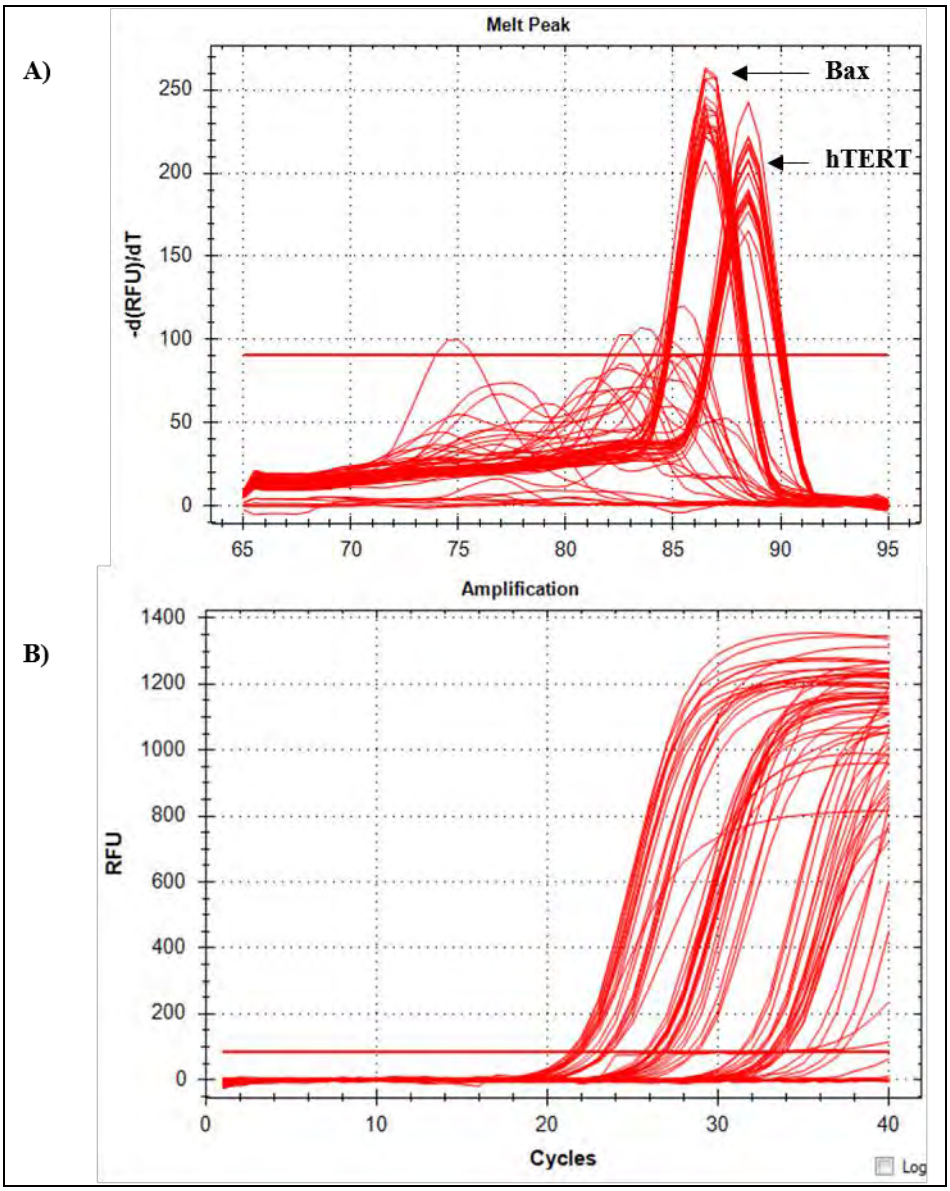


Figure A8: Melt peak and amplification curve analysis of Bax and TERT genes. A) Representative qPCR melt peak analysis of Bax and hTERT genes in non-transfected, esiRNA-RLUC control-transfected and Human-RPSA-transfected DLD-1 cell samples. B) Amplification curve following real-time PCR amplification of Bax and TERT genes in non-transfected, esiRNA-RLUC control-transfected and Human-RPSA-transfected DLD-1 cell samples. The No template controls (NTC's) and reverse transcriptase controls (RTC's) did not amplify only displaying noise/background.

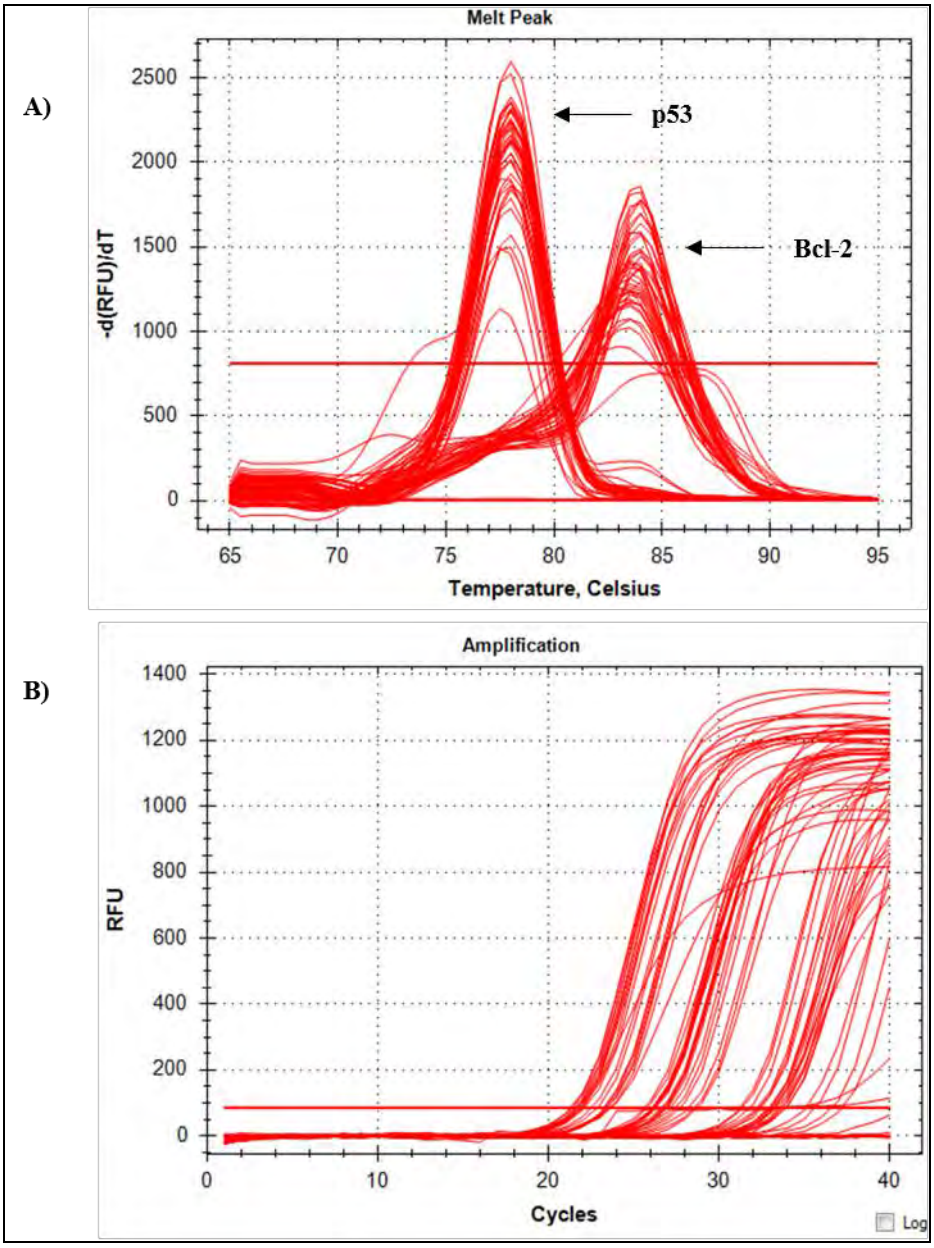


Figure A9: Melt peak and amplification curve analysis of p53 and Bcl-2 genes. A) Representative qPCR melt peak analysis of p53 and Bcl-2 genes in non-transfected, esiRNA-RLUC control-transfected and Human-RPSA-transfected DLD-1 cell samples. B) Amplification curve following real-time PCR amplification of p53 and Bcl-2 genes in non-transfected, esiRNA-RLUC control-transfected and Human-RPSA-transfected DLD-1 cell samples. The No template controls (NTC's) and reverse transcriptase controls (RTC's) did not amplify only displaying noise/background.

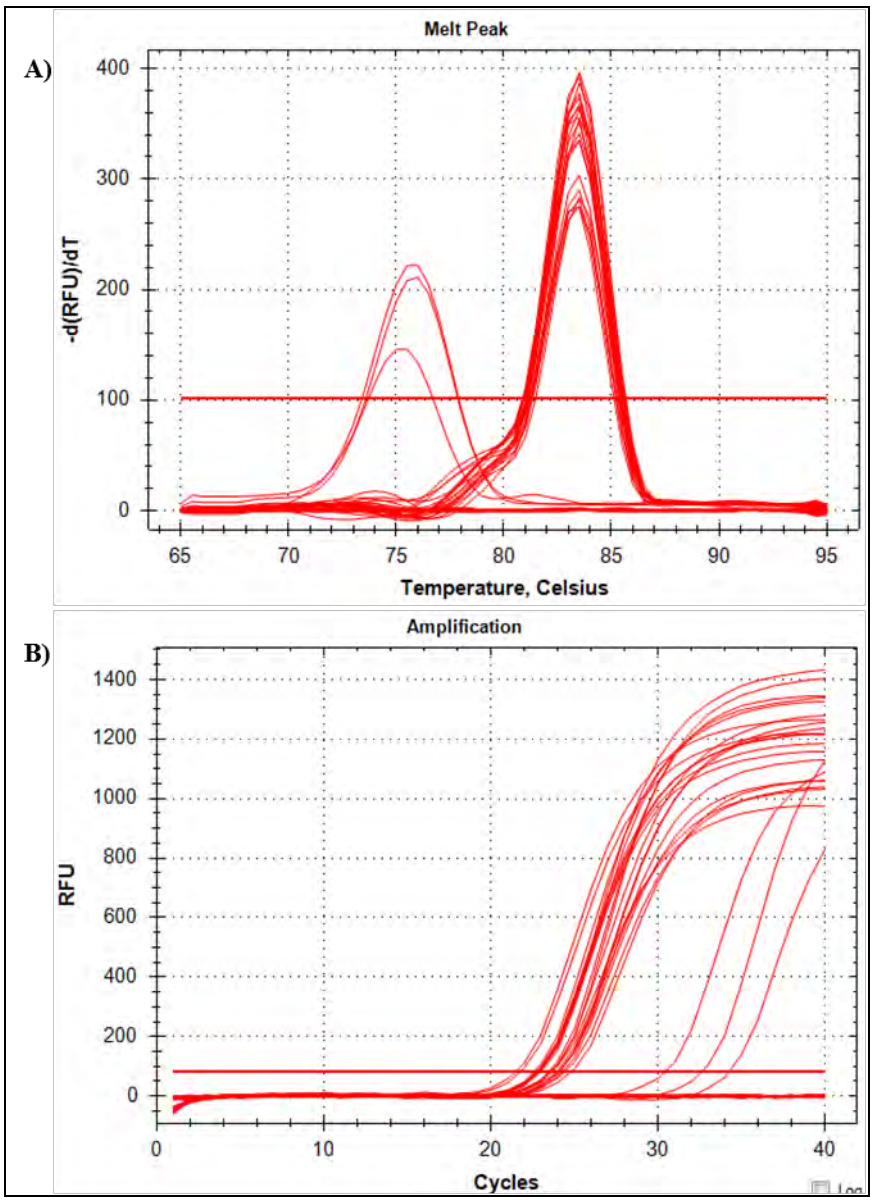


Figure A10: Melt peak and amplification curve analysis of the CREB gene. A) Representative qPCR melt peak analysis of the CREB gene in non-transfected, esiRNA-RLUC control-transfected and Human-RPSA-transfected DLD-1 cell samples. B) Amplification curve following real-time PCR amplification of the CREB gene in non-transfected, esiRNA-RLUC control-transfected and Human-RPSA-transfected DLD-1 cell samples. The No template controls (NTC's) and reverse transcriptase controls (RTC's) did not amplify only displaying noise/background.

Pearson's correlation co-efficient analysis for Human-RPSA:

Table A12: Assessment of correlation between levels of Human-RPSA siRNA-mediated LRP knock-down and viability, apoptotic levels and caspase-3 activity of early (SW-480) and late (DLD-1) stage colorectal cancer cells, using Pearson's correlation co-efficients (R).

Cell line	SW-480	DLD-1
Correlation between total LRP levels before and after Human-RPSA transfection (R-value)	0.91	0.96
Correlation between Human-RPSA-mediated LRP knockdown and reduction in cell viability (R-value)	0.99	0.98
Correlation between Human-RPSA-mediated LRP knockdown and reduction in cell viability (R-value)	0.99	0.93
Correlation between total levels of apoptosis and total LRP levels after Human-RPSA transfection (R-value)	0.99	0.98
Correlation between increases in caspase-3 activity and total LRP levels after Human-RPSA (R-value)	0.94	0.93

Pearson's correlation co-efficient analysis for esiRNA-RPSA:

Table A13: Assessment of correlation between levels of alternate esiRNA-RPSA siRNA-mediated LRP knock-down and cell viability

Cell line	SW-480	DLD-1
Correlation between total LRP levels before and after esiRNA-RPSA transfection (R-value)	0.98	0.99
Correlation between esiRNA-RPSA-mediated LRP knockdown and reduction in cell viability (R-value)	0.95	0.98

Appendix B

Reagents and Materials

- Media (DMEM)- GE Healthcare Life sciences (Massachusetts, USA)
- BSA, Ethanol -VWR Life sciences (Pennsylvania, USA)
- Molecular weight marker, OneTaq® HotStart Taq Polymerase - New England Biolabs Inc. (Massachusetts, USA)
- Penicillin/streptomycin, PBS, Opti-MEM – Gibco Life Sciences (California, USA)
- Tween-20, DMSO, lysis buffer - Merck Millipore (Johannesburg, RSA)
- Ethanol, Methanol, Paraformaldehyde, Acetic acid -Associate chemical enterprise (ACE) (Johannesburg, RSA)
- MTT powder - Duchefa Biochemie (Haarlem, The Netherlands)
- Trypsin/EDTA, Pen/Strep Amphoteracin B 100X – Lonza (Basel, Switzerland)
- HEPES – Thermo Fisher Scientific (Massachusetts, USA)
- Hoechst-33258 - Invitrogen (Oregon, USA)
- iRT peptide standards – Biognosys (Schlieran, Switzerland)
- Agarose Csl-AG100 LE - Cleaver Scientific Ltd, (Warwickshire, UK)
- Non-essential amino acids, BCA reagents, APS, acrylamide, TEMED, fluoromount mounting fluid, SDS, Triton-X, CHAPS lysis buffer, Agarose gel stain, Glycine, Loading buffer, 2ml centrifugation tubes, β-mercaptoethanol, Bisacrylamide, Trypan Blue, PCA , FCS – Sigma Life science (Missouri, USA)
- PVDF membrane – Pall corporation (New York, USA)
- Chemiluminescent substrate, Cell counting slides – Biorad (California, USA)
- Microscopy slides and coverslips – Labocare (Gauteng, RSA)
- Matrigel Matrix, 24-well, 6-well plates, 15ml and 50 ml falcon tubes – Corning Inc. (New York, USA)
- 25 and 75 cm^3 cell culture flasks - NEST Biotechnology Co. Lt (Wuxi, China)
- 0.2 ml PCR tubes – STARLAB (Hamburg, Germany)
- Guava® cell cycle reagent - Luminex Corporation (Texas, USA)

Antibodies and siRNAs

- IgG1-iS18 (1:1000) – Affimed Therapeutics (Heidelberg, Germany)
- Anti-human IgG-HRP, anti-human PE, anti-rabbit APC– Abcam (Cambridge, UK)
- Anti-rabbit pTERT (1:1000), Anti-β actin peroxidise (1:1000), esiRNA-RPSA (200ng/ul), esiRNA-RLUC (200 ng/ul) – Sigma Life Sciences (Missouri, USA)
- Human-RPSA siRNA(5 nmol/20nmol) and DharmaFect1 transfection reagent – GE Life Sciences (Massachusetts, USA)
- Anti-rabbit TRF-1 (1:1000), Anti-rabbit IgG-HRP (1:2000), Anti-mouse IgG-HRP (1:2000) – Cell Signalling Technology® (Massachusetts, USA)
- Anti-rabbit TRF-2 (1:1000) – Merck Millipore (Gauteng, RSA)
- Anti-mouse hTERT (1:1000) – Invitrogen (California, USA)

Kits

- Annexin V-FITC/ PI kit – BD Biosciences (New Jersey, USA)
- Caspase 3,-8 and -9 kits and cell cycle kit – Merck Millipore (Gauteng, RSA)
- MAPK, Phospho-MAPK and Apoptosis Proteome Profiler Antibody Array™ kits – R&D Systems (Minnesota, USA)
- SensiFAST SYBR™ No-ROX kit, SensiFAST™ cDNA synthesis kit – Bioline (London, UK)
- KAPA Taq PCR kit – Kapa Biosystems (Massachusetts, USA)
- Quick-RNA™ MiniPrep Plus kit – Zymo Research (California, USA)

Equipment list

- Flow cytometer BD Accuri C - BD Biosciences (California, USA)
- Confocal microscope – Zeiss LSM 710 3-channel (images were captured using the blue laser, 63X magnification, and using Zen 2011 software). (Oberkochen, Germany)
- Nanodrop® ND-1000 - NanoDrop Technologies (Detroit, USA)
- KingFisher™ Flex magnetic particle processing robot, Dionex Ultimate 3000 RSLC system coupled to an AB Sciex 6600 TipleTOF mass spectrometer, Bright field microscope/EVOS Floid cell imaging station (Massachusetts, USA)
- Laminar flow – Labotec (Gauteng, RSA)
- ELISA reader – Tecan (using Magellan software) (Shanghai, China)
- Eppendorf 5417C - Merck Millipore (Gauteng, RSA)
- Pipettes and micropipettes – Eppendorf research (Hamburg, Germany)
- TC20 cell counter, gel casting and running apparatus, Chemidoc imaging machine, Trans-Blot® Turbo™ Transfer System, CFX Maestro™ thermo cycler – Biorad (California, USA)
- pH meter – Eutech instruments (Singapore, Republic of Singapore)

Software

- ImageJ (version 1.8)
- BD Sciences flow cytometry software - BD Biosciences (California. USA)
- CFX Maestro™ (version 1.0), Image Lab (version 5.1) Image acquisition and analysis software -BioRad (California, USA)
- BindIt Software 3.0 - Thermo Fisher scientific (Massachusetts, USA)
- Protein Pilot (version 5.0.1)
- Skyline (version 4.1.1.18179) spectral library builder
- Proteo Wizard MS Convert
- GraphPad prism (version 5.03)
- Microsoft® Excel 365

Appendix C

Ethics clearance

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2015/02/04/C

APPLICANT: Professor SFT Weiss

SCHOOL: Molecular & Cell Biology
DEPARTMENT:
LOCATION:

PROJECT TITLE: Verification of the therapeutic potential of anti-LRP/LR antibody IgG1-IS18 for the treatment of metastatic colorectal cancer and malignant melanoma in MF1 Nude mice

Number and Species

36 MF1 Nude mice

Approval was given for the use of animals for the project described above at an AESC meeting held on 2015/02/24. This approval remains valid until 2017/02/23.
Unreported changes to the application may invalidate the clearance given by the AESC
An annual progress report must be provided

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

A pilot project, using six mice (three for each cancer cell line), should first be carried out and the results reported to the AESC
Weight loss should be monitored
Analgesic and euthenase to be specified

Signed: _____ (Chairperson, AESC) Date: 25 October 2017

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 25 October 2017

cc: Supervisor: N/A
Director: CAS

Works 2000/lain0015/AESCCert.wps

University
of the Witwatersrand,
Johannesburg

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical)

Secretariat: Research Office, Room SH10005, 10th floor, Senate House • Telephone: +27 11 717-1234 • Fax: +27 11 339-5708
Private Bag 3, Wits 2050, South Africa

Ref: W-CJ-110707-1 07/07/2011

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: **Professor Stefan F T Weiss.**

Project title: **Cancer therapy targeting LRP/LR.**

Reason: This is a laboratory study using commercial cell lines including - A549, HeLa, MDA-MB453, SW-480, LNCaP, KYSE-140, BC-1, AGS, SK HEP1, Capan-1, Human Umbilical Vein Endothelial Cells, HUVEC Cells, Invitrogen Catalogue Number C-015-5C. Techniques to be used include MTT-assays, Annexin-V assays, nuclear morphological changes assay, western blot analyses and PARP levels. The role of LRP/LR in tumour angiogenesis will be studied. No humans are involved.
This waiver also applies to the use of any other cell lines or laboratory techniques that may be appropriate during this in-vitro study.

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON-JONES
HREC (MEDICAL)
2011 -07- 07
JOHANNESBURG

Professor Peter Cleaton-Jones
Chair: Human Research Ethics Committee (Medical)

copy: Anisa Keshav, Research Office, Senate House, Wits

University
of the Witwatersrand,
Johannesburg

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical)

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Ref: W-CJ-110316-1 16/03/2011

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: **Professor Stefan F T Weiss.**

Project title: **Therapeutic antibodies targeting the 37 kDa/67 kDa laminin receptor for the treatment of metastatic cancer.**

Reason: This is a laboratory study using commercial cell lines including - A549, HeLa, MDA-MB453, HT-29, LNCaP, KYSE-140, BC-1, AGS, HEP G2, HPAC. No humans are involved.

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PROF PE CLEATON-JONES
HREC (MEDICAL)
2011 -03- 16
JOHANNESBURG

Professor Peter Cleaton-Jones
Chair: Human Research Ethics Committee (Medical)

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of the Witwatersrand,
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Ref: W-CJ-130916-1 16/09/2013

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: **Prof S F T Weiss.**

Project title: **Antibodies and shRNA targeting the 37kDa/67kDa LRP/LR for the treatment of metastatic cancer.**

Reason: This is a laboratory study using commercial cell lines including SHSY5Y, A375, MDA-MB231, HT-29, LNCaP, HEP G2, HPAC, HUVEC and one established at Wits - WHCO1. There are no human participants

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HREC (MEDICAL)
2013 -09- 16
JOHANNESBURG

Professor Peter Cleaton-Jones
Chair: Human Research Ethics Committee (Medical)

copy: Anisa Keshav, Zanele Ndlovu, Wits Research Office

Human Research Ethics Committee (Medical)

Research Office Secretariat: Senate House Room SH10005, 10th floor. Tel +27 (0)11-717-1252
Medical School Secretariat: P V Tobias Building, Room 304, 3rd floor Tel +27 (0)11-717-2700
Private Bag 3, Wits 2050, www.wits.ac.za. Fax +27 (0)11-717-1265

Ref: W-CJ-140804-1 04/08/2014

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: **Prof ST Weiss.**

Project title: **Antibodies and shRNA targeting the 37kDa LRP/LR for cancer treatment.**

Reason: This is a laboratory study using commercially available or established cell lines at Wits including USHSY5Y, A375, MDA-MB231, HT-29, LNCaP, WHCO1, HEP G2, HPAC, HUVEC, MCF-7, HeLa and HEK-293 or similar. There are no human participants

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2014 -08- 04
JOHANNESBURG

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy - HREC(Medical) Secretariat : Anisa Keshav, Zanele Ndlovu.

Original publications

Part of PhD by Thesis

Vania, L., Rebelo, T. M., Ferreira, E., & Weiss, S. F. T. (2018). Knock-down of LRP/LR promotes apoptosis in early and late stage colorectal carcinoma cells via caspase activation. *BMC Cancer*, **18**:602. http://doi.org/10.1186/s12885-018-4531-2.

Vania, L., Morris, G., Otgaar, T.C., Bignoux, M.J., Bernert, M., Burns, J., Gabathuse, A., Singh, E., Ferreira, E. and Weiss, S.F.T. (2019). Patented therapeutic approaches targeting LRP/LR for cancer treatment. *Expert Opinion on Therapeutic Patents*, 29(12):987-1009. http://doi.org/10.1080/13543776.2019.1693543.

Not part of PhD by Thesis

Vania, L., Chetty, C. J., Ferreira, E., & Weiss, S. F. (2016). Anti-LRP/LR–Specific antibody IgG1-iS18 significantly impedes adhesion and invasion in early- and late-stage colorectal carcinoma cells. *Molecular Medicine*. **22**:664–673. http://doi.org/10.2119/molmed.2016.00169.

Rebelo, T.M., Vania, L., Ferreira, E. and Weiss S.F.T. (2018). siRNA-mediated LRP/LR down-regulation reduces cellular viability of malignant melanoma cells through the activation of apoptotic caspases. Exp. Cell. Res. 368(1):1-12. http://doi: 10.1016/j.yexcr.2018.04.00.

Vania et al. BMC Cancer (2018) 18:602
https://doi.org/10.1186/s12885-018-4531-2

BMC Cancer

RESEARCH ARTICLE Open Access

Knock-down of LRP/LR promotes apoptosis in early and late stage colorectal carcinoma cells via caspase activation

Leila Vania, Thalia M. Rebelo, Eloise Ferreira and Stefan F. T. Weiss*

Abstract

Background: Cancer remains one of the leading causes of death around the world, where incidence and mortality rates are at a constant increase. Tumourigenic cells are characteristically seen to over-express the 37 kDa/67 kDa laminin receptor (LRP/LR) compared to their normal cell counterparts. This receptor has numerous roles in tumourigenesis including metastasis, angiogenic enhancement, telomerase activation, cell viability and apoptotic evasion. This study aimed to expose the role of LRP/LR on the cellular viability of early (SW-480) and late (DLD-1) stage colorectal cancer cells.

Methods: siRNA were used to down-regulate the expression of LRP/LR in SW-480 and DLD-1 cells which was assessed using western blotting. Subsequently, cell survival was evaluated using the MTT cell survival assay and confocal microscopy. Thereafter, Annexin V-FITC/PI staining and caspase activity assays were used to investigate the mechanism underlying the cell death observed upon LRP/LR knockdown. The data was analysed using Student's t-test with a confidence interval of 95%, with *p*-values of less than 0.05 seen as significant.

Results: Here we reveal that siRNA-mediated knock-down of LRP led to notable decreases in cell viability through increased levels of apoptosis, apparent by compromised membrane integrity and significantly high caspase-3 activity. Down-regulated LRP resulted in a significant increase in caspase-8 and -9 activity in both cell lines.

Conclusions: These findings show that the receptor is critically implicated in apoptosis and that LRP/LR down-regulation induces apoptosis in early and late stage colorectal cancer cells through both apoptotic pathways. Thus, this study suggests that siRNA-mediated knock-down of LRP could be a possible therapeutic strategy for the treatment of early and late stage colorectal carcinoma.

Keywords: Colorectal cancer, Small interfering RNAs, Apoptosis, 37 kDa/67 kDa laminin receptor, LRP/LR, Therapeutics

Background

Cancer remains one of the main causes of death around the world, where incidence and mortality rates are at a constant increase. According to the World Health Organisation (WHO), over 14 million new cases were diagnosed in 2015, and 8.8 million cancer related deaths were reported [1]. The current study focuses on a particular cancer type known as colorectal cancer. In South Africa, colorectal cancer is found to be the 5th most common cancer [2]. However, globally, it has been ranked as the 3rd most common cancer type with over 1.4 million new cases in the year 2015 – contributing to 9.7% of the total number of cancer cases diagnosed, including 774,000 cancer related deaths [1]. Due to the increasing prevalence and mortality rates of colorectal cancer, it is crucial to develop a novel treatment strategy to combat this disease.

There are several intrinsic and extrinsic factors which contribute to normal cells transforming into cancerous cells. Due to the complexity and diversity of neoplastic diseases, the collective term known as "the hallmarks of cancer" came about in order to provide a better understanding of this disease [3]. These hallmarks show that

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tumour cells acquire several capabilities that their normal counterparts do not have, including: independent of growth signals, resistance to anti-growth signals, unlimited replicative potential, tissue invasion and metastasis, continuous angiogenesis and apoptosis evasion [3]. In addition, recent studies have shown that cancerous cells also require the help of a particular receptor known as the 37 kDa laminin receptor precursor/67 kDa laminin receptor (LRP/LR) to maintain their tumourigenic state [4–9].

LRP/LR, also known as RPSA, is known to assist in numerous physiological processes [10, 11] . Moreover, the receptor possesses a strong binding affinity for laminin-1, a ligand found in several non-collagenous glycoproteins and is said to play critical roles in cell attachment, cell growth and differentiation [12], cell migration [13] and angiogenesis [14]. Hence, the interaction between LRP/LR and laminin-1 in is seen as an enhancement of tumour growth and progression [15]. In addition, LRP/LR has also been seen to play several other roles such as maintaining ribosomal processing of RNA [16], protein synthesis [17], cell cycle regulation [17] and importantly, cell survival [18].

Several studies have shown that LRP/LR contributes to many other pathological conditions such as microbial infections [19], neurological diseases including Alzheimer's disease [20–22], prion-related diseases [23], as well as numerous other cancer types [10]. Furthermore, Naidoo et al. has also shown that LRP/LR mediates telomerase activity by enhancing hTERT activity, thus, illustrating a novel role for the receptor [24, 25]. Due to LRP/LR being involved in several of the aforementioned tumourigenic processes, this prompted the investigation of the receptor's role in cellular viability and cell survival. One study has revealed that through silencing LRP/LR via siRNA technology, the viability of cervical (HeLa) [26], liver (Hep3B) [27] and lung (A549) [26] cancer cells was reduced by means of apoptotic induction. Other studies indicated that the viability of breast (MCF-7 and MDA-MB231) [28], oesophageal (WHC01) [28], neuroblastoma (IMR-32) [29], pancreatic (AsPC-1) [29] as well as malignant melanoma cancer cells [30] was also reduced through siRNA-mediated LRP/LR knockdown. Therefore, these studies show the vital role of LRP/LR in apoptosis and maintaining tumour cell survival.

Apoptosis is essential for several other processes within organisms including: tissue homeostasis maintenance, normal development preservation, as well as damaged cell elimination – all involving cells actively committing suicide. Once cells undergo apoptosis, they undergo several morphological and biochemical changes [31]. A biochemical change of importance to apoptosis is the activation of caspases. These caspases may become active through two key pathways and as a result induce apoptosis i.e. the intrinsic mitochondrial pathway and the extrinsic death receptor pathway [31].

Hence, the current study investigated whether siRNA-mediated knock-down of LRP/LR will reduce the viability of early (SW-480) and late (DLD-1) stage colorectal cancer cells. This study revealed that knock-down of LRP/LR using siRNA technology significantly reduces the viability of early and late stage colorectal cancer cells, and proposes that apoptosis is the cause for the notable decreases in cellular viability.

Methods

A detailed list of suppliers/manufacturers of antibodies, reagents and equipment used to carry out the following experiments is given in the supplementary data section.

Cell culture and conditions

Authenticated colorectal cancer cell lines SW-480 and DLD-1 were obtained from American Tissue Culture Collection (ATCC) with catalogue numbers ATCC® CCL-228 and ATCC® CCL-221, respectively. Both cancer cell lines were cultured in DMEM/Ham's F-12 (1:1) (GE Lifesciences) together with 10% Fetal Calf Serum (FCS) (Capricorn Scientific) and 1% penicillin/streptomycin (Biowest). All cells remained at 37 °C with 5% CO_2 in a humidified incubator.

siRNA-mediated knock-down of the laminin receptor (LRP/LR)

Cell counts were performed with the TC20™ cell counter (Biorad) and cells were seeded at a density depending on the experiment being performed. Cells were allowed to reach 50–70% confluency prior to transfection. Both cancer cell lines were transfected with ON-TARGETplus SMARTpool Human-RPSA (GE Dharmacon) (targeted towards LRP/LR) – this siRNA will be referred to as siRPSA #1 and esiRNA-RLUC (serving as the negative control) (Sigma). The appropriate amounts of Dharma-Fect transfection reagent (GE Dharmacon) and Mission transfection reagent (Sigma) were added to the cells, respectively. The cancer cell lines were likewise transfected with esiRNA-RPSA (Sigma) which is also targeted to LRP/LR (this siRNA will be referred to as siRPSA #2). Thereafter, cells were incubated for 72 h at 37 °C. This procedure was performed before any further experiments took place.

SDS-PAGE and western blotting

To determine siRNA-treated LRP/LR levels in the colorectal cancer cell lines, western blotting was performed. Cell lysates containing 10 μg of protein were separated on 12% sodium dodecyl sulphate polyacrylamide gels via electrophoresis (SDS-PAGE) (Bio-Rad). Thereafter, PVDF membranes (Pall Corporation) were soaked in methanol

(Associate Chemical Enterprise, ACE) for 2 min followed by a 5-min incubation in transfer buffer. Proteins were transferred at 300 V via electro-blotting. The membranes were then blocked for an hour in 0.1% PBS-Tween in 3% BSA. Thereafter, the membranes were incubated with the IgG1-iS18 primary antibody which was diluted in blocking buffer (1:5000). The membranes were then washed three times in PBS-Tween (10 min for each wash) followed by an incubation in the appropriate secondary antibody diluted in blocking buffer (1:10000) for 1 h. The membrane was washed three more times with PBS-Tween before adding the chemiluminescent substrate (Biorad) to the membrane in order to detect proteins. In addition, 42 kDa β-actin served as a loading control. Finally, densitometric analysis was completed in order to quantify protein levels using ImageLab™ software.

MTT assay

The MTT assay is a valid assay for determining cell viability employed in various cancer studies [28–30, 32–35]. Before transfections took place, 1×10^4 cells/ml SW-480 and DLD-1 cells were seeded on 24-well plates. After transfection, cells were incubated at 37 °C for 72 h, followed by the addition of 1 mg/ml of MTT [100 μg of MTT (Duchfei Biochemic) being dissolved in 1 X PBS (Gibco)] to all wells. This was followed by a further incubation at 37 °C for 2 h. Thereafter, the media from each well containing MTT was removed and 500 μl DMSO was added to dissolve the residual formazan crystals (Merck Millipore). The resultant absorbance was measured at 570 nm. This procedure was performed for controls as well which included: untreated cells, PCA (Protocatechuic acid) positive control (Aldrich Chemistry) treated cells as well as esiRNA-RLUC negative control treated cells.

Assessment of nuclear morphological changes – Confocal microscopy with Airyscan

In order to evaluate nuclear morphology post knock-down of LRP/LR via siRNA technology, confocal microscopy was used. Early and late stage colorectal cancer cells were seeded onto coverslips at a cell density of 1×10^5 cells/ml. After transfection, the cells were fixed in 4% PFA (Associated Chemical Enterprise, ACE) for 15 min prior to 3 washes with PBS. Once remaining PBS was blotted off after washing, cells were then incubated with 0.1% Triton-X for 20 min for permeabilization of the cell membrane. The cells were then washed twice followed by the addition of DAPI nuclear stain (Sigma) diluted in PBS (1:100) onto each coverslip and incubated for 8 min in the dark. Once stained, the coverslips were washed twice in PBS prior to each coverslip being mounted on a microscope slide with fluoromount (Sigma). The microscope slides were left to set for 45 min in the dark, after which they were maintained at 4 °C. Note: untreated cells were used as a control, esiRNA-RLUC as a negative control and PCA as a positive control. Airyscan is a technique used to enhance confocal laser scanning microscopy. It has been shown that total resolution is improved by a factor of 1.7 in all spatial directions i.e. a resolution of 140 nm laterally and 400 nm axially can be achieved. The Airyscan was used to further analyse the nuclear morphological changes observed after LRP/LR down-regulation (Zeiss LSM 780).

Annexin V-FITC/7AAD assays

This experiment was performed as per the manufacturer's directions (Beckman Coulter). Both SW-480 and DLD-1 cells were seeded at a cell density of 2×10^6 cells/ml before transfection. After 72 h incubation at 37 °C, cells were subjected to trypsinization with trypsin/EDTA (Biowest) followed by washes with cold PBS. Thereafter, cells were centrifuged at 5000 rpm for 5 min was performed, after which pellets were resuspended in 1X annexin-binding buffer (BD Sciences). Thereafter, 10 μl of Annexin V-FITC (BD Sciences) solution and 5 μl of PI viability dye were added to each cell suspension which was followed by a 15-min incubation on ice in the dark. Subsequently, 400 μl of ice-cold 1X annexin binding buffer was added to the samples for 30 min and all resulting cell suspensions were reviewed using the BD Accuri C6 flow cytometer. Note: esiRNA-RLUC was the negative control and PCA was the positive control.

Caspase-3, – 8 and – 9 activation assays

Caspase-3,-8 and – 9 assays were completed as per the manufacturer's directions (Merck Millipore). Cells were seeded at a cell density of 1×10^6 cells/ml before transfection. Cells were then centrifuged at 1200 rpm for 10 min, followed by pellet resuspension in 50 μl of lysis buffer. The samples were then incubated for 10 min on ice, followed by a further centrifugation at 10000 x g for 5 min. While the pellet was discarded, the supernatant was placed into a new microcentrifuge tube and put on ice. Thereafter, a BCA™ assay was performed in order to obtain the supernatant's protein concentration. This was followed by 200 μg of protein being diluted per sample, prior to being added to wells of a 96-well plate. Once this was completed, 20 μl of 5X assay buffer was added to every sample. Thereafter, 10 μl of peptide substrate was added followed by incubation at 37 °C for 2 h. Finally, the absorbance was read at 405 nm. Note: esiRNA-RLUC treated cells served as a negative control and PCA treated cells were used as a positive control.

Statistical evaluation

In order for accurate data analysis, Student's t-test had to be utilized, with a confidence interval of 95%.

Furthermore, p-values greater than 0.05 were seen as non-significant. To measure the degree of association between LRP/LR levels and apoptotic induction as well as cellular viability, Pearson's correlation coefficient was calculated. A positive coefficient shows a directly proportional relationship between the two variables (where values close to 1 indicates a highly positive correlation).

Results

siRNA technology successfully results in knock-down of LRP expression and reductions in cellular viability in early and late stage colorectal cancer cells

To understand the effect LRP/LR expression has on early and late stage colorectal cancer cell viability, down-regulation of the receptor had to be performed. Once early (SW-480) and late (DLD-1) cells were transfected with siRPSA #1 (targeted towards the 37 kDa LRP mRNA), evaluation of Western blotting and densitometry was performed. Densitometry showed that LRP was significantly knocked down in both SW-480 and DLD-1 cells when transfected with siRPSA #1. The SW-480 and DLD-1 transfected cells exhibited a 75 and 78% decrease in LRP expression, respectively, when compared to cells that were not transfected, since these LRP levels were set to 100% (Fig. 1a and b). Additionally, to determine whether the reduction in LRP expression had resulted due to siRPSA #1-mediated LRP knock-down and not just an off-target effect, an alternative siRNA that targets another region of LRP was utilised. When comparing them to the non-transfected cells, both early (SW-480) and late (DLD-1) stage colorectal cancer cells displayed a knock-down of 72 and 61% in LRP expression, respectively (Fig. 1c and d). SW-480 and DLD-1 cells transfected with the esiRNA-RLUC showed no significant LRP knock-down when comparing them to non-transfected cells. Once LRP expression was successfully down-regulated using siRNA technology, its effect on the viability of both cell lines were observed. MTT assays were performed which showed that when SW-480 and DLD-1 cells were transfected with siRPSA #1, cell viability was significantly decreased in contrast to cells that were not transfected, indicating that siRNA-mediated knock-down of the receptor leads to reductions in cell viability in both early and late stage colorectal cancer cell lines. The MTT assays revealed that SW-480 and DLD-1 cells treated with siRPSA #1 displayed a 60 and 55% decrease, respectively, in comparison to the cells that were not transfected (Fig. 1g). Additional MTT assays were performed to evaluate whether siRPSA #2-mediated LRP knock-down also influenced the viability of SW-480 and DLD-1 cells. Post treatment of cells with siRPSA #2 was found to reduce the viability significantly by 44 and 89% in both SW-480 and DLD-1 cells, respectively, when comparing them to the cells which were not transfected (Fig. 1g). Additionally, treating SW-480 and DLD-1 cells with the negative control siRNA, esiRNA-RLUC, indicated no notable change in cell viability in comparison to cells that were not transfected (Fig. 1g).

Knock-down of LRP expression via siRNA technology leads to changes in nuclear morphology signifying apoptosis

To determine whether the decreases in cell viability after treatment with siRPSA #1 was caused by apoptotic induction, nuclear morphological changes were studied by confocal microscopy and Airyscan. siRPSA #1 treated early (SW-480) stage colorectal cancer cells exhibited nuclear morphological changes in the form of condensed nuclei and reduced nuclear size (Fig. 2d), when compared to the nuclei of cells that were not transfected (Fig. 2a). Late (DLD-1) stage colorectal cancer cells transfected with siRPSA #1 presented nuclear morphological changes such as cellular fragmentation into membrane-bound bodies, weakened membrane integrity and membrane blebbing and (Fig. 2h), in contrast to nuclei of cells that were not transfected (Fig. 2e). Membrane blebbing was confirmed for the DLD-1 cells by bright field microscopy (Additional file 1: Figure S1). Results obtained for siRPSA #1 treated cells were consistent with those of the positive control, PCA (Fig. 2c and g). In addition, upon treatment with esiRNA-RLUC, both cell lines did not reveal any morphological changes in nuclei, when comparing them to cells that were not transfected (Fig. 2b and f).

siRNA-mediated knockdown of LRP expression induces apoptosis in early and late stage colorectal cancer cells

Due to confocal microscopy proposing that the knock-down of LRP expression in early (SW-480) and late (DLD-1) stage colorectal cancer cells leads to morphological changes of the nuclei (a key feature of cells undergoing apoptosis), Annexin-V/PI assays had to be performed to confirm this result quantitively.

SW-480 cells treated with siRPSA #1 revealed 36.6% of cells undergoing early apoptosis, while 44.1% of cells underwent late apoptosis (Fig. 3.1d), in contrast to cells that were not transfected (Fig. 3.1a). Transfected DLD-1 cells with siRPSA #1 resulted in 10.0% of cells undergoing early apoptosis while 74.3% of cells underwent late apoptosis (Fig. 3.1h) when compared to cells that were not transfected (Fig. 3.1e) Additionally, upon treatment with esiRNA-RLUC, SW-480 and DLD-1 cell lines did not undergo apoptosis (Fig. 3.1b and f). PCA positive control showed most cells underwent late apoptosis (Fig. 3.1c and g. Further statistical analysis confirmed

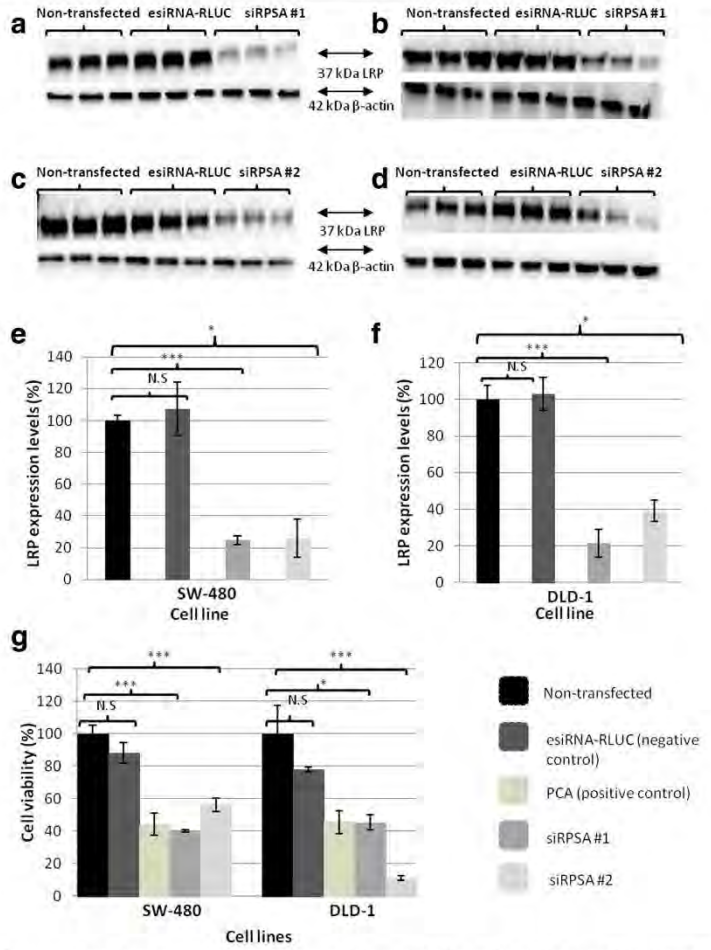

Fig. 1 The effect of siRNA-mediated knock-down on LRP expression and the viability of early (SW-480) and late (DLD-1) stage colorectal cancer cells. **a**), **c**) and **e**) Upon transfection of SW-480 with siRPSA #1 and siRPSA #2, significant 75 and 72% decreases in LRP expression levels was revealed, respectively, in contrast to cells that were not transfected. Densitometric analysis of LRP levels was performed where levels of the non-transfected cells were set to 100%. $^{***}p = 0.0001$, $^{*}p = 0.02$ N.S: $p > 0.05$. **b**), **d**) and **f**) siRPSA #1 and siRPSA #2 transfection in DLD-1 cells caused significant 79 and 61% decreases in LRP expression levels, respectively, when comparing them to cells that were not transfected. Densitometry displayed no significant difference in LRP expression between non-transfected and negative control esiRNA-RLUC transfected cells for both cell lines. $^{***}p = 0.0005$, $^{*}p = 0.02$ N.S: $p > 0.05$. **g**) MTT assays were performed to assess SW-480 and DLD-1 cell viability, upon treatment with siRPSA #1 and siRPSA #2. Non-transfected cell value were set to 100%. It was found that upon siRPSA #1 transfection, SW-480 and DLD-1 cells exhibited a significant decrease of 60 and 55% in cellular viability, respectively, in contrast to cells that were not transfected. It was also revealed that when the SW-480 and DLD-1 were transfected with siRPSA #2, there were significant reductions of 44 and 89% in cellular viability, respectively, when compared to non-transfected cells. Both cell lines showed no noteworthy differences in cell viability when treated with the negative control esiRNA-RLUC. PCA was used as the positive control. SW-480: siRPSA #1:$^{***}p = 0.0008$, siRPSA #2:$^{***}p = 0.0004$ DLD-1: siRPSA #1:$^{*}p = 0.01$, siRPSA #2:$^{***}p = 0.0009$, N.S. $p > 0.05$, non-significant. All graphs represent an average of three biological and three technical repeats

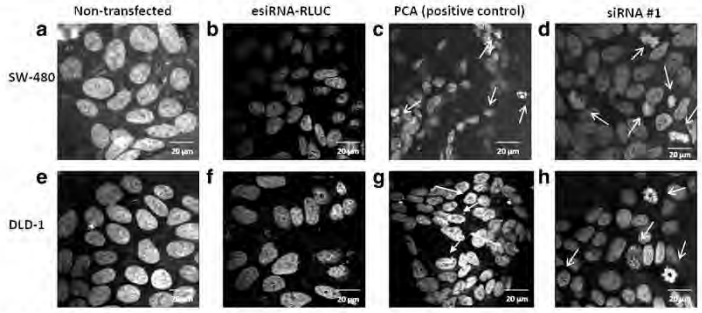

Fig. 2 The effect of siRNA-mediated knock-down of LRP on nuclear morphology of early (SW-480) and late (DLD-1) stage colorectal cancer cells. Confocal microscopy with Airyscan analysis was completed to investigate changes in nuclear morphology upon siRNA treatment. **a** and **e**) Non-transfected SW-480 and DLD-1 cells displayed large nuclei with healthy membrane integrity. **b** and **f**) SW-480 and DLD-1 cells transfected with negative control esiRNA-RLUC showed parallel characteristics to the non-transfected cells with no changes in nuclear morphology. **c**) SW-480 cells treated with positive control PCA revealed nuclei that underwent apoptotic body formation. **d**) SW-480 cells transfected with siRPSA #1 exhibited nuclear shrinkage and condensed nuclei, proposing induction of apoptosis. **g**) DLD-1 cells treated with PCA displayed weakened membrane integrity and condensed nuclei. **h**) DLD-1 cells transfected with siRPSA #1 showed formation of apoptotic bodies and weakened membrane integrity. Images were obtained at a 630X magnification and Airy scan analysis was applied to each image. Scale bars are indicative of 20 μm

a significant increase in apoptotic cells when treated with siRPSA #1, in contrast to non-transfected cells in both cell lines (Fig. 3.2).

siRNA-mediated knock-down of LRP expression causes a notable increase in caspase-3 activity

For additional validation of apoptosis occurring in early (SW-480) and late (DLD-1) stage colorectal cancer cells once LRP has been down-regulated, caspase-3 activity assays were completed. Post transfection with siRPSA #1, SW-480 cells were found to have a significant 4-fold increase in caspase-3 activity, in comparison to cells that were not transfected (Fig. 4a). DLD-1 cells treated with siRPSA #1 displayed a 5-fold increase in caspase-3 activity in contrast to non-tranfected cells (Fig. 4a). Furthermore, no differences in caspase-3 activity was seen when both cell lines were treated with esiRNA-RLUC (Fig. 4a). PCA positive control displayed a 3-fold and 5-fold increase in caspase-3 activity was observed in SW-480 and DLD-1 cells, respectively (Fig. 4a).

siRNA-mediated knock-down of LRP results in significant increases in caspase-8 and caspase-9 activity in early and late stage colorectal cancer cells

Caspase-3 activation occurs through both apoptosis pathways (intrinsic and extrinsic) hence, further insight of how the receptor aids in tumourigenic cell survival was required; therefore caspase-8 and -9 activity assays were performed. These assays determine whether treatment with siRPSA #1 leads to the activation of the extrinsic pathway (facilitated through caspase-8) or the intrinsic pathway (facilitated through caspase-9) in each of the cell lines. SW-480 and DLD-1 cells transfected with siRPSA #1 were found to undergo a 4-fold increase in caspase-8 activity, in comparison to cells that were not transfected (Fig. 4b). Moreover, SW-480 cells and DLD-1 cells indicated a significant 7-fold and 4-fold increase in caspase-9 activity, respectively, in comparison to cells that were not transfected (Fig. 4c). Moreover, both cell lines transfected with esiRNA-RLUC displayed no differences in caspase-8 and -9 activity in comparison to cells that were not transfected (Fig. 4b).

Discussion

LRP/LR has gained a large amount of interest due to the many roles it plays in the cell. Particularly, the receptor's over-expression in several cancer cell types as well as its contribution in tumourigenesis has become a target area for research. LRP/LR has been seen to assist with several tumourigenic processes including tumour adhesion and invasion (metastasis), angiogenic enhancement as well as apoptotic evasion [3]. Additionally, since LRP/LR is not limited to the cell surface but also localized in the perinuclear region, cytosol and nucleus, it is able to perform many intracellular and extracellular physiological roles including maintaining cell viability, cell adhesion, cell growth and migration, cell cycle regulation, ribosomal anchorage to microtubules, pre-rRNA processing and protein synthesis. Thus, cancerous cells are found to over-express LRP/LR, thereby exploiting these functions, resulting in the development of the abovementioned tumourigenic processes. Moreover, a recent study

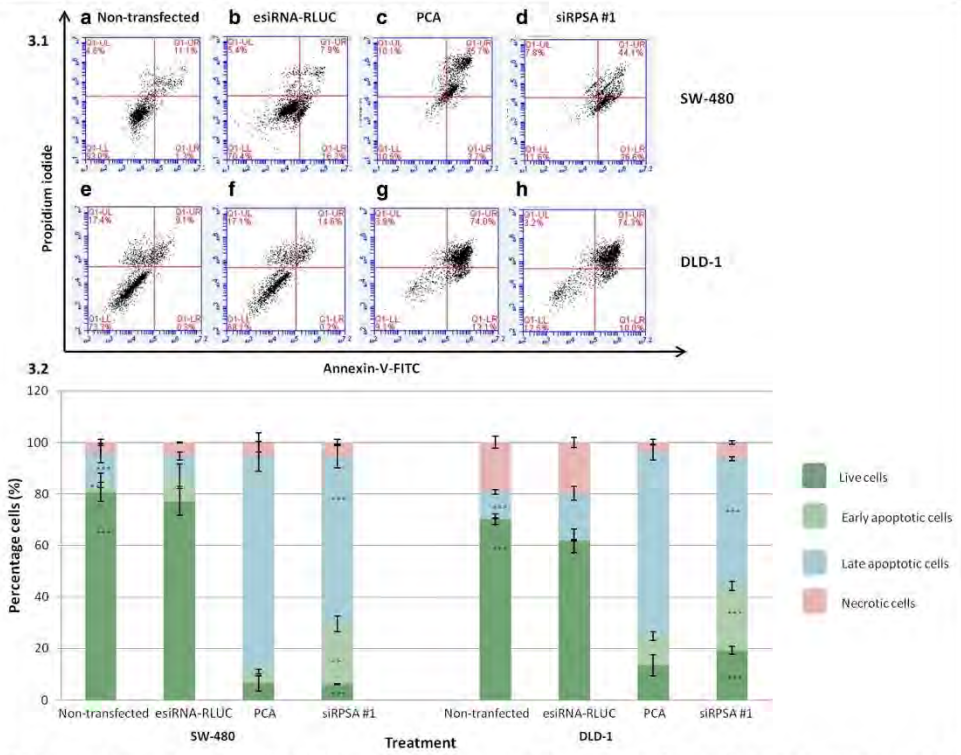

Fig. 3 3.1 Apoptotic induction in early (SW-480) and late (DLD-1) stage colorectal cancer cells post siRNA transfection. It was revealed that most of the non-transfected **a**) SW-480 and **e**) DLD-1 cells fall in the lower quadrant (Q1-LL) which is known to represent normal live cells. **b**) and **f**) SW-480 and DLD-1 cells treated with negative control esiRNA-RLUC mostly appeared in the lower quadrant indicating live cells. **c**) and **g**) Positive control PCA, revealed that most cells were found in the upper right quadrant (Q1-UR), representing SW-480 and DLD-1 cells undergoing late apoptosis, respectively. **d**) and **h**) Cells transfected with siRPSA #1 resulted in 36.6% of SW-480 cells (**d**) and 10% of DLD-1 cells (**h**) undergoing early apoptosis, which is depicted in the lower right quadrant (Q1-LR); while 44.1% of SW-480 cells and 74.3% of DLD-1 cells underwent late apoptosis. This indicates that upon treatment with siRPSA #1, a total of 80.7% of SW-480 cells and 84.3% DLD-1 cells underwent apoptosis. 3.2 Bar graph illustrating apoptotic induction in early (SW-480) and late (DLD-1) colorectal cancer cells after siRNA transfection. This graph displays an average of three experiments completed in triplicate. Percentages for each quadrant were pooled together and compared to one another for both cell lines. It was found that SW-480 and DLD-1 cells had a significant increase in early and late apoptosis when treated with siRPSA #1, in contrast to cells that were not transfected, and both cell lines were seen to undergo more late apoptosis than early apoptosis. SW-480: $^{***}p = 8.92029E\text{-}06$ (live), $^{***}p = 0.0001$ (early apoptosis), $^{**}p = 0.002$; DLD-1: $^{***}p = 1.97127E\text{-}05$ (live), $^{***}p = 3.72195E\text{-}05$ (early apoptosis), $^{***}p = 1.08522E\text{-}06$ (late apoptosis)

performed by Vania et al. showed that there were significantly higher levels of the receptor in late (DLD-1) stage colorectal cancer cells, compared to the early (SW-480) stage – indicating that LRP expression also increases in the course of malignant transformation [4].

To gain insight into how LRP/LR maintains cell viability, siRNA technology was used to knock-down LRP expression (Fig. 1) and evaluate this effect on cell viability of SW-480 and DLD-1 cells. Due to siRPSA #1 only targeting the mRNA of the 37 kDa laminin receptor precursor (LRP) form, it was employed to down-regulate LRP in this study (see Additional file 1: Table S1). Cells treated with siRPSA #1 resulted in significant decreases in LRP down-regulation. Furthermore, a high correlation of 0.91 for SW-480 and 0.96 for DLD-1 was observed between total levels of LRP before and after siRPSA #1 transfection (Table 1). This high and positive correlation suggests that the level of LRP expression is indeed influenced by siRNA

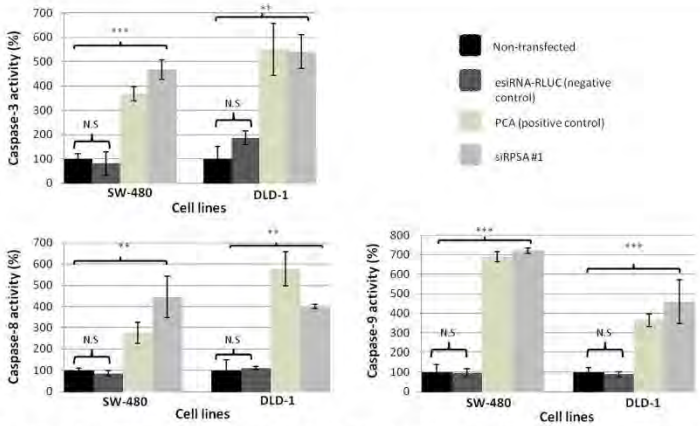

Fig. 4 The effect of siRNA-mediated LRP knock-down on caspase-3, − 8 and − 9 activity in early (SW-480) and late (DLD-1) stage colorectal cancer cells. **a)** Upon treatment of SW-480 and DLD-1 cells with siRPSA #1, a significant 4-fold and 5-fold increase in caspase-3 activity was revealed, respectively, in comparison to cells that were not transfected (set to 100%). Both cell lines showed no significant difference in caspase-3 activity between cells transfected with negative control esiRNA-RLUC and non-transfected cells. PCA was used as a positive control. SW-480:***$p = 0.0007$ and DLD-1: **$p = 0.0059$. N.S: $p > 0.05$, non-significant. **b)** siRPSA #1 transfected SW-480 and DLD-1 cells both displayed a 4-fold significant increase in caspase-8 activity, compared to cells that were not transfected (set to 100%). Both cell lines showed no significant difference in caspase-8 activity between cells transfected with the esiRNA-RLUC and non-transfected cells. SW-480: **$p = 0.0083$ and DLD-1: **$p = 0.002$. N.S: $p > 0.05$, non-significant. **C)** SW-480 and DLD-1 cells transfected with siRPSA #1 showed a significant 7-fold increase and 4-fold increase, respectively, in contrast to cells that were not transfected. Both cell lines showed no significant difference in caspase-9 activity between cells transfected with the esiRNA-RLUC and non-transfected cells. SW-480:***$p = 0.0001$ and DLD-1: ***$p = 0.0008$. N.S.: $p > 0.05$, non-significant. This data represents three biological replicates which were completed in triplicate

treatment i.e. lower levels of LRP expression prior to treatment with siRNA leads to more LRP knockdown post treatment with siRNA.

To validate that the observed knock-down was not due to off target effects, SW-480 and DLD-1 cells were both treated with an alternative siRNA, siRPSA #2. This siRNA targets a specific region of 37 kDa LRP mRNA i.e. nucleotides 521–929 (Table 1). Upon treatment with siRPSA #2, both cell lines showed significant decreases in LRP knock-down in contrast to cells that were not transfected (Fig. 1). These results validated that LRP was being down-regulated and was not just an off-target effect. In addition, the correlation between total LRP levels before and after siRPSA #2 treatment was found to be high (sees Additional file 1: Table S2).

To further investigate the receptor's role in maintaining cell viability, an MTT assay was employed to investigate the effect of treatment with siRPSA #1 and siRPSA #2 on cell viability. A significant decrease in viability was observed for both SW-480 and DLD-1 cells after LRP down-regulation (Fig. 1). These reductions in cellular viability correlate with the decreased levels of LRP observed after siRNA-mediated down-regulation, signifying the receptor's vital role in the survival of SW-480 and DLD-1 cells (Table 1). It has been discovered that LRP/LR localised in the nucleus allows for chromosome stability maintenance via interactions with the Midkine heparin-binding growth factor; well-known for enhancing cell proliferation, migration and survival [36]. In addition, several cancer types are showed to have an

Table 1 Assessment of correlation between levels of siRNA-mediated LRP knockdown and viability, apoptotic levels and caspase-3 activity of early (SW-480) and late (DLD-1) stage colorectal cancer cells, using Pearson's correlation co-efficients (R)

Cell line	SW-480	DLD-1
Correlation between total LRP levels before and after siRPSA #1 transfection (R-value)	0.91	0.96
Correlation between siRPSA #1-mediated LRP knockdown and reduction in cell viability (R-value)	0.99	0.98
Correlation between siRPSA #2-mediated LRP knockdown and reduction in cell viability (R-value)	0.99	0.93
Correlation between total levels of apoptosis and total LRP levels after siRPSA #1 transfection (R-value)	0.99	0.98
Correlation between increases in caspase-3 activity and total LRP levels after siRPSA #1 (R-value)	0.94	0.93

up-regulated expression of Midkine, which results in the promotion of cell survival factors as well as obstruction of apoptosis through caspase-3 inhibition [37]. However, by targeting LRP expression through siRNA technology, LRP/LR-Midkine interactions may decrease, and as a result decrease cell viability.

To establish whether the observed decrease in SW-480 and DLD-1 cell viability post LRP knockdown was due to cell death caused by apoptosis, confocal microscopy was used to evaluate nuclear morphology. Both cell lines revealed several changes in nuclear morphology which were all characteristic of apoptosis including: nuclear condensation, reduced nuclear size and the formation of membrane-bound bodies – when treated with siRPSA #1 (Fig. 2). It is known that nuclear structures are maintained via the binding of histones to perinuclear and nuclear LRP/LR, thus when the receptor is down-regulated, loss of membrane integrity and distorted nuclear morphology is evident [38].

Although confocal microscopy provided a visual indication that apoptosis was occurring, additional quantification and affirmation of apoptotic induction was needed. This was made possible by means of Annexin V-FITC/PI assays. Non-transfected and negative control esiRNA-RLUC-transfected SW-480 and DLD-1 cells showed negative staining for Annexin-V – indicating live cells. On the other hand, siRPSA #1-treated and PCA-treated SW-480 and DLD-1 cells exhibited positive staining for Annexin-V or Annexin-V and PI, indicating early and late stage apoptosis, respectively. This shift of Annexin-V staining from negative to positive shows that siRPSA #1-mediated down-regulation in SW-480 and DLD-1 cells activates membrane asymmetry loss and a membrane-flip reaction involving the externalization of phosphatidylserine (PS) on the outer leaflet of the plasma membrane – allowing these cells to be taken up by phagocytes [39]. Thus, these findings together with the nuclear morphological changes observed, confirm that siRNA-mediated down-regulation of LRP/LR leads to the induction of apoptosis in SW-480 and DLD-1 cells. In addition, the correlation (Table 1) between total levels of LRP after siRPSA #1 treatment and total levels of apoptosis for both cell lines was found to be high (Figs. 1 and 3.1), further reiterating LRP expression affects cell viability and apoptosis.

Sustantad et al. found that when LRP/LR is down-regulated via siRNA technology, not only did the cell viability of cervical cancer (HeLa) cells and lung (A549) cancer cells decrease but caspase-3 activity was increased in both cell lines [27]. Caspase-3, an effector caspase, is responsible in executing the hallmarks of apoptosis which includes the afore-mentioned nuclear morphological changes by cleaving substrates [40]. Therefore, upon apoptotic induction, caspase-3 activity is found to be significantly increased. Hence, to further establish that apoptosis was indeed occurring and whether caspases were activated after treatment with siRPSA #1, caspase-3 assays were performed. Down-regulation of LRP caused a distinct increase in caspase-3 activity in SW-480 and DLD-1 cells when compared to the cells that were not transfected. Furthermore, the correlation between the total levels of LRP after siRPSA #1-mediated knockdown (Fig. 1) and increases in caspase-3 activity (Fig. 4) was high in both cell lines (Table 1). These results noticeably point to the induction of apoptosis in SW-480 and DLD-1 cells due to the silencing of LRP though siRNA technology.

It has previously been shown that interactions between LRP/LR and focal adhesion kinase (FAK) are made possible via the binding of the receptor to laminin-1. Furthermore, LRP/LR-FAK interactions were seen to be involved in activating cell signalling cascades such as MEK/ERK 1/2 and PI3-kinase/AKT as well as up-regulating the anti-apoptotic protein, Bcl-2 [41]. This ultimately leads to the inhibition of apoptosis of cancerous cells. Hence, we suggest that silencing LRP/LR through the use of siRNA technology as performed in the current study, impedes the LRP/LR-FAK interaction, and in this way apoptosis is induced. Furthermore, LRP/LR has been shown to have a direct relationship with the MAPK signalling pathway – where decreased levels of LRP causes a response in the pathway – resulting in cell stress and ultimately, cell death [10]. Another reason which could have led to apoptotic induction through siRNA-mediated knock-down of LRP is the receptor's role in ribosomal processing. Research has shown that LRP/LR is involved in processing 21S pre-rRNA into mature 18S rRNA, also known as biogenesis of ribosomes [16]. LRP/LR has also been shown to associate with pre-40S ribosomal subunits, providing nucleolar exits for the subunits, thereby facilitating protein synthesis [42]. We propose that the LRP down-regulation performed in this study may have hampered formation of the ribosome as well as the resultant translation of proteins required for correct cellular functioning, ultimately leading to apoptosis. Furthermore, LRP has also been seen to play a key role in the cell cycle thus, down-regulating the receptor could have resulted in the induction of G1-phase arrest in the colorectal cancer cells, aiding in apoptosis [10].

Since both the MEK/ERK 1/2 and PI3-kinase/AKT cell signalling cascades are known to inhibit both apoptotic pathways, caspase-8 and caspase-9 assays were performed to determine if these caspases are activated upon siRPSA #1-mediated LRP/LR knock-down. Both early and late stage colorectal cancer cell lines were found to have higher caspase-8 activity post transfection with siRPSA #1, in contrast to cells that were not transfected.

Caspase-8 plays a vital role in the extrinsic apoptotic signalling pathway through death receptors, thus it is suggested that siRNA-mediated LRP knock-down induces the apoptotic process in SW-480 and DLD-1 cells extrinsically. Moreover, this study revealed that SW-480 cells and DLD-1 also have increased in caspase-9 activity after LRP down-regulation, in contrast to cells that were not transfected. Caspase-9 plays a critical role in the intrinsic apoptotic signalling pathway, proposing that siRNA-mediated LRP knock-down also initiates apoptosis in SW-480 and DLD-1 cells through the intrinsic pathway.

The intrinsic and extrinsic pathways interconnect with each other at several levels and can both be influenced by similar factors. In fact, one study showed that activated extrinsic caspase-8 stimulated the release of cytochrome *c* and apoptosome formation and ultimately activation of the intrinsic pathway [43, 44]. A potential reason as to why SW-480 and DLD-1 cells experience apoptosis through both apoptotic pathways may be that these colorectal cancer cells undergo a mechanism known as retaliatory caspase activation where the two apoptotic pathways are found to use a feedback amplification loop in order to activate one another [45]. Specifically, activated caspase-9 initiates and proteolytically cleaves caspase-3, also leading to caspase-8 activation [45, 46]. Moreover, due to SW-480 and DLD-1 cells undergoing both apoptotic pathways, it can be said that down-regulated LRP/LR possibly hampers both anti-apoptotic signalling pathways on account of the reduced interaction of phosphorylated FAK and LRP/LR.

Conclusions

This study shows that down-regulating LRP via siRNA technology significantly decreases the viability of early (SW-480) and late (DLD-1) stage colorectal cancer cells through the induction of apoptosis. Moreover, SW-480 and DLD-1 cells underwent apoptosis through both apoptotic pathways. It is possible that cell signalling cascades are involved in inducing apoptosis, however, the exact mechanism is unclear. These findings demonstrates the critical function LRP/LR plays in maintaining the viability of both early and late stage colorectal cancer cells. In addition, these findings emphasize the therapeutic potential of siRNAs targeted against LRP, which could be used as a possible tool in treating early and late stage colorectal cancer.

Additional file

Additional file 1: Figure S1. Late stage (DLD-1) colorectal cancer cells show membrane blebbing and reduced nuclei post transfection with siRPSA #1 using bright field microscopy. A) and B) Non-transfected and esiRNA-RLUC (negative control) transfected cells are found to be large with uncompromised membrane integrity. C) and B) siRPSA #1-transfected and PCA (positive control) treated cells are found to have a reduced size together with compromised membrane integrity i.e. membrane blebbing and condensed nuclei – all indicative of apoptosis occurring. Images were obtained at 200X magnification. Scale bars are indicative of 20 μm. **Table S1.** Sequence of Human-RPSA, esiRNA-RPSA and control siRNA-RLUC used for down-regulation of LRP/LR. **Table S2.** Pearson's correlation co-efficients (R) between total LRP levels prior to and post transfection with esiRNA-RPSA (DOCX 425 kb)

Abbreviations

ATCC: American type culture collection; BCA: Bicinchoninic acid; BSA: Bovine serum albumin; CO_2: Carbon dioxide; DMEM: Dulbecco's Modified Eagle's medium; DMSO: Dimethyl sulfoxide; ERK: Extracellular signal-regulated kinases; FAK: Focal adhesion kinase; FCS: Fetal calf serum; FITC: Fluorescein isothiocyanate; HRP: Horseradish peroxidase; IgG: Immunoglobulin G; kDa: Kilodaltons; LRP/LR: Laminin receptor precursor/ laminin receptor; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PAGE: Polyacrylamide gel electrophoresis; PBS: Phosphate buffered saline; PCA: Protocatechuic acid; PI: Propidium Iodide; PI3K: Phosphoinositide 3-kinase; PS: Phosphatidyl serine; PVDF: Polyvinylidene fluoride; RLUC: Renilla luciferase; RNA: Ribonucleic acid; Rpm: Revolutions per minute; RPSA: Ribosomal protein SA; SDS: Sodium dodecyl sulfate; siRNA: Small interfering RNA; TEMED: Tetramethylethylenediamine

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Availability of data and materials

All data generated or analysed during this study are included in this published article (and its Additional file 1).

Authors' contributions

SFTW conceptualised and designed the study. LV performed experiments. LV and TMR analysed the data. EF and SFTW edited the manuscript. All authors have read and approved this version of the manuscript and confirm that this is the case.

Ethics approval and consent to participate

Not applicable

Competing interests

The authors declare that they have no competing interests.

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REVIEW

Patented therapeutic approaches targeting LRP/LR for cancer treatment

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ABSTRACT

Introduction: The ubiquitously expressed 37 kDa/67 kDa high-affinity laminin receptor (laminin receptor precursor/laminin receptor, LRP/LR) is a protein found to play several roles within cells. The receptor is located in the nucleus, cytosol and the cell surface. LRP/LR mediates cell proliferation, cell adhesion and cell differentiation. As a result, it is seen to enhance tumor angiogenesis as well as invasion and adhesion, key steps in the metastatic cascade of cancer. Recent findings have shown that LRP/LR is involved in the maintenance of cell viability through apoptotic evasion, allowing for tumor progression. Thus, several patented therapeutic approaches targeting the receptor for the prevention and treatment of cancer have emerged.

Areas covered: The several roles that LRP/LR plays in cancer progression as well as an overview of the current therapeutic patented strategies targeting LRP/LR and cancer to date.

Expert opinion: Small molecule inhibitors, monoclonal antibodies and small interfering RNAs might act used as powerful tools in preventing tumor angiogenesis and metastasis through the induction of apoptosis and telomere erosion in several cancers. This review offers an overview of the roles played by LRP/LR in cancer progression, while providing novel patented approaches targeting the receptor as potential therapeutic routes for the treatment of cancer as well as various other diseases.

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1. Introduction

1.1. Cancer – a worldwide crisis

Cancer is a collective term used for the group of diseases best defined by the abnormal growth of cells, which have the potential to invade and ultimately, spread to numerous sites in the body [1]. All tumors are abnormal masses of tissue which may either be fluid filled or solid [2]. Tumors which are confined to the site of origin and have a normal presence are known as benign and non-tumourigenic while tumors that proliferate rapidly and invade nearby sites are termed as malignant and tumourigenic [3]. Moreover, cancers can be classified according to where the original tumor developed i.e. sarcomas are normally malignant tumors that originate in soft tissue while carcinomas are malignant tumors which have originated from epithelial tissue [4]. The causes of cancer are multifactorial. Uncontrolled cell growth is normally caused by successive mutations in vital genes such as tumor suppressor genes (e.g. Rb and p53) and protooncogenes (e.g. Ras) which are used for cell growth under normal conditions [5]. Deactivated tumor suppressor genes and activated oncogenes trigger cells to bypass cell cycle checkpoint regulators and alter the DNA repair system, ultimately resulting in uncontrolled cell proliferation and transformation into tumourigenic cells [6]. There are several key contributors to this transformation. This includes both internal factors such as genetic mutations and epigenetic alterations, as well as external factors including tobacco intake (which contains numerous carcinogenic chemical compounds), exposure to radiation or infectious organisms as well as obesity [7]. Additionally, although cancer is non-communicable, there are several factors which can contribute to the formation of this disease such as bacteria and viruses e.g. cervical cancer is able to arise from subtypes of Human Papilloma Virus (HPV) [8]. Due to the various contributors to the formation of cancer, incidence and mortality rates of this disease are at a constant rise every year.

1.2. Epidemiology

In 2015, the World Health Organization reported cancer amongst the top four leading causes of death in 113 of 172 countries globally [9]. In fact, if cancer were considered a single entity, it would be the leading cause of death worldwide, with a higher number of deaths than caused by ischemic heart disease [10]. As a result in 2017, the World Health Assembly implemented a new cancer specific resolution: Cancer prevention and control in the context of an integrated approach (WHA70.12) [11]. The resolution recognized the significant impact of cancer on lifespan, health and socio-economic well-being of individuals and populations affected by cancer and urged governments to integrate cancer care across all health plans.

In its latest report on global cancer incidence and mortality, the International Agency for Research on Cancer (IARC) estimated 18 million new cases of cancer and 9.6 million cancer

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Appendix C

Article highlights

- Synthetic and natural small molecule inhibitors have been shown to have a positive effect on metastasis in several cancer cell types.
- Monoclonal anti-LRP/LR specific antibody, IgG1-iS18, has been shown to decrease adhesion and invasion in several cancer types.
- Small interfering RNAs targeting LRP/LR are found to decrease cell survival through the induction of apoptosis.
- Potential avenues of targeting LRP/LR include investigating the formation of the 67 kDa LR form, as well as its relationship with proteins parkin, telomerase and RNF8.

This box summarizes key points contained in the article.

deaths for 2018 (Figure 1) [9]. Of these, just under half of cancer cases and over 50% of cancer deaths occurred in Asia, in part, due to the large proportion of the world population residing on that continent. The highest age-standardized cancer incidence rates (ASR) for cancer were recorded for Australia/New Zealand at 571/100 000 in men and 362/100 000 in women. Globally, the lifetime risk of developing cancer before the age of 75 as well as the cancer death rate is higher for men than woman. With an incidence of 1:5 and 1:6 and a death rate of 1:8 and 1:10 for men and women respectively [9].

Of the 9.5 million new cases of cancer in men, 5.4 million cases resulted in death. The most commonly diagnosed cancer was lung cancer (accounting for 14.5% of all cancers in males) followed by prostate cancer (13.5%) and colorectal cancer (10.9%). Lung cancer was also the leading cause of cancer related death in men (22%), followed by liver (10.2%) and stomach cancer (9.5%) [9].In women, there were 8.6 million incident cancer cases and 4.2 million cancer deaths. Breast cancer was the leading cause of cancer incidence (accounting for 24.2% of all cancers in females) followed by colorectal (9.5%) and lung cancer (8.4%). Breast cancer was also the leading cause of cancer mortality (15%) followed by lung (13.8%) and colorectal cancer (9.5%) [9]. The differences in cancer types between males and females show the need for broader cancer treatment options.

1.2.1. *Africa*

Broader treatment options would also be beneficial due to the differences in cancer causes between developed and developing countries and the healthcare burden in Africa displays the unique characteristics of transitioning countries [12]. On the one hand, the contribution of infection-related cancers is higher in Africa compared to elsewhere in the world. Globally, 15.4% of cancer cases were attributed to infectious agents in 2012 compared to 31.3% for sub-Saharan Africa [13]. In addition, although population structures are gradually transforming toward a larger proportion of aging individuals (longer life expectancy), there still remains a significant pediatric population. As a result, Africa has a larger proportion of pediatric cancers contributing to total cancer burden compared to developed countries [14]. On the other hand, the aging population suggests that the number of new cancer cases in Africa will increase by 70% between 2012 and 2030 due to demographic changes alone [15].

In 2018, according to the Global Cancer Observatory, there were 1 055 172 new cases of cancer diagnosed in individuals living on the African continent with 693 487 deaths from cancer [9]. Globally, Africa accounted for 5.8% of cancer cases and 7.3% of deaths. While the cumulative risk of developing cancer is 49% in men from Australia/New Zealand and 22% in Southern African men, the risk of men dying from these cancers is only 10% in Australia/New Zealand and 13% in Southern Africa. Therefore, Africans bear a disproportionate cancer mortality compared to the rest of the world, highlighting the need for early detection, better care and access cancer therapeutic options.

In most African countries, the most common cancer diagnosed in men was prostate cancer. Notable exceptions were some countries in West Africa where liver cancer predominated, sub-Saharan Africa with Kaposi's sarcoma, and lung cancer in North Africa [14]. For women, there was less variation with breast and cervical cancers being the most common cancers reported for almost all African countries [14].

Summary statistic 2018

	Males	Females	Both sexes
Population	3 850 719 284	3 782 099 828	7 632 819 272
Number of new cancer cases	9 456 418	8 622 539	18 078 957
Age-standardized incidence rate (World)	218.6	182.6	197.9
Risk of developing cancer before the age of 75 years (%)	22.4	18.3	20.2
Number of cancer deaths	5 385 640	4 169 387	9 555 027
Age-standardized mortality rate (World)	122.7	83.1	101.1
Risk of dying from cancer before the age of 75 years (%)	12.7	8.7	10.6
5-year prevalent cases	21 014 830	22 826 472	43 841 302
Top 5 most frequent cancers excluding non-melanoma skin cancer (ranked by cases)	Lung Prostate Colorectum Stomach Liver	Breast Colorectum Lung Cervix uteri Thyroid	Lung Breast Colorectum Prostate Stomach

Figure 1. Summary of global cancer incidence and mortality rates for 2018. Highlighted are the most common types of cancer, where lung cancer can be seen to have the highest amount of cases in both sexes (Accessed from: https://www.iarc.fr/) [9].

Moreover, causes of cancer mortality on the continent largely mirrored incidence and included preventable and treatable cancers. Most countries reported prostate cancer as the leading cause of cancer death in men with Kaposi's sarcoma also noted. However, the leading cause of cancer deaths in sub-Saharan and East African women was cervical cancer with the rest of Africa mainly reporting breast cancer as the commonest cause of female cancer mortality. Liver cancer in East Africa and leukemia in West Africa were reported as the number one cause of male cancer death, with lung cancer being most prevalent in some Northern African countries and South Africa [9]. Interestingly, the overall number of cancer cases in South Africa has been on an alarming increase each year.

1.2.2. South Africa

For many years South Africa has elected to track cancer incidence trends using a pathology based National Cancer Registry (NCR) established in 1985. The latest countrywide incidence estimates produced from the NCR for the year 2014 are described below (Figure 2) [16].

For the population of 54 million, there were 74 577 new cancers diagnosed in private and public laboratories in South Africa with 50.7% of cancers diagnosed in women. Overall lifetime risk for cancer was 1:8 for women and 1:7 for men. Breast cancer accounted for 21% of all female cancers followed by cervical cancer (15.1%); and colorectal cancer (4.3%). Prostate cancer was the leading cancer amongst men and a lifetime risk of 1:19, followed by colorectal and lung cancers [16]. Thus, there is a growing need for new therapeutic tools to combat these cancer types not only in South Africa but around the world.

1.3. Diagnosis

Early detection and diagnosis are of great importance since it allows for elimination of the cancer cells, before metastasis can occur – where curative treatment is no longer as effective. There are several tools used to diagnose cancers, all starting with tissue biopsies and genetic testing [7]. Imaging technology is routinely utilized, which includes magnetic resonance imaging (MRI), computed tomography (CT) scans, X-rays as well as ultrasounds [17]. Other methods to diagnose cancers include common screening tests particular to cancer types such as mammograms for breast cancer or colonoscopies [18] for colon cancer as well as serology for specific cancer biomarkers [19]. However, there are limitations to existing diagnostic tools such as insensitivity and non-specificity to several cancer biomarkers, making these tools not as reliable. Moreover, most cancer deaths are unfortunately caused by metastatic tumors thus, the lack of effective diagnostic and therapeutic options is an ever-motivating factor for many research efforts worldwide.

1.4. Metastasis

Metastasis is known as the spread of tumourigenic cells from the site of origin to local tissues and distant organs [20]. Metastasis is a complex, multi-step process involving several sequential steps. The process is initiated by tumourigenic cells detaching from the primary site and intravasating into the circulatory system. This is then followed by extravasation at distant capillary beds, ultimately leading to invasion of the tumourigenic cells in local as well as regional organs [20]. These tumourigenic cells are then able to establish an ideal microenvironment that allows for angiogenesis, resulting in the formation of secondary tumors. In addition, it has been proven that tumourigenic cells are able to adhere to and invade secondary sites through the mediation of integrin and non-integrin receptors [21].

2. 37 kDa/67 kDa laminin receptor precursor/ laminin receptor (LRP/LR)

An important protein found to play a role in cancer and metastasis, is the highly conserved ribosomal protein SA (RPSA) found in both pro- and eukaryotes. The *RPSA* gene is located on the short arm of chromosome 3 and consists of 1700 base pairs, encoding a protein of 295 amino acids with a mass of 33 kDa that appears as ~37 kDa when viewed by SDS-PAGE. There are a total of 64 pseudogenes that have been cataloged for the *RPSA* gene in humans, suggesting that the gene is ancient. This is supported by the high sequence conservation in 37 kDa LRP across phyla [22]. Moreover, the RPSA protein has been found to be a vital component of the 40S ribosomal subunit consequently making it a necessary protein for cell viability. As with many ribosomal proteins, RPSA performs several extra-ribosomal functions; most notably as a cell surface non-integrin high affinity laminin-1 receptor. Therefore, RPSA is often referred to as the 37 kDa laminin receptor precursor/ 67 kDa/high affinity laminin receptor (LRP/LR) [22]. LRP/LR is known by several other pseudonyms including: laminin

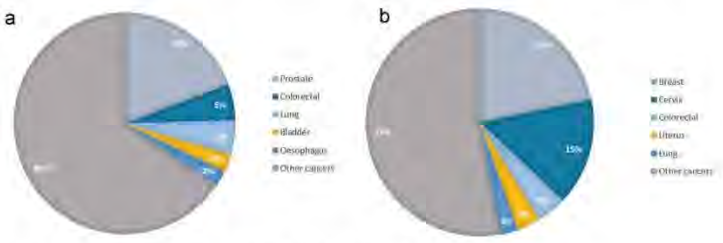

Figure 2. Percentage contribution of the top five cancers in (a) Males and (b) Females in South Africa in 2014 [16].

receptor 1 (LAMR1), ribosomal protein SA (RPSA) and p40. For the sake of clarity, we shall refer to the precursor form of the *RPSA* gene product as 37 kDa LRP and the mature high affinity laminin receptor form as 67 kDa LR for the remainder of this review.

2.1. Structure and function of LRP/LR

LRP/LR has been found to comprise of two domains: an RPS2-like globular N-terminal domain and an intrinsically disordered C-terminal domain. Although each domain has individual functionality, the two domains are required for laminin binding [23]. Phylogenetic analysis confirms that LRP/LR is a ribosomal protein that gained laminin-binding functionality [24]. Some controversy surrounds the identity of the laminin interaction interface. Attempts to map the laminin binding surface have identified several possibilities: a buried peptide (peptide G) and a surface peptide directly involved in the interaction with laminin-1 [25]; mutagenesis studies of recombinant 37 kDa LRP suggest that laminin-1 binding involved Phe32 (N-terminal), Glu35 (N-terminal) and Arg155 (C-terminal) [26]; finally, kinetics experiments revealed that some structure is contextually induced in the disordered C-terminal to maximize contacts between LR and laminin-1 [23,27]. In addition, the C-terminus contains several binding sites for immunoglobulins, carbohydrates, microorganisms, elastin and importantly, laminin-1, as well as also allowing for anchorage of 37 kDa LRP to the 40S ribosomal subunit via a LRP-18S rRNA interaction [28]. Thus, it can be seen that the receptor's structure has been extensively studied however, questions on how the precursor becomes a mature cell surface receptor remain.

It has been hypothesized that since the amino acid sequences of the 37 kDa and 67 kDa forms are the same [29], homodimerization could be forming the mature receptor [22]. However, it is also known that 37 kDa LRP is acylated with oleate, palmitate and stearate, which are thought to promote fatty acid-fatty acid interactions between 37 kDa LRP monomers or between 37 kDa LRP and other acylated partner proteins, resulting in the observed higher molecular weight mature receptor [22]. Thus, the composition of the 67 kDa LR is still a point of contention as in vitro studies show that the translated 37 kDa LRP is monomeric and the expression of RPSA cDNA does not show any 67 kDa LR production [27]. To date, the maturation of the 37 kDa LRP to the 67 kDa LR has yet to be resolved.

Despite many unknowns, LRP/LR has interestingly been found to play several physiological roles within the cytoplasmic, perichromosomal and perinuclear areas as well as on the plasma membrane of cells [22]. As mentioned above, LRP/LR aids ribosomal processing and protein translation in the cytoplasm [28]. In addition, the receptor has been seen to interact with histones in the nucleus, thereby aiding in nuclear structure maintenance [30,31]. LRP/LR is also found to interact with components of the cytoskeleton such as actin and tubulin, by acting as a linker for ribosomes to microtubules, resulting in functional protein synthesis [32]). LRP/LR is also found to be predominantly located in the plasma membrane, specifically in lipid rafts, where it acts as a receptor for various viruses

Figure 3. The pathological roles of the 37 kDa/67 kDa laminin receptor (LRP/LR). Over the years, research has shown that LRP/LR is involved in several pathologies. This is due to LRP/LR having several binding sites thus, serving as a receptor for numerous viruses, bacteria and infectious prions. Furthermore, LRP/LR has been found to associate with enzymes responsible for the formation of the neurotoxic amyloid-beta peptide as well as internalization of the peptide in AD. It has also been shown to play a role in aging and other age-related disorders due to its interaction with the enzyme, telomerase. In addition, overexpression of LRP/LR is a key characteristic of cancerous cells which has a correlation with angiogenic enhancement [51], metastasis [6,42,44,45] and cell survival [43,52,53,55] of neoplastic cells.

including Dengue, Sindbis [33], Adenoviruses and Venezuelan equine encephalitis virus [34]. It also acts as binding partner for PrP^{c} [35,36] and as a receptor for prion proteins PrP^{c} and PrP^{sc} [37], respectively, bacteria including the cytotoxic necrotizing factor-1 expressing *Escherichia coli* K1 [38] as well as several other proteins part of the extracellular matrix such as laminin-1, elastin and heparin sulfate proteoglycans [39]. Recently, it has been found that LRP/LR has a link to aging, since it to co-localizes with telomerase, an enzyme involved in regulating the aging process [40] (Figure 3). Specifically, increased LRP/LR has been found to enhance telomerase activity and reduce senescence markers, making LRP/LR a potential novel telomerase activator and anti-aging drug [40] (Figure 3). LRP/LR is also found to be involved in several diseases including cancer [6,41–46], micro-organism infections [47], Transmissible Spongiform Encephalopathies (TSEs) [39], prion disorders [36], Alzheimer's Disease (AD) [48–50] and age-related disorders [40]. However, the receptor's primary roles have been identified as enhancing adhesion of tumor cells to the basement membrane and disseminating these tumors, which are both fundamental events of metastasis [6,42–46], promoting tumor angiogenesis [51] and finally, allowing for cancer cells to survive through apoptotic evasion [52–55]. Thus, this review will focus on LRP/LR and its role in cancer, due to its significant influence on tumor progression and aggressiveness as well as therapeutic options targeting the receptor that may provide potential therapeutic applications.

2.2. Oncofoetal antigen/immature laminin receptor protein

A recent study demonstrated that oncogenic tissues are enriched in an immunogenic proteoform of LRP/LR, known as oncofoetal antigen/immature laminin receptor protein (OFA/iLRP) [56,57]. Protein and cDNA sequence analysis revealed that OFA/iLRP shares 99.9% sequence identity with LRP/LR [57]. In addition, it was found that the 37 kDa OFA/iLRP allows for tumourigenic cells to penetrate laminin, thereby facilitating tumor progression [56]. Thus, it is apparent that 37 kDa OFA/iLRP and 37 kDa LRP represent the same protein species and overall function; however, during development, 37 kDa LRP undergoes an unknown alteration to become the mature 67 kDa LR. The mature 67 kDa LR does not induce an autoimmune response whereas OFA/iLRP appears to facilitate induction of a trained pool of T and B lymphocytes recognizing specific epitopes located in the N-terminal and C-terminal domains [58]. Considering that the mature 67 kDa LR retains all the epitope sequences shown to induce a strong immunogenic response against 37 kDa iLRP, there must be some form of shielding of these epitopes. Some likely possibilities include: the epitopes form a part of the dimer interface – which is the current hypothesis concerning maturation of 37 kDa LRP to 67 kDa LR; or that the 37 kDa LRP undergoes significant structural changes during membrane integration that conceals the epitopes within the core of the protein or within the interior of the cell. Because 37 kDa LRP and 37 kDa iLRP are the same protein, we have included all novel therapeutic patents regarding OFA/iLRP in this review.

3. The role of LRP/LR in tumourigenesis

3.1. LRP/LR and metastatic cancer

LRP/LR has been found to be over-expressed on the surface of several cells including various cancers such as pancreatic (AsPC-1) cancer cells [59], lung (A549), prostate (LNCaP) cervical (HeLa) [6], esophageal (WHCO1) breast (MDA-MB231) [43] liver (HUH-7) cancer cells [42] as well as early (SW-480) and late (DLD-1) stage colorectal cancer [44] and malignant melanoma (A375SM)cells [46]. Furthermore, a correlation between LRP/LR expression and tumor development has been reported [60]. This over-expression of LRP/LR might be a consequence of its interaction with the laminin-1 glycoprotein. Laminin-1 is found in the basement membrane of cells and is normally involved in biological processes such as cell adhesion [61], differentiation, growth and migration [62]. Thus, it is no surprise that laminin-1 is said to promote the invasive phenotype of tumourigenic cells. Studies have shown that the LRP/LR-laminin-1 interaction is essential in promoting two main steps in the metastatic cascade – adhesion and invasion [6,43–45,63] (Figure 4). Upon the binding of LRP/LR, laminin-1 undergoes a conformational change and becomes more efficient in interacting with integrins of the basement membrane, which leads to increased adhesion [39]. This as a result, increases the sensitivity of laminin-1 to proteolytic enzymes such as type IV collagenase which hydrolyzes collagen in the basal lamina, leading to degradation of the basement membrane and invasion of the tumourigenic cells [64]. In addition, the LRP/LR-laminin-1 interaction may also contribute to angiogenic enhancement by providing these cancer cells with oxygen and nutrients, making it possible for the cells to metastasize to new sites of the body [51]. Thus, it might be concluded that up-regulated LRP/LR may serve as molecular marker for metastasis in many cancer types.

3.2. LRP/LR and tumor angiogenesis

Angiogenesis is an essential process needed for growth and development whereby new blood vessels grow from preexisting ones [65]. Additionally, it plays a vital role in wound healing as well as granulation tissue formation [66]. This is exploited by cancer cells to aid in the conversion of tumors from benign to malignant, because as they grow and move away from blood vessels, they become deprived of nutrients and oxygen. The cancer cells perform this by emitting signals known as angiogenic factors e.g. VEGF to stimulate the growth of new blood vessels to receive oxygen and nutrients [67] which results in rapid growth of the tumor cells, allowing for the cells to become malignant. Thus, angiogenesis favors tumourigenic enhancement as well as aiding in the migration and metastasis of cancer cells through the circulatory system.

As reported before, 67 kDa LR has been shown to be vital in the neovascularisation and migration of hemopoietic stem cells (HSCs) which are known to encourage angiogenesis by producing new endothelial cells [68]. It has also been shown that high levels of LRP/LR on the surface of human umbilical vein endothelial (HUVE) cells could play a role in enhancing their invasive potential [51] (Figure 4). Consequently, this may allow them to move toward pro-angiogenic stimuli by degrading important constituents of the extracellular matrix, and ultimately lead to the development of tubular structures needed for the formation of blood vessels. Moreover, it was found that when HUVE cells were treated with the anti-LRP/LR specific antibody, W3, tubular formation was fully halted [51]. Thus, LRP/LR specific antibodies such as W3 may be a potential therapeutic tool for the treatment of cancer through impediment of tumor angiogenesis.

3.3. LRP/LR and evasion of apoptosis

Alongside angiogenesis, LRP/LR has been linked to a variety of cancer promoting processes, including cell viability maintenance and apoptotic evasion. By blocking apoptosis, several diseases such as autoimmune lymphoproliferative syndrome as well as neurodegenerative diseases like Parkinson's disease and Alzheimer's disease are able to arise, and specifically cancer [69]. Here, changes in the apoptotic process have been reported to enhance the progression of tumors and, several cancers are thought to favor these changes and evade apoptosis [21]. One of the reported changes include mutations in the p53 tumor suppressor gene which may result in the loss of regulation in several pro-apoptotic genes such as Bax and Bid, ultimately leading to increased cell growth [21].

Due to evidence indicating that LRP/LR plays a role in apoptosis [70], several *in vitro* cancer studies have been performed to determine whether down-regulating the receptor may have an effect in inhibiting tumourigenesis. LRP/LR down-regulation via siRNA technology resulted in a significant decrease in the cell viability of

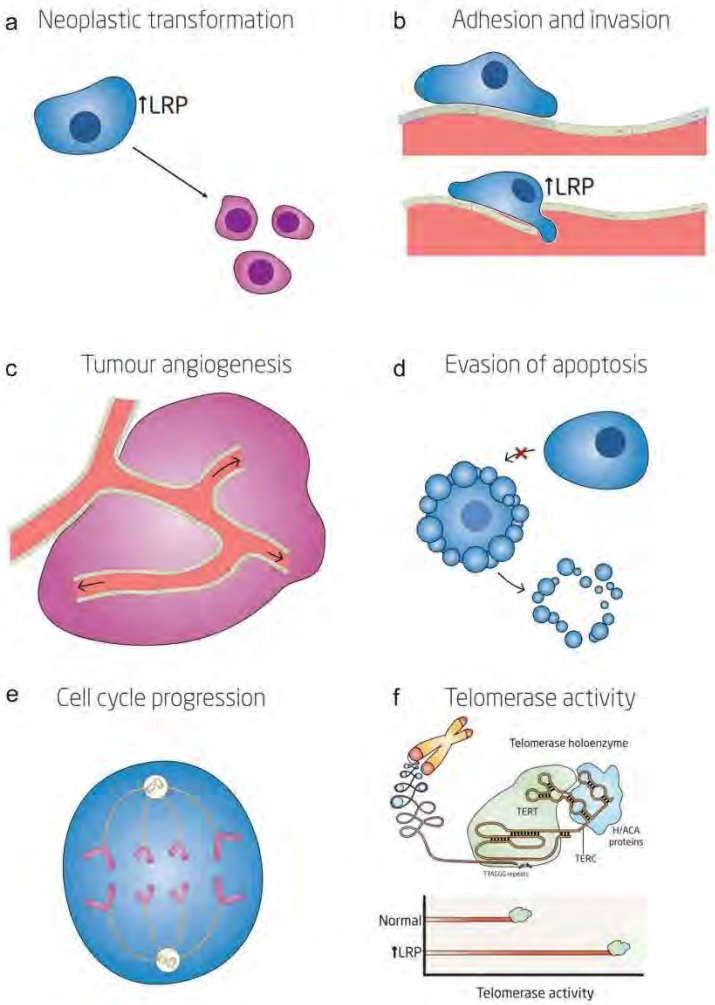

Figure 4. Roles of LRP/LR in cancer. (a) One of the main characteristics of tumourigenic cells is that the 37/67 kDa laminin receptor (LRP/LR) is overexpressed when compared to their normal cell counterparts, thus LRP/LR is said to play a role in neoplastic transformation [6]. (b) LRP/LR expression levels have been found to have a direct correlation with the adhesive and invasive potential (primary features in metastasis) of tumourigenic cells [6,42–46]. (c) LRP/LR has been shown to play a key role in the formation of tumor angiogenesis via endothelial tube establishment [51]. (d) LRP/LR has been shown to cause tumourigenic cells to have increased cell survival through apoptotic evasion [52–55]. (e) LRP/LR is said to be involved in the cell cycle by halting the G1 phase, and as a result exposes its role in tumor protein translation and progression in specific cancer cell lines [145].It has also been found that through siRNA-mediated knockdown of LRP/LR, the receptor is linked to telomerase, an enzyme involved in cancer progression [70,143].

tumorigenic breast (MDA-MB-231) [54], pancreatic (AsPC-1) [53], neuroblastoma (IMR-32) [53], early (SW-480) and late (DLD-1) stage colorectal carcinoma [55] as well as early (A375) and late (A375SM) stage malignant melanoma [52] cells (Figure 4). Furthermore, a high correlation between the total levels of LRP before siRNA treatment to the levels post siRNA treatment was observed. This high correlation proposes that the expression levels of LRP/LR is in fact influenced by siRNA treatment i.e. lower levels of LRP/LR expression prior to treatment with siRNA leads to more LRP knockdown post treatment with siRNA [52,53,55]. Another high correlation was seen between total levels of LRP after siRNA treatment and total levels of cell viability and apoptosis for cancer cell lines i.e. the higher the level of LRP expression, the higher the amount of cell viability, which as a result leads to lower levels of apoptosis. This result therefore further reiterates that LRP/LR expression affects cell viability and apoptosis.

LRP/LR is not only located in the plasma membrane and the cytosol but has also been found in the nucleus. Here, LRP/LR

maintains chromosome stability via contact with the Midkine heparin-binding growth factor; well-known for promoting cell migration, proliferation and survival [71]. As a result, the growth factor assists LRP/LR in maintaining cellular viability. Several cancer types are reported to have increased levels of Midkine, leading to the enhancement of cell survival factors as well as the impediment of apoptosis through caspase-3 inhibition [72]. Caspase-3, an effector caspase required for the cleavage of specific substrates, ultimately leads to key hallmarks of apoptosis, including biochemical and nuclear morphological changes [73].

However, by targeting LRP/LR expression through siRNA technology, LRP/LR-Midkine interactions may decrease, which results in decreased cell viability. In fact, one study showed that siRNA-mediated knock-down of LRP/LR not only reduced cell viability of cervical cancer (HeLa) cells and lung (A549) cancer cells but also increased caspase-3 activity in both cell lines [70]. This has been confirmed in several other tumourigenic cell lines including pancreatic (AsPC-1) [53], nueroblastoma (IMR-32) [53] early (SW-480) and late (DLD-1) stage colorectal carcinoma [55] as well as early (A375) and late (A375SM) stage malignant melanoma [52] cells, further confirming that siRNA technology, through the knockdown of LRP, is a promising tool in combating cancer.

The interaction of LRP/LR with focal adhesion kinase (FAK) might be diminished after LRP/LR down-regulation which might further contribute to apoptosis induction [74,75]. A study performed by Lu et al (2016) demonstrated that siRNA-mediated knockdown of LRP/LR reduced FAK phosphorylation, leading to the knockdown of the anti-apoptotic protein Bcl-2 while an up-regulation of the pro-apoptotic Bax protein, reiterating that the knockdown of LRP/LR induces apoptosis [74]. Moreover, another study performed by Sun et al (2014) also showed that found that the LRP/LR-laminin-1 interaction may allow for LRP/LR's interaction FAK, leading to the activation of the PI3-kinase/AKT and MEK/ERK 1/2 cellular survival pathways as well as an increased expression of the [75]Bcl-2 protein. Both the MEK/ERK 1/2 and PI3-kinase/AKT cell signaling cascades are found to inhibit the caspase-8 and –9 apoptotic pathways. Caspase-8 plays a vital role in the extrinsic apoptotic signaling pathway known to initiate apoptosis through transmembrane receptor-mediated interactions which involve death receptors of the tumor necrosis factor (TNF) receptor gene superfamily [69]. On the other hand, caspase-9 is critical in the intrinsic apoptotic signaling pathway which initiates apoptosis through a diverse collection of non-receptor-mediated stimuli – which include toxins, free radicals and hypoxia – that all produce intracellular signals that act directly on cellular targets [69]. These events are mitochondrial-dependent. Thus, investigations on both apoptotic pathways are needed to determine whether caspase-8 and –9 become activated once LRP/LR is down-regulated. Upon siRNA-mediated down-regulation, recent studies show that pancreatic (AsPC-1) [53], neuroblastoma (IMR-32) [53], early (SW-480) and late (DLD-1) stage colorectal cancer [55] cells as well as early (A375) and late (A375SM) stage malignant melanoma [52] cells either undergo both the extrinsic and intrinsic apoptotic pathways or either one of them. This variance suggests the significance of the different interactions between the expression levels of LRP/LR and the constituents of the ECM, specifically laminin in different cancer cell types. More particularly, on the one hand both early and late stages of malignant melanoma experience apoptosis through different pathways [52]. This could be as a result of a change in specific apoptotic protein expression levels implicated in the two different apoptotic pathways i.e. as the transition from early to late stage occurs, the proteins involved in the extrinsic pathway decrease while the proteins in the intrinsic pathway increase. This may occur as apoptotic proteins can be subject to the extent of caspase-mediated cleavage, with the cleavage of caspase substrates being linked to signature alterations of apoptotic cells [76]. On the other hand, early and late stage colorectal cancer cells were found to undergo both caspase-8 and –9 activation [55]. This is also highly likely as the extrinsic and intrinsic pathways are able to interconnect at numerous levels and as a result, become influenced by similar factors. In fact, a study showed that activated extrinsic caspase-8 stimulates the release of cytochrome c and apoptosome formation, respectively, and ultimately activation of the intrinsic pathway [77,78]. Another possible reason as to why both apoptotic pathways are activated may be that these colorectal cancer cells undergo a mechanism known as retaliatory caspase activation where the two apoptotic pathways are found to use a feedback amplification loop in order to activate one another [79]. Specifically, activated caspase-9 initiates and proteolytically cleaves caspase-3, also leading to caspase-8 activation. These findings have given insight into LRP/LR and its mechanism in cancer and apoptosis however, more information is still needed in order to fully understand the role LRP/LR plays in the evasion of apoptosis in cancer.

3.4. LRP/LR and telomerase

It has very recently been observed that in addition to its various functions and interactions in cancer and neurodegenerative disorders, LRP/LR plays a role in mediating telomerase activity in breast cancer cells [70] (Figure 4). Telomerase is principally responsible for elongating telomeres; tandemly repeated TTAGGG repeats found on the ends of chromosomes which help prevent chromosomal degradation [80]. Telomerase is a ribonucleoprotein with reverse transcriptase activity. It is made up of two essential components, namely, in humans: the reverse transcriptase catalytic component, hTERT, which is responsible for the addition of telomeric repeats; and the functional RNA component, hTERC, which serves as the telomeric template [80].

Telomeres have an associated protein complex, the shelterin complex, which forms distinct t-loop structures. Chromosomes are protected from degradation and end-to-end fusions by these structures which prevent chromosome ends from being recognized as double-stranded DNA breaks [81]. Chromosome ends, or telomeres, are predisposed to shorten with each successive cell division, due to a process well-known as the 'end replication problem' [82,83]. To this effect, when telomeres shorten to a critical length, the dysfunctional telomere is recognized as damaged DNA and cellular quiescence is triggered [83]. Cells can remain in this senescent state for years, provided no other changes occur and as such, this is considered to be a type of tumor suppressor mechanism in humans.

Conversely, however, human carcinomas circumvent inhibitory pathways induced by DNA damage, as well as senescence, through the upregulation of telomerase activity [84]. The initiation and maintenance of the senescent state involves cell cycle checkpoint proteins, including p53/p21 and pRB/p16. When these proteins are inactivated, cells can bypass senescence and subsequently proliferate, causing genetic instability, leading to cell death. However, cells can survive this by activating telomerase, where the cells then gain the capability to maintain the length of these unstable telomeres, where after malignancy occurs [84]. Interestingly, it has been shown that cancer cells that have escaped senescence, can be reversed to the senescent state by restoring the tumor suppressor, p53 [85]. Therefore, it is generally believed that telomeres and telomerase are the connection between cellular senescence and immortalization [86].

There is an increasing amount of evidence to suggest that telomerase is also implicated in cancer and aging by actions beyond its function in maintaining telomeres [87]. It is known that the inhibition of telomerase, and the resultant loss of telomere maintenance, results in apoptosis. This effective the loss of telomerase activity causes progressive telomere shortening and leads to the inactivation of the telomere capping function of the shelterin complex, eventually leading to cell death [88]. The catalytic subunit of telomerase, hTERT, has been found to play a role in regulating apoptosis, independent of telomere maintenance and telomerase activity. It has been demonstrated both *in vitro* and *in vivo*, that there is a direct association between TERT and an increased resistance to apoptosis [89]. In addition, hTERT overexpression has been found to make cells more resistant to apoptosis [90]. The study showed that telomerase expression did not prevent stress-induced senescence in normal human fibroblasts, but rather protected the cells from apoptosis [90]. Moreover, a recent study has shown that downregulating hTERT through RNA silencing, activated Bax, which triggered a mitochondrial cell death pathway [91]. Furthermore, this did not occur as a result of telomerase inhibition, as there was no telomere erosion, ultimately suggesting a role of TERT in preventing apoptosis [91].

Increased telomerase activity has been detected in 85–90% of malignant human tumors [92] and therefore, telomerase has become an attractive target for potential cancer therapeutics. Further to the role in cancer progression, it has even been shown that telomere dysfunction and elevated levels of telomerase activity are associated with cancer recurrence and chemotherapeutic resistance [93], outlining the important role that telomerase has in cancer progression. Moreover, what makes the inhibition of telomerase particularly appealing, is that most somatic cells do not possess detectable telomerase activity and thus, inhibiting telomerase should have little off-target effects. Taken together, these factors have enticed an array of varying patented research into the inhibition or down regulation of telomerase to serve as potential therapeutics for the treatment of cancer. A few of the most recent patents regarding telomerase and cancer include: the use of telomerase peptides that target telomerase (WO/2010/003520A2) [94], using modified oligonucleotides or aptamers for telomerase inhibition (WO/2005/023994A2) [95], using plant- or chemical-based compounds to inhibit telomerase activity/induce telomere shortening and dysfunction (WO/2016/111530A1 [96]; WO/2014/168947A2 [97]) and lastly, combinatorial treatments that focus on inhibiting both telomerase activity and other tumorigenic processes (WO/2006/113470A2) [98]. With regard to the telomerase peptide treatment, it is an immunization approach, whereby telomerase epitopes are recognized by CD8+ or CD4 + T lymphocytes, which elicit an immune response for the targeted killing of cancer cells (WO/2010/003520A2) [94]. The aptamer-based treatment adopts a different approach, whereby a modified oligonucleotide attached to a lipid moiety is used to target the RNA component of the enzyme to inhibit telomerase activity and ultimately cancer cell proliferation (WO/2005/023994A2) [95]. Finally, there have been a range of both chemical and plant-based compounds developed that target telomerase for anti-cancer properties as well as for the treatment of other diseases. Two such recent patents have focused on using Luterion, a plant-based compound for inhibition of both telomerase and proliferation, in tumorigenic cells only (WO/2016/111530A1) [96]. The other patent focuses on the use of Mercaptopurine ribonucleoside and analogues for the induction of telomere shortening and/or dysfunction. This occurs where the compound is converted into a telomere substrate containing 2'-deoxyguanosine 5'-triphosphate and is incorporated by telomerase into the cell's telomeres. The modified nucleotides within the substrate then alter the telomeric structure, causing DNA chain termination as it is recognized as DNA damage and ultimately, causes the induction of cell death (WO/2014/168947A2) [97]. Although there have been numerous patented approaches in which telomerase and its associated functions have been the main target for the progression, recurrence and chemoresistance of most cancers it remains vital to find new ways to efficiently target telomerase and the processes in which it is involved.

In this regard, the potential relationship shared between LRP and TERT/Telomerase offers a unique avenue in therapeutic study. Since LRP functions as a receptor for extracellular molecules and further shares a functional relationship with TERT, it can be used as a target for drug delivery against telomerase, in a similar manner as the previously mentioned plant-derived EGCG compound [99]. Once EGCG gains access intracellularly via LRP, it subsequently influences telomerase and its consequent functions [99,100]. In relation, it was found that a long term non-cytotoxic treatment with EGCG was able to effectively induce cellular senescence in cancer cells by the stimulation of telomere dysfunction caused by the direct inhibition of telomerase activity [99,100]. Therefore, this relationship could aid in the delivery of unique telomerase affecting therapeutics.

Aside from LRP's use as a target for drug delivery against telomerase, both proteins are also commonly implicated in multiple shared tumorigenic functions. In fact, a patented study (WO/2017/077516A3) showed that telomerase and LRP/LR co-localize in the perinuclear compartment of breast cancer cells [70,101] and it has recently been found that LRP/LR enhances telomerase activity [40,102]. Furthermore, the knockdown of LRP/LR with LRP-specific siRNA significantly reduced telomerase activity [70,101] and should this state be maintained it could effectively prevent the preservation of the shorter telomeres characteristic of tumorigenic cells. This would therefore lead to widespread telomere erosion, which could not only increase the

susceptibility for the occurrence of crisis in the cells but also further enhance chemosensitivity [93]. This enhanced chemosensitivity would arise from the loss in genomic protection that the telomeres convey, thereby allowing chemotherapeutic drugs to induce widespread genomic instability and damage more effectively. Therefore, suggesting that the downregulation of LRP/LR may be a potential therapeutic intervention for cancer through the resultant downregulation of telomerase activity, owed to the relationship between LRP/LR and TERT.

In addition to telomeric dysfunction, the knockdown of LRP may further affect TERT levels affecting its ability to carry out extra-telomeric functions, especially regulation of pathway and gene expression [103]. This could lead to a profound effect on the cancer cell's physiology, as shared pathways between LRP and TERT would become affected. One such pathway that could be affected is the p53-dependent apoptotic pathway, where both proteins have been found to be profoundly influenced by p53 [104,105]. In this regard, p53 a protein commonly dysregulated or mutated in cancers has been found to influence expression of both TERT and LRP by transcriptional repression to limit proliferation [104,105]. Moreover, p53 has been found to not only affect TERT but also downregulates telomerase activity for apoptotic induction or cell cycle arrest [106]. Consequently, a decrease in both of these proteins (which share an antagonistic relationship with p53) may allow the elevation of p53 and subsequent induction of apoptosis in the cancerous cells. Furthermore, as both LRP and TERT have been intricately linked to proliferation pathways like the MAPK pathway [107,108], knockdown of LRP and subsequently TERT, could severely impact the aggressive and rapid proliferation observed in cancer cells. This said, targeting LRP with its corresponding effect on TERT/telomerase, will compromise multiple key cancer hallmarks influenced by both proteins, including: invasion, apoptotic and growth suppressor evasion as well as replicative immortality.

4. Current patented studies targeting LRP/LR for the treatment of cancer

It is known that the most widespread treatment strategy for cancer is surgery. However, if the cancer has already reached the late stages, additional treatments such as chemotherapy and radiotherapy, or a combination of the two is recommended. Unfortunately, chemotherapy and radiation may damage epithelial cells and cause swelling to soft tissue [109]. Furthermore, these treatments are often ineffective, cause severe side effects which may lead to other conditions, and potential relapse may still occur from remaining cancer cells, thus, other therapeutic approaches have been explored [110].

Several strategies including hormone therapy and targeted therapy have been considered to reduce cancer progression. Targeted therapy, which can be either natural or drug-based, is particularly used specifically to deliver agents that block essential routes needed for cancer cells to survive [111]. In particular, agents such as small molecule inhibitors (SMIs), small interfering RNAs (siRNAs) and monoclonal antibodies are mostly used to target tumors while anti-angiogenic drugs are employed to target angiogenesis of tumor cells.

4.1. Chemotherapeutic strategies

These agents are usually delivered to target molecules using nanoparticles which can either be synthetic lipoproteins [112] or natural-based polymers such as hyaluronic acid [113] and chitosan [114]. In addition, glycosylation-targeted drug delivery is also becoming an increasing popular method whereby carbohydrates are used as ligands that bind to surface receptors of target molecules, resulting in the sustained and controlled release of the agent together with reduced toxicity and biostability [115].

4.1.1. Small molecule inhibitors (SMIs)

SMIs have also become increasingly favorable as their small size (approximately 500 Da in size) enables easy translocation from the cell membrane via their interaction with several cell surface receptors [109]. Due to their size, many SMIs are used to target extracellular as well as intracellular proteins which play a role in cancer progression. Cancer markers such as receptor tyrosine kinases (RTKs), as well as important growth factor receptors such as EGF and VEGF, are primarily targeted by SMIs through blocking kinase activity intracellularly, resulting in downstream signaling inhibition [109]. Studies also show that the proteosome can be targeted within cells in order to induce apoptosis of cancer cells. In particular, the small molecule proteosome inhibitor known as Bortezomib, was synthesized to activate apoptosis in tumor cells by inducing the expression of the cell cycle regulator p21 [116] as well as dysregulating the anti-apoptotic protein, survivin, both of which are known to be regulated by the p53 tumor suppressor gene [117]. As a result, Bortezomib was found to treat multiple myeloma, renal carcinoma as well as subtypes of lymphoma [118]. Other SMIs from the Bcl-2 family of proteins including Navitoclax and Obatoclax are known for inducing apoptosis in several cancer cells by blocking their anti-apoptotic functions [119,120]. In particular, both Navitoclax and Obatoclax were found to induce Bax translocation, facilitate the release of cytochrome C as well as simultaneously liberating pro-apoptotic proteins Bim and Bak. This in turn allows for the formation of the active pro-apoptotic Bak/Bax complex, ultimately leading to apoptosis through the intrinsic pathway [119,121].

Since 2001, multiple SMIs have been approved including Tarceva, which targets EGFR, a receptor required for cell growth and survival. This mechanism involves selective and reversible inhibition of the tyrosine kinase activity of EGFR by competing with ATP for the ATP-binding site [122]. Tarceva was successful in treating non-small cell lung cancer and pancreatic cancer [123]. Another SMI, Lapatinib, also targets EGFR as well as HER2/neu for the treatment of breast cancer [124]. Sorafenib on the other hand targets tyrosine protein kinases such as VEGFR, leading a decrease in Bcl-2 expression and subsequent apoptotic induction, ultimately treating renal cell and hepatocellular carcinoma [125]. Trametinib, an ATP noncompetitive SMI is found to target the MEK pathway. Trametinib binds MEK next to the ATP binding site, leading to the inhibition of dual phosphorylation of MEK, required for MEK activation. As a result, tumor cell proliferation is impeded [126]. Trametinib has been used to treat several cancers including metastatic melanoma [127].

Furthermore, a recently patented study (WO/2019/012030) used the PF477736 checkpoint kinase-1 (Chk-1) inhibitor to hamper dihydroorotate dehydrogenase (DHODH), a mitochondrial enzyme expressed in highly proliferative cells [124,128]. It was found that there was increased cytotoxicity and cell death within several triple negative breast cancer cell lines which are known to be aggressively metastatic and resistant to several treatments [128]. Hence, this confirms that DHODH and Chk-I inhibitions may be a potential therapeutic tool for triple negative breast cancer as well as other cancer types [128]. An advantage to SMIs is that they can be administered orally and they are found to be much more cost effective than monoclonal antibodies [109]. However, the disadvantage of these molecules include have a short half-life within the body and due to their ability to bind to multiple targets, the risk of toxicity is increased [109].

4.1.2. Smis targeting LRP/LR

SMIs targeting LRP/LR have been shown to be very efficient in blocking its function. A study aimed to determine whether any small synthetic molecules are able to target LRP/LR and block the binding to laminin, due to this interaction enhancing key steps of metastasis [129]. Pasepane et al (2015) used an *in silico* method to find small molecules that were able to inhibit LRP/LR, by focusing on the peptide G sequence of 37 kDa LRP which binds specifically to laminin-1. Each chemical structure was docked into the 67LR/laminin-1 binding site to find the best-scoring ligands. This was followed by *in vitro* testing of each ligand, until the study concluded that one cell permeable molecule, NSC47924, was able to selectively inhibit LRP/LR cell adhesion to LM [129].Consequently, the adhesive and invasive potential of human fibrosarcoma (HT1080) and breast cancer (MDA-MB231) cells was decreased [129]. NSC47924 was also able to inhibit LRP/LR-mediated cell invasion and migration, recommending it as a potential therapeutic agent targeting LRP/LR-dependent malignancies (Figure 7).

Another study investigated the small molecule, BC-K-YH16899, targeting lysyl-tRNA synthetase (KRS) (Figure 5). High levels of KRS have been detected in cancer cells and the enzyme is also known to interact with LRP/LR, making it a favorable target [130]. The study screened numerous synthetic compounds using a yeast two-hybrid (Y2H) assay, aimed at finding a compound that was not only able to inhibit cell proliferation but also specifically inhibit the interaction between LRP/LR and KRS, without impacting on other interacting pairs. Based on its metabolic stability, efficiency and low cytotoxicity *in vivo*, the BC-K-YH16899 compound was selected for further confirmation studies [130]. The study showed that BC-K-YH16899 was able to significantly block the interaction between KRS and LRP/LR, and consequently suppressed metastasis in breast cancer mouse models. Effective inhibition of the metastasis-promoting interaction of LRP/LR and KRS was observed. This suggests that this inhibition could be a possible alternative for metastasis reduction without hampering normal LRP/LR functions [130].

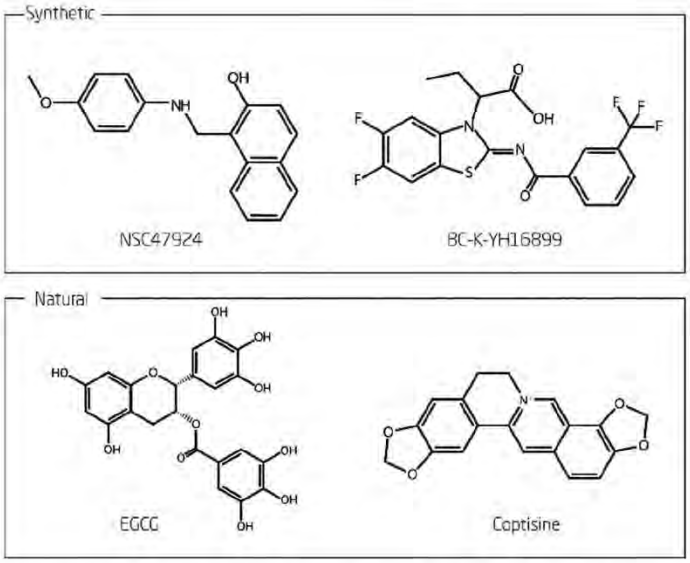

Figure 5. Small molecular inhibitors targeting LRP/LR for the treatment of cancer. The plant-derived compounds (EGCG and Coptisine) bind to the mature laminin receptor on the cell surface where it acts as a death receptor inducing apoptosis in tumorigenic cell lines [136]. The interaction between LRP/LR and the lysyl-tRNA synthetase (KRS) is disrupted by BC-KYH16899 effectively preventing translocation of KRS to the cell surface resulting in reduced metastatic potential in breast cancer cells [130]. Similar to EGCG and Coptisine, NSC47924 also binds LR but does not result in apoptosis. Instead, NSC47924 results in internalization of the 67 kDa LR preventing laminin-1 binding and a decrease in invasion potential of tumourigenic cell lines [129].

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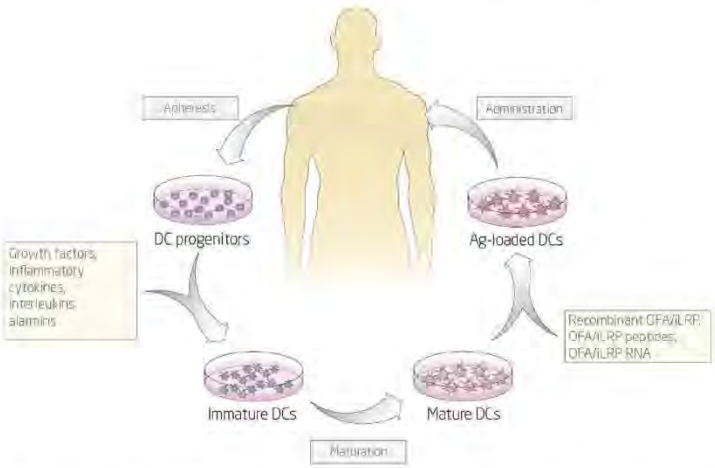

Figure 6. Immunotheraputic strategies targeting immature laminin receptor protein (iLRP). There are at least three immunotherapies targeting iLRP. Two of the patented immunotherapies utilize autologous dendritic cells (DC) primed with iLRP encoding RNA or iLRP-derived antigenic peptides [170,173,174]. One of the therapies primes the Mature DCs with the full-length iLRP recombinantly produced in bacterial cultures [57].

Several natural molecules showed promising results in targeting LRP/LR in cancer. One such molecule is EGCG as mentioned above, has been found to directly bind to LRP/LR with high specificity (Figure 5) [131]. EGCG is one of many polyphenols used to inhibit carcinogenesis. In addition to EGCG being able to hamper cancer progression, EGCG has apoptotic, anti-proliferative, and anti-matrix metalloproteinase properties [99,132]. Interestingly, Tachibana et al (2004) aimed to identify agents through which EGCG would be able to inhibit cell proliferation. Using a subtraction cloning method involving the construction of cDNA libraries, the authors were able to isolate one target that allowed EGCG to bind to the surfaces of cells. This target was LRP/LR, which was able to bind to EGCG with a Kd value of 39.9 nM [131]. As a result, a supposed binding site for EGCG was identified which corresponded to the area between the 161 and 170 residues of LRP/LR [133]. Moreover, Yu et al (2009) used a virtual docking program (MVD) to identify a binding site for EGCG with the identical sequence of the laminin peptide. This sequence corresponded to the of β1 chain sequence of 67LR [134]. This LRP/LR-EGCG interaction allowed for EGCG to bind to the cell surface in human lung cancer (A549) cells. These lung cancer cells were used to investigate whether LRP/LR causes EGCG-mediated cell growth inhibition. It was concluded that LRP/LR is able to mediate the anti-cancer activity of EGCG, resulting in an effect similar to that of a tumor suppressor gene.

Another study used nanoparticles to improve the interaction between EGCG and LRP/LR and they found that the nanoparticles were able to bind to LRP/LR with high affinity as well as improve the anti-cancer activity of EGCG, without affecting normal cells [135]. Together, these studies ultimately not only provided a novel role for LRP/LR as an anti-cancer mediator through its interaction with EGCG, but also a new avenue in preventing and treating cancer.

Another natural molecule, Coptisine, an isoquinoline alkaloid isolated from *Coptis chinensis*, was found to specifically activate external/death receptor pathway of apoptosis in hepatocellular carcinoma (HCC) through 67 kDa LR (Figure 5) [136]. The study found that Coptisine was able to promote apoptosis in cells by increasing LR, and in the event that LR expression was inhibited via anti-67 LR antibodies and sh67LR lentiviruses, coptisine-induced apoptosis and reductions in cellular viability was hampered. Here, the 67 kDa LR acts as a specific death receptor in cancer cells overexpressing LR. Interestingly, both Coptisine and EGCG induce apoptosis through the same pathway (PKCδ/aSMase) suggesting that they both bind to a similar site of action on 67 kDa LR which might be specific for cell-death signaling. If the precise site can be located, it may be possible to design potent therapeutics to target the 67 LR as a death receptor in cancer (Figure 7).

4.1.3. Small interfering RNAs (siRNAs)

The use of RNA interference (RNAi) to down-regulate or to silence genes involved in cancer development is becoming an increasingly popular route regarding cancer treatment. RNA silencing is used by double-stranded RNA molecules called siRNAs which aid in degrading sequences found in the target mRNA as well as those sequences that are complimentary to it [137]. These double-stranded molecules play key roles in inducing and activating the RNAi process since siRNAs are able to cleave inducer molecules as well as degrade target mRNA [137]. Thus, most siRNAs aim to interfere with metastasis and angiogenesis of cancer cells.

Since siRNA technology is a fairly new route of targeting cancer cells, most siRNA-based drugs are only at the clinical stage. This is due to a serious obstacle in siRNA development involving controlled and effective delivery of siRNAs *in vivo*. For siRNAs to work effectively, they have to first undergo

degradation by nucleases followed by hydrolysis in lysosomes [138]. Therefore, several siRNAs require chemical modification or highly specific delivery systems in order to improve their efficiency.

There are several advantages of using siRNAs including their unrestricted target specificity and high efficacy. However, there are also disadvantages to this method including the instability of siRNAs at physiological conditions and thus, the need for efficient packaging into vesicles for effective delivery to cancer cells. Nanoparticles protect the siRNA from degradation within the circulatory system and represent an effective delivery method to targeted tumor cells [138]. One such example is the siRNA known as Atu027, which uses lipid-based nanoparticles to down-regulate the expression of protein kinase N3 (PKN3), an important protein that regulates growth, adhesion and survival in the PI3K cell pathway. Animal studies showed that Atu027effectively down-regulated the PKN3 gene, ultimately inhibiting metastasis [139]. Further clinical studies performed in 2014, revealed that Atu027 was able to treat several advanced solid tumors and 41% of patients were found to have no further tumor progression post treatment (Clinical trial: NCT00938574) [140,141]. A recent patent (WO2016/177343) filed by Zhang et al. in 2016 disclosed an anti-siRNA expression vector incorporated in an efficient carrier for targeted delivery [142]. The patent focuses on simultaneously targeting EGFR and K-RAS, a member of the RAS family known to play a role in several signaling pathways involved in tumor formation, proliferation and migration [142]. Zhang et al believe that by inhibiting the pathways of both proteins through siRNA/miRNA technology, existing EGFR-targeted drugs will have an increased therapeutic effect on cancer patients with K-RAS mutations [142]. Furthermore, the patent describes the design of a pharmaceutical formulation containing an expression vector for expressing the siRNA together with a pharmaceutically accepted carrier for the siRNA to be effectively transported. Thus, in conjunction with a delivery system, the use of siRNA technology is a promising avenue for cancer therapy.

4.1.4. siRNAs targeted against LRP/LR

Besides antibodies and SMIs, siRNAs have also become popular agents to target LRP/LR. Moodley and Weiss (2014) showed that siRNA-mediated knockdown of LRP/LR significantly decreased the viability of in lung (A549) and cervical (HeLa) tumor cells [70,143] through the induction of apoptosis, via increased levels of caspase-3. This result was confirmed in several cancer cell lines including breast (MDA-MB231) [54], pancreatic (AsPc-1) [53], neuroblastoma (IMR-32) [53] early (SW-480) and late (DLD-1) stage colorectal carcinoma [55] as well as early (A375) and late (A375SM) stage malignant melanoma [52] cells. siRNAs directed against 37 kDa LRP were patented in WO 2013/042053 A2 [144]. Another research group also used siRNAs to target LRP/LR and they found that by down-regulating the receptor, the G_1 phase of the cell cycle is arrested in fibrosarcoma (HT1080) cells [145] (Figure 4). It is known that protein translation is needed for cells to progress from the G_1 phase to the S phase of the cell cycle. By halting the G_1 phase, translation is also inhibited thus exposing the ribosomal function of LRP/LR to regulate cell growth and consequently, tumor progression.

Due to the promising *in vitro* results, LRP/LR knockdown strategies were performed *in vivo*. However, as mentioned above, siRNAs are limited due to the lack of an effective delivery system to target tumors. Vectors and nanoparticles are routinely used to attain effective and specific targeting *in vivo*. Thus, Meruelo et al (2010) aimed to investigate the role of LRP/LR role in tumor-cell proliferation *in vivo* using both lentiviral and Sindbis viral vectors – since LRP/LR acts as a receptor for viruses (WO/2002/076468) [145,146]. The generated Sindbis/Lenti pseudotype vectors were able to target and impede the growth of ovarian cancer developing mice by 50% through binding and down-regulating LRP/LR, which supports siRNAs targeting LRP/LR as potential anti-cancer therapeutic agents.

siRNAs used to target LRP/LR have been shown to reduce tumourigenesis through the induction of apoptosis [52–55] and inhibition of telomerase activity [70,143]. Furthermore, monoclonal antibodies directed against LRP/LR are known to inhibit angiogenesis [51] and metastasis [6,43–46,63] thus, combining siRNAs and antibodies targeted against LRP/LR would be a potential therapeutic option for the treatment of cancer. Combination treatment would be highly beneficial as siRNAs could be tailored to target various oncogenic molecules with tumor specifications all within one antibody construct. Ultimately, LRP/LR antibody-siRNA conjugates may be able to provide powerful, invaluable therapeutics for cancer therapy (Figure 7).

4.2. Immunotherapeutic strategies

4.2.1. Monoclonal antibodies

Other promising therapeutic molecules that could target the LRP/LR include monoclonal antibodies. Monoclonal antibodies have become a prominent source of treatment as they act mainly through blocking specific functions or modifying signaling pathways involving a target molecule [147]. The Food and Drug Administration (FDA) has approved several antibodies for treating solid and hematological cancers. Well-known antibodies such as Iressa® and Erbitux® target the epidermal growth factor receptor (EGFR) while Avastin® targets vascular endothelial growth factor (VEGF), and are used for the treatment of colorectal carcinoma and non-small cell lung cancer. In addition, Yervoy® aids in treating melanoma by targeting the receptor, CTLA-4, known to down-regulate the immune system, thereby allowing tumor cells to thrive [148]. Adcetris®, Zevalin® and Rituxan® are all used for treating non-Hodgkin's lymphoma by selectively targeting cancerous cells that express the CD20 [149,150] or CD30 antigens [151], biological markers of non-Hodgkin's lymphoma. Finally, Herceptin® and Kadcyla® antibodies are commonly used by binding to the HER2 receptor and inhibiting the receptor's signaling, ultimately leading to the inhibition of cell signaling pathways for the treatment of breast cancer [152].

There are several ways in which antibodies can exert their function; for example, Alemtuzumab® which is used to treat chronic lymphocytic leukemia (CLL), binds to the CD52 antigen found on lymphocytes and attacks them [153] while Trastuzumab®, an antibody targeted against HER2 on breast cancer cells, attaches to and blocks antigens on tumor cells [154]. Moreover, a recently patented study (WO/2013/170263) showed that by using bispecific anti-ErbB2/anti-ErbB3

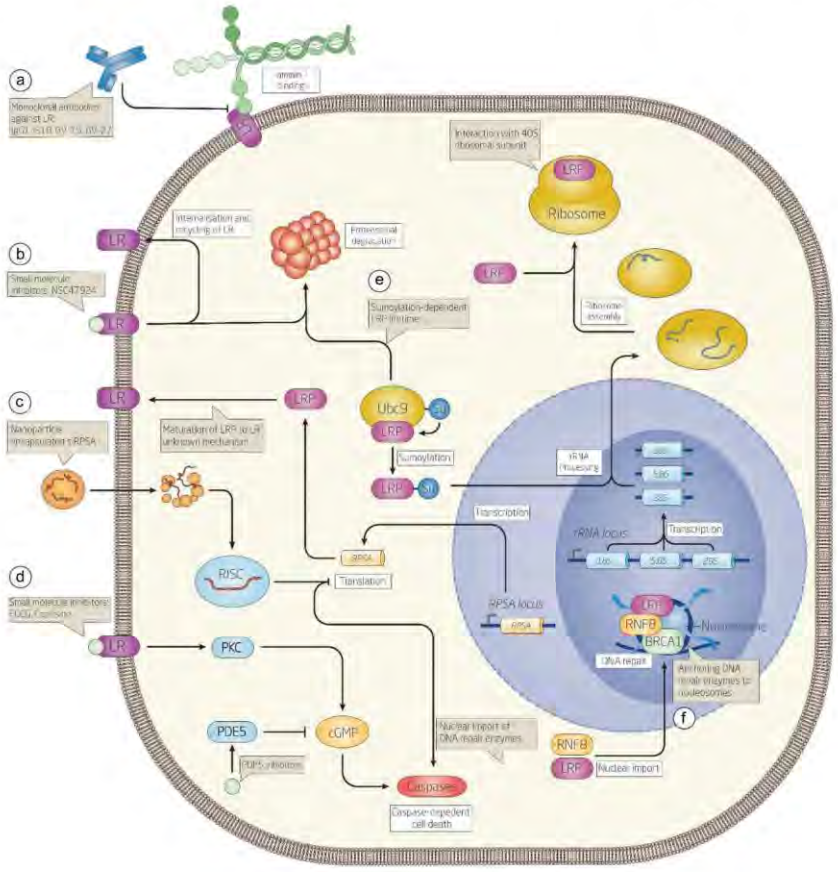

Figure 7. Summary of current and potential drug-based therapeutic strategies targeting LRP/LR in cancer. The *RPSA* gene encodes the immature 37 kDa laminin receptor precursor (LRP) that is processed, by an unknown mechanism, into the mature 67 kDa form (LR) [22]. (a) Monoclonal antibodies such as IgG1-iS18, BV15, BV-27 have been shown to exert powerful effects in significantly preventing cell adhesion, resulting in an anti-proliferative action on cancer cells [6,42–45]. (b) and (d) Small inhibitor molecules, including the synthetic molecule NSC47924 has been shown to decrease the adhesive and invasive potential of cancer cells, while plant-derived inhibitors such as EGCG and Coptesine result in apoptosis of cancer cells [129,136]. (c) siRNA-mediated knock-down of LRP/LR has a significant effect on the viability of tumor cells. By encapsulating siRNAs, efficient delivery to the target cells can be achieved [52–54]. (e) Presented here are potential avenues for targeting 37 kDa LRP in anti-cancer therapeutics. Blockage of the 37 kDa LRP sumoylation sites may prove to be a potent anti-cancer therapy [176]. (f) Finally, disruption of the 37 kDa LRP-RNF8 interaction would prevent the loading of RNF8 in the nucleolus resulting in impaired or absent DNA-damage response in the cancer cell [177].

antibodies, late stage HER2 positive tumors can be treated and it is said to show promising results in patients [155].

Antibodies can also be conjugated to nanoparticles, radioactive particles or chemotherapeutic agents, allowing the antibody to enter the cancer cells directly [156]. One such antibody known as Brentuximab Vedotin® known to treat Hodgkin lymphoma, is able to target the CD30 antigen on lymphocytes by first attaching to a chemotherapy drug [157]. In addition, a patented study (WO/2019/011918) showed that by combining the pro-apoptotic antibody anti-CD19 with the chemotherapeutic agent Rituxan®, the levels of diffuse large B cells lymphoma (DLBCL) were found to significantly decrease [158]. The advantages of monoclonal antibodies as therapeutic agents are that they have a long half-life in the body, especially if they are full-length antibodies, and their intravenous administration guarantee a direct and rapid action. However, Disadvantages of monoclonal antibodies include their high cost to be produced and the fact that they are only able to act on the cell surface [109].

4.2.2. *Anti-LRP/LR specific antibodies*

As mentioned previously, a high correlation between LRP/LR levels and adhesive and invasive potential, key hallmarks of cancer and metastasis, has been shown in several tumourigenic cell lines [6,42–46]. In 2004, a patent was filed (WO/2005/035580) stating that single-chain anti-LRP/LR specific antibody scFv iS18

may potentially be used for diagnosing and treating of cancer [159]. Another patent was filed in 2005, stating that SLAC-23, a monoclonal antibody, that is able to bind to the 37 kDa LRP, may be used to diagnose and treat cancer [160]. This was followed by a separate patent (WO/2006/105954) filed in 2006 which stated that LRP/LR-specific antibodies such as W3 have the ability to halt the angiogenic process, thus suggesting the possibility of using said antibodies as potential therapeutic tools for the treatment of cancer [161]. Patent WO/2006/105954 was filed in 2006 stating that the 37 kDa LRP or the 67 kDa LR could be targeted by an immunoglobulin antibody in order to aid diagnosing and treating cancer [161]. In 2008, a patent was filed claiming that by administering the IgG1-iS18 antibody targeting the 67 kDa LR, the proliferative capacity of tumourigenic cells was decreased thus, another approach in potentially treating cancer was made [162].

However, current research has been mainly focused on the effect of the monoclonal, anti-LRP/LR specific antibody IgG1-iS18. The antibody targets the LRP/LR-laminin-1 interaction, revealing a significant decrease in adhesive and invasive potential of several tumourigenic cell lines [42–46] (Figure 7). It was also found that upon comparison with a poorly invasive breast cancer cell line, MCF-7; the tumourigenic cell lines showed much higher total and cell surface levels of LRP/LR which correlated with the adhesive and invasive potential of these cells [42–46]. Furthermore, it was revealed that upon adhesion inhibition with IgG1-iS18, invasion was also hindered in several cancer cell lines [42,44–46]. The hindered effects seen on the adhesive and invasive potential of cancer cells upon treatment with IgG1-iS18, confirms that the LRP/LR-laminin-1 interaction is certainly needed for the two main steps in metastasis – adhesion to the basement membrane into the circulatory system. This was affirmed by several other studies showing that either high cell surface or total LRP/LR levels increase the LRP/LR-laminin-1 interaction, resulting in heightened adhesive and invasive potential, ultimately leading to increased aggressiveness in tumors [42,44–46]. Moreover, Vania et al (2016) showed that when early (SW-480) and late (DLD-1) stage colorectal cancer cells were compared to one another, there was a significant increase in total LRP levels in the late stage. As mentioned before, LRP/LR also plays a role in the cytosol where it helps with translation and protein synthesis [44]. Thus, these findings imply that with increased levels of total LRP/LR, tumor cells obtain increased levels of protein synthesis which are vital for processes involved in angiogenesis, tumor viability and metastasis.

Several studies have explored the LRP/LR-laminin-1 interaction using anti-LRP/LR tools including pentosan sulfates [163], transdominant negative mutants [60] and monoclonal antibodies [164]. These approaches confirmed that the reductions observed in the adhesive and invasive potentials of selected cancers were due to the inhibition of the LRP/LR-laminin-1 interaction. Moreover, it was suggested that the interaction also stimulates the activation of specific enzymatic substances such as type IV collagenase and matrix metalloproteinases, resulting in the proteolytic cleavage of the basal lamina, consequently aiding in invasion of tumor cells [165].

The exact mechanism IgG1-iS18 inhibits adhesion and invasion is however, not clearly understood. It is proposed that the two laminin binding domains on the 37-kDa/67-kDa laminin receptor LRP/LR (amino acid 160–180 and 205–229) may be directly blocked by the antibody or indirectly. This leads to conformational changes of the laminin binding domains, thereby changing the affinity of the binding domains for laminin.

Although the above-mentioned studies have demonstrated that the LRP/LR-laminin-1 interaction can be hindered in several cancer types through the use of the anti-LRP/LR specific antibody IgG1-iS18, it is imperative to investigate the antibody's effect on LRP/LR *in vivo*, using cancer mouse models. A previous study revealed a significant reduction in the growth of human fibrosarcoma (HT1080) tumor cells in the lungs of BALB/c *nu/nu* nude mice[166]. However, these findings were obtained using the P4G polyclonal antibody directed against the 37 kDa LRP which is only known to treat metastasis [166]. Since the LRP/LR-laminin receptor is greatly involved in the process of invasion and therefore metastasis, targeting LRP/LR with LRP/LR specific antibodies is a promising approach for combating cancer.

In addition to the above, a series of novel monoclonal antibodies targeting the C-terminal of OFA/iLRP have been patented (WO/2015/017113) [167,168]. Of the 12 antibodies two (BV-15 and BV-27) were assessed in syngeneic BALB/c mice intravenously injected with A20 B-cell leukemia or B16 melanoma cells. For both antibodies a small but significant decrease in the A20 blood tumor load and number of liver tumors (A20) or lung tumors (B16) were observed (Figure 7). Like IgG1-iS18, BV-27 can prevent cell adhesion to laminin and shows potent anti-proliferative action on A20 cells *in vitro*. The mechanism by which BV-15 or BV-27 alters metastatic potential of A20 or B16 cells *in vivo* has yet to be determined.

4.2.3. Dendritic cell therapies

Interestingly, it has been found that several oncogenic tissues are enriched with an immunogenic proteoform of LRP/LR, OFA/iLRP, prompting investigations using OFA/iLRP in immunotherapies targeting both hematologic and epithelial derived malignancies [57]. For example, syngeneic BALB/c mice were immunized with liposomes doped with recombinant human OFA/iLRP prior to intravenous injection of fibrosarcoma cells. Treated mice showed a significant reduction in tumor attachment and growth [57]. Currently, two phase I clinical trials are being conducted for the use of OFA/iLRP as a cancer vaccine: the first (Clinical trial: NCT00715832) aims to assess the efficacy of the recombinant OFA/iLRP-liposomes as a vaccine against breast cancer; and the second (Clinical trial: NCT00879489) aims to make use of autologous dendritic cells pulsed with OFA/iLRP as an immunotherapy against metastatic breast cancer. Since the discovery of iLRP as a potent tumor-associated antigen (TAA), several novel anti-cancer immunotherapeutic approaches have been patented (Figure 6).

Modern immunotherapies use autologous dendritic cells (DC), antigen presenting immune cells capable of activating cytotoxic T-lymphocytes (CTL) and natural killer cells (NK), to initiate an immune response capable of locating and destroying tumor cells Autologous DC progenitor cells are harvested from the patient by apheresis and cultured with various inflammatory cytokines, alarmins and growth factors to form mature DCs [169]. The mature DCs are then loaded with an

appropriate antigen before being administered to the patient (Figure 6). Discovery of cancer specific antigens presents one of the major challenges to DC-based immunotherapy. An appropriate tumor-associated antigen (TAA) ideally must be expressed predominantly in tumor cells at high levels. One such antigen is the OFA/iLRP. Mapping of the immunogenic epitopes of OFA/iLRP revealed several peptide sequences located in both the N- and C-terminal domains with a high immunogenic activity [170]. Mice vaccinated with autologous murine DCs pulsed with intact OFA/iLRP showed that a combination of CTL-activating OFA/iLRP-derived peptides, or a combination of T_S cell-activating OFA/iLRP-derived peptides resulted in a significant decrease in the number and size of lung tumor colonies from MCA-1315 cells injected two weeks following vaccination [170]. The vaccine methodology from the study has been patented (WO/2011/006084) [171]. Rorher, Barsoum, and Coggin currently also hold the patent for use of OFA/iLRP derived peptides in cancer vaccines (WO/2004/012681) [172]. The success of the OFA/iLRP immunotherapy has encouraged a phase I clinical trial that is currently recruiting patients to assess the efficacy of autologous DCs pulsed with OFA/iLRP against metastatic breast cancer (Clinical trial: NCT00879489).

A similar immunological study utilizing healthy donor-derived DCs pulsed with a modified OFA/iLRP peptide (iLR-ASK-A2) induced polyclonal CTLs capable of specifically killing malignant cells endogenously overexpressing OFA/iLRP [173,174]. The CTLs had a high specificity for tumor cells presenting iLR-ASK-A2 in the HLA-A*0201 complex [173]. Siegal and Zeis (2011) have patented iLR-ASK-A2 for use in potential cancer immunotherapeutics (EP2330122A1) [175]. The modified peptide VLIENPADV, differs slightly from a naturally derived peptide VALENPADV, and appears to occur as a part of the natural processing of OFA/iLRP in PBMCs. The patent covers the peptide sequence; stereoisomers of the peptide; formulations for therapeutic applications of the peptide; T-cell receptors (TCR) capable of identifying cells expressing the peptide epitope on the cell surface; and CTLs capable of specifically killing malignant cells expressing the peptide. The polyclonal CTLs induced by patient-derived iLR-ASK-A2-pulsed PBMCs are capable of lysing tumor cells presenting the modified epitope with high specificity. Many of these peptide epitopes, that elicit an immune response, are found in LRP/LR. However, LRP/LR does not induce an immunogenic response suggesting that significant modification of LPR/LR is required to prevent cytotoxic immunogenicity seen in malignancies overexpressing immunogenic OFA/iLRP proteoform.

5. Potential avenues for targeting LRP/LR in cancer

From the afore-mentioned studies, it can be seen that LRP/LR plays several roles within the cell and therefore can be targeted in many different ways. Moreover, these studies have encouraged several potential avenues for targeting the receptor in ways which have not yet been explored. This section of the review will discuss potential approaches for targeting the receptor itself regarding the formation of the mature 67 kDa LR form as well as specific proteins that influence the function of LRP/LR directly or indirectly in tumourigenesis.

5.1. Sumoylation of 37 kDa LRP affects protein lifetime

Despite the extensive research on LRP/LR, there are still many avenues that can be explored to target the receptor. In particular, the formation of the mature 67 kDa LR has been of great interest. At first, sumoylation was thought to contribute to the maturation of 37 kDa LRP to LR, however due to the difficulty in obtaining tagged mature LR directly; evidence of the involvement of sumoylation in 37 kDa LRP maturation is inconclusive. Despite this, recent work has shown that sumoylation does play a significant role in regulating the life-time of 37 kDa LRP in the cell [176]. The sumoylation sites play antagonistic roles where sumoylation at K11 results in an increased 37 kDa LRP lifespan and targeted proteosomal degradation of 37 kDa LRP when K42 is sumoylated. In addition, sumoylation of several putative sumoylation sites is necessary for efficient processing of 18S, 45S, 47S and 5.8S rRNAs. This presents a novel two-pronged avenue of targeting 37 kDa LRP in a therapeutic capacity. By targeting the putative sumoylation sites it may be possible to control sumoylation of 37 kDa LRP thereby altering the life expectancy of 37 kDa LRP and potentially preventing its maturation to the oncogenic LR (Figure 7). Given this, the prevention of sumoylation may reduce rRNA processing hindering ribosomal assembly and subsequently protein synthesis rates in cancerous cells, ultimately leading to a reduction in these cancer cells.

5.2. 37 kDa LRP in DNA damage

As mentioned above, targeting proteins involved in enhancing LRP/LR's tumourigenic function may be a potential therapy against cancer. It is known that LRP/LR has several roles within the nucleus including maintaining nuclear structures. The receptor is also found to play a role in importing and anchoring the E3 protein-ubiquitin ligase RNF8, to nucleosomes in the nucleolus [177]. Studies have shown that RNF8 plays a central role in the DNA damage response to double stranded breaks (DSBs) [110]. In the absence of DNA damage, RNF8 is localized to the nucleosome. In the event of DNA damage, the DNA-damage repair enzyme BRCA1 (breast cancer susceptibility protein 1) translocates to nucleosomes bound by RNF8, which is stabilized by LRP/LR [177] (Figure 7). During the DNA damage response, RNF8 and BRCA1 are then released into the nucleoplasm where they coordinate DSB DNA damage response (DDR). Therefore, disrupting the interaction between LRP/LR and RNF8 in the cytosol, may result in an impaired DNA repair response in tumorigenic cells. Consequently, this may lead to DNA-damage induced cell-cycle arrest and apoptosis, ultimately aiding as a therapeutic approach for the treatment of cancer.

5.3. LRP/LR and Parkin

Another potential target in the treatment of cancer could be the parkin protein. Parkin plays a crucial role in Parkinson's Disease and, as reported more recently, in some cancer cases [178–181]. Parkin is an E3 ubiquitin ligase that together with the E1 ubiquitin-activating enzyme and the E2 ubiquitin-conjugating enzyme is involved in ubiquitination of a number of cytosolic proteins as

well as some outer mitochondrial membrane proteins following mitochondrial membrane depolarization [182]. Polyubiquitination of the mitochondria results in the recruitment of autophagosomes as well as proteasomes that initiate mitophagy and thus parkin plays a vital role in the degradation of damaged mitochondria [183]. However, when the parkin protein is mutated it results in a nonfunctional protein and thus leads to autosomal recessive juvenile parkinsonism (AR-JP) [184].

As mentioned before, LRP/LR plays a role in metastasis as well as the evasion of apoptosis. Although the main function of parkin is to induce mitophagy it also has many neuroprotective roles through the promotion of mitochondrial integrity, promoting degradation of toxic substances as well as regulating non-degradative ubiquitin signaling within cell viability and death pathways [185]. Furthermore, parkin is also involved in the repair of mildly damaged mitochondria as well as promotion of mitochondrial synthesis [186,187]. However, more recent studies have shown that parkin also influences several of the hallmarks of cancer including metastasis as well as apoptosis [188]. Parkin knockout mice unexpectedly developed macroscopic tumors, resulting in a higher probability of cancer development [189]. It has also been shown that some breast cancer cell lines have lower parkin mRNA and protein levels [178]. Moreover, when parkin was overexpressed in these breast cancer cell lines a decrease in proliferation and migration was seen [178]. These findings could be evidence that parkin is indeed a tumor suppressor gene. Another study, looking at the correlation between parkin expression and its impact of the survival of patients with advanced colorectal cancer found higher levels of the parkin protein within the deep colorectal tumor region, correlated with prolonged survival of these patients [190]. This seems to be further supported by a recent study which attempts to describe the mechanism behind the tumor-suppressing nature of the parkin protein. This study showed that cervical cancer (HeLa) cells overexpressing parkin revealed low levels of LRP/LR [191]. This decrease in LRP/LR expression could possibly explain the tumor suppressor activity of parkin observed in cancer cell lines [189]. Additionally, Poulogiannis et al (2010) showed an increase in the tumorigenesis of intestinal adenoma as well as an increase in the polyp multiplicity, when parkin heterozygous knockout mice were interbred with Apc^{Min} mice (spontaneously form benign tumors in the colon), supporting an important role of parkin in colorectal carcinoma [192]. Due to these factors, parkin could be used as a target for cancer treatment since by lowering LRP/LR levels. Moreover, the link between LRP/LR and Parkinson's Disease is covered in a recent patent in which it describes a novel compound, that includes LRP/LR, for the treatment and/or prevention of Parkinson's Disease (Provisional South Africa patent application NO. 2018/08025) [193].

Parkin has also been shown to ubiquitinate the hypoxia inducing factor 1 alpha (HIF-1α) and thus HIF-1α is degraded, which ultimately leads to an inhibition of migration as well as invasion in breast cancer cells [194]. Hypoxia is a common phenomenon in many different solid tumors, where oxygen supply is low in the center of these solid tumors [194]. HIF-1α is activated in these solid tumors in response to low oxygen levels and plays an important role in the survival of cancer by activation of over 100 genes that are vital in the survival of the tumor [194]. These genes include glucose metabolism, cell proliferation, migration as well as angiogenesis among others [194]. Therefore, not only does this provide evidence for the tumor-suppressing nature of parkin but this finding could also provide a possible mechanism of action.

Parkin can also promote apoptosis by sensitizing cells to chemotherapeutic drugs such as paclitaxel [195]. Paclitaxel is a chemotherapeutic drug that is used to treat many cancer types by targeting microtubules and thus preventing cell division [196]. Parkin was found to bind to microtubules where it increases and stabilizes the interaction between the microtubule and paclitaxel [195]. Additionally, researchers also demonstrated a correlation between parkin expression and paclitaxel sensitivity in breast cancer cells [195]. In summary, parkin seems to display numerous anti-tumorigenic effects, including the decrease in LRP/LR expression, therefore, elevated parkin levels may serve as an alternative therapeutic strategy for cancer treatment.

6. Current and potential drug-based therapeutic strategies targeting LRP/LR in cancer

In summary, the *RPSA* gene encodes for the immature 37 kDa laminin receptor precursor (LRP) that is processed, by an unknown mechanism, into the mature 67 kDa form (LR) [22]. Additionally, the presence of increased levels of the 67 kDa LR on the cell surface has been observed in several human cancers. Therefore, the maturation mechanism presents an avenue for further therapeutic target exploration. Although both proteoforms of LRP/LR are present on the cell surface, current drugs (sythentic and plant-derived) appear to selectively target the 67 kDa LR [124,176].

Recently, there have been several approaches to cancer therapeutics by targeting LRP/LR. In particular, existing synthetic SMIs such as NSC47924 are able to disrupt LRP/LR-laminin-1 activity by binding to laminin-1 and selectively targeting 67 kDa LR [129], resulting in decreased adhesive and invasive potential. Plant-derived SMIs such as EGCG and Coptisine have also been used for cancer therapy since the 67 kDa LR has been seen to act as a death receptor for these molecules [136]. Furthermore, it was found that combination therapy of EGCG and PDE5 inhibitors, such as Sildenafil, are able to amplify the cytotoxic effect of EGCG (Figure 7) resulting in apoptosis of cancer cells [136]. Alternatively, present siRNA studies have also shown favorable effects in cancer therapeutics since siRNA-mediated knockdown of LRP/LR has a significant effect on the viability of tumor cells, through the induction of apoptosis [52–54]. Lastly, current monoclonal antibodies including the IgG1-iS18 antibody have also shown promise in combating cancer, due to the antibody being able to target the LRP/LR-laminin-1 interaction, resulting in a significant decrease in adhesive and invasive potential of several tumourigenic cell lines [6,42–45]. In addition, BV-15 and BV-27 antibodies have been shown to significantly decrease blood tumor loads in multiple liver (A20) and lung (B16) tumors [167]. Moreover, as with IgG1-iS18, BV-27 can prevent cell adhesion and shows potent anti-proliferative action on cancer cells [167] (Figure 7). In addition to the promising nature of the above approaches, there are many other potential approaches in which LRP/LR can be targeted for the treatment of cancer.

One such approach is the sumoylation of the 37 kDa LRP form. As previously mentioned, sumoylation is key to the activity of 37 kDa LRP in rRNA processing and regulation of laminin-1 binding. Thus, by blocking the 37 kDa LRP sumoylation sites, it may prove to be a potent anti-cancer therapy [176]. Furthermore, the interaction between LRP/LR and RNF8 is has been found to be essential for DNA-damage repair. LRP/LR is responsible for importing and anchoring RNF8 in the nucleolus, allowing RNF8 to coordinate DNA-damage repair alongside BRCA1 [177]. Thus, disruption of the 37 kDa LRP-RNF8 interaction would prevent the loading of RNF8 in the nucleolus resulting in impaired or absent DNA-damage response in the cancer cell. Finally, the protein Parkin, known to play a crucial role in Parkinson's disease, has recently been found to function in cancer. Parkin not only regulates metastasis and apoptosis but also prevents tumor enhancement, thus the protein has been deemed as a tumor suppressor gene [178]. In particular, a study showed that overexpressed parkin resulted in reduced levels of LRP/LR in cervical (HeLa) cancer cells [191]. Therefore, increasing parkin levels in neoplastic cells might be an efficient and promising avenue for the treatment of cancer.

7. Conclusion

Despite efforts to provide millions of cancer patients with powerful, efficient and cost-effective and anti-cancer therapeutics, the mortality rate for both female and male cancer patients remains constant at approximately 50%. Thus, worldwide, there is an increasing need for therapeutic regimens for the efficient treatment of various cancer types. LRP/LR represents a tumor-promoting receptor molecule for many, if not all, cancer types. It favors metastatic processes, angiogenesis and telomere extension while impeding the apoptotic processes. Progress has been made in the development of SMIs, antibodies and siRNAs in the blocking or down-regulation of LRP/LR for the impediment of various tumor types. In vitro studies have shown great success, however more in vivo studies must first be completed for conclusions to be made.

Since cancer is a multifactorial process, further development of therapeutics that involve blocking or downregulating LRP/LR have shown great promise. Antibodies and siRNAs might be among the most powerful tools, especially when delivered through targeted delivery systems including nanotechnology. By targeting at least four processes crucial in cancer development, i.e. metastasis, angiogenesis, telomere extension and apoptotic inhibition, this may favor regimens targeting LRP/LR over other anti-neoplastic strategies. However, a combination of therapeutic approaches targeting both various pro-neoplastic molecules and LRP/LR may prove to be the most successful.

8. Expert opinion

Cancer is a worldwide burden affecting all races, genders and societies. The number of patients suffering from cancer is increasing despite decades of intensive research on cancer diagnostics and therapeutics. This is due to the complexity of multiple molecular mechanisms responsible for cancer development. Cancer therapy is much more demanding and, depending on the type of cancer and the state of detection, may nevertheless result in mortality, despite applied combination therapies such as chemotherapy, radiation therapy and surgery.

This review teaches us that any person can be diagnosed with any cancer type, but depending on lifestyle, ethnicity and gender as well as the geographic location, the patient might develop certain cancers over others. Regular smokers come to mind, as they have a far greater risk of developing lung, esophageal and oral cancers. Similarly, when broken down by sex, 24% of all female cancer patients suffer from breast cancer and 22% of males from lung cancer. Cancer statistics also teach us that the mortality rates in males and females are roughly 50%. Due to these alarming statistics, there is a tremendous need for novel treatments to combat this disease. These shocking statistics clearly highlight the need for more cancer research, specifically in identifying more targets for the development of anti-neoplastic drugs. This review focuses on a promising target, the well-known 37 kDa/67 kDa laminin receptor, first identified in 1983. We learn that this receptor plays several roles in promoting cancer development. It supports metastasis, the key event for the spread of cancer cells throughout the body, but also supports angiogenesis, blocks apoptosis and – as recently identified – enhances telomerase activity resulting in extended telomeres supporting uncontrolled cell proliferation and tumor progression. The receptor seems to support many if not all cancer types. This identifies it as a possible target for the efficient impediment and control of various types of cancer.

Several research groups around the world have identified LRP/LR as a promising target for cancer therapy and have developed drugs for the inhibition or downregulation of the receptor. A series of SMIs, well known to regulate RTKs and apoptosis associated proteins such as p21, survivin and Bcl-2 family members, have been developed for LRP/LR impediment, which resulted in reduced metastatic potential and increased apoptotic induction for some cancer types. Antibodies might still be the most potent family of cancer therapeutics. We learn that many of them are patented and on the market for treatment of specific cancer types. Most of them target RTKs, while others are directed against cell surface antigens, which promote cell proliferation. LRP/LR-specific antibodies have been reported and patented for metastatic and angiogenic impediment. Some antibodies target OFA/iLRP, a proteoform of LRP, which was similarly detected in high levels in tumorigenic tissues. OFA/iLRP was recently recommended and patented for immunotherapeutic strategies. Two phase 1 clinical trials were reported using OFA/iLRP for vaccination and immunotherapy. A series of peptides encompassing sequences of OFA/iLRP have been patented as possible immunotherapeutics.

siRNAs are well-known for knocking down proteins supporting cancer development. LRP/LR is a prominent target for siRNA-mediated knockdown and a series of studies resulted in the impediment of cell proliferation through the induction of apoptosis in various cancer cell lines. Some siRNAs were effective *in vivo*, however, clinical studies engaging siRNAs for LRP/LR knockdown have not yet been reported. One patented and published study reported a decrease in telomerase activity after siRNA-mediated LRP knock-down in breast cancer cells, which extends the effect of siRNAs targeting LRP/LR. Impediment of

telomerase activity resulting in telomere shortening is in our opinion a key strategy to combat cancer. siRNA-mediated LRP/LR knockdown attacks cancer on four levels: blockage of metastasis and angiogenesis, induction of apoptosis as well as the impediment of telomere extension. Therefore, targeted delivery of siRNAs, possibly through nanotechnology, may result in a high success rate for therapy of various cancer types.

In this review we learn about three alternative avenues for cancer impediment through LRP/LR modification. In cases where 37 kDa LRP becomes sumoylated, the impediment of sumoylation might shorten the lifetime of 37 kDa LRP, which is beneficial for tumor development. In this context, we believe that more research is needed to elucidate the 37 kDa/67 kDa polymorphism of the receptor, since it is still unclear how 67 kDa LR is formed from the primary 37 kDa LRP translation product. Secondly, a disruption of the LRP/RNF8 interaction in the cytosol may result in a hampered DNA repair response in neoplastic cells and consequently induce cell cycle arrest and apoptosis through the resultant DNA damage. Thirdly, and this is of particular interest, LRP/LR could link cancer to neurodegenerative diseases, especially Parkinson's disease through the well-known tumor suppressor parkin. Parkin, in its non-mutated form, is neuroprotective by supporting mitochondrial integrity, promoting degradation of toxic substances and regulating ubiquitin signaling within cell viability and death pathways. Parkin also regulates metastasis and apoptosis and prevents tumor progression. One possible reason for this could be that high levels of the anti-carcinogenic parkin seem to reduce the levels of pro-neoplastic LRP/LR in some cancer types. We therefore believe that increasing parkin levels in neuronal and neoplastic cells might be an efficient and promising avenue for patients suffering either from cancer and/or Parkinson's Disease. Thus, it can be seen that there are several agents targeting LRP/LR, which may provide powerful, potential therapeutic approaches to aid the fight against cancer.

Author contributions

L Vania: leading author on review, wrote the majority and contributed to several of the sections of the review. She was involved in conceptualizing the figures and edited the review. G Morris: wrote the sections 'Structure and Function of LRP/LR', 'Oncofoetal antigen/immature laminin receptor protein' 'Dendritic cell therapies', and some sections for 'SMIs targeting LRP' and 'Potential avenues for targeting LRP/LR in cancer'. He constructed the figures. TC Otgaar: contributed to the 'LRP/LR and Telomerase' section and edited the review. MJ Bignoux: contributed to the 'LRP and Telomerase' section. M Bernert: edited the review. J Burns: wrote the 'LRP and Parkin' section. A Gabathuse: provided a list of existing patents. E Singh: wrote the 'Epidemiology' section. E Ferreira: edited the review. SFT Weiss: wrote with J Burns the LRP and Parkin section. He wrote the 'Conclusion' and 'Expert Opinion' sections, and edited the review. All authors finally approved the version to be published; all authors agree to be accountable for all aspects of the work.

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Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Anti-LRP/LR–Specific Antibody IgG1-iS18 Significantly Impedes Adhesion and Invasion in Early- and Late-Stage Colorectal Carcinoma Cells

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Cancer is a highly complex disease that has become one of the leading causes of death globally. Metastasis, a major cause of cancer deaths, requires two crucial events, adhesion and invasion. The 37kDa/67kDa laminin receptor (laminin receptor precursor/high-affinity laminin receptor (LRP/LR)) enhances these two steps, consequently aiding in cancer progression. In this study, the role of LRP/LR in adhesion and invasion of early-stage (SW-480 and HT-29) and late-stage (DLD-1) colorectal cancer cells was investigated. Western blotting revealed that early- and late-stage colorectal cancer cells contained significantly higher total LRP/LR levels compared with poorly invasive MCF-7 breast cancer control cells. Flow cytometry revealed that both stages of colorectal cancer displayed significantly higher cell surface LRP/LR levels. Furthermore, upon treatment of colorectal cancer cells with the anti-LRP/LR–specific antibody IgG1-iS18, adhesion to laminin-1 was significantly reduced in both stages. Each stage's invasive potential was determined using the Matrigel™ invasion assay, showing that invasion was significantly impeded in both colorectal cancer stages when the cells were incubated with IgG1-iS18. In addition, Pearson's correlation coefficients propose that both total and cell surface LRP/LR levels are directly proportional to the adhesive and invasive potential of both stages of colorectal cancer. Hence, these findings indicate potential for use of the IgG1-iS18 antibody as a promising therapeutic tool for colorectal cancer patients at both stages.

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INTRODUCTION

Cancer, a highly complex disease, has become one of the leading causes of death globally. The World Health Organization predicts a 75% increase in total cancer cases worldwide by the year 2030 (1). More relevantly, South Africa ranks 50th in highest cancer incidences, and a recent study suggests that South Africa could face a 78% increase in the number of cancer cases by 2030 (2).

The present study focuses on colorectal cancer, the third most common cancer type, with over 1.4 million new cases diagnosed in 2012, including 600,000 deaths (http://www.wcrf.org/int/cancer-facts-figures/worldwide-data). Untreated colorectal cancer is the second most fatal type after lung cancer, yet if diagnosed in its early stages, it can be effectively treated (2). Colorectal cancer can be classified into four primary stages: early (stage I), middle (stages II and III) and late (stage IV), which results in metastasis.

According to Hanahan and Weinberg (3), there are several well-known hallmarks of cancer. These include activation of growth pathways, suppression of growth-inhibiting pathways, inhibition of apoptosis, enhancement of angiogenesis, and tissue invasion and metastasis, the latter being the focus of the present study. It has also been proven that cancerous cells are able to adhere to and invade secondary sites through the mediation of integrin and nonintegrin receptors (4). More specifically, the nonintegrin receptor 37kDa laminin receptor precursor/67kDa high-affinity laminin receptor (LRP/LR) has been shown to be notably overexpressed in various cancer types (4). This overexpression is found to have a direct correlation to the degree of adhesive and invasive potential of several cancer types (5).

LRP/LR is a nonintegrin cell surface receptor located in the extracellular matrix of mammalian cells (6,7). While LRP/LR predominantly functions as a transmembrane receptor (8), it has also been found in the nucleus, where it interacts with histones and chromatin (9), as well as in the cytosol, where it aids in translation and ribosomal

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biogenesis (10). Its physiological functions include cellular growth, adhesion, invasion, movement and viability (10). LRP/LR has been found to be a major contributor to the pathogenesis of neoplastic cancers (10), angiogenesis enhancement (12), prion disorders (13–15) and neurodegenerative diseases such as Alzheimer's disease (16–20). In addition, upregulation of the receptor has been seen to be implicated in telomerase activity (21). Research has shown that LRP-mRNA encodes for the 37kDa laminin receptor precursor, which is the precursor protein for the 67kDa high-affinity laminin receptor (22). However, the exact mechanism by which the 67kDa LR is formed is not known.

When LRP/LR is located on the cell surface, it is known to aid in organization of the basement membrane (22). Furthermore, it has been found that LRP/LR exhibits a high affinity for laminin-1, an essential element of the basement membrane (22). Laminin-1 is a noncollagenous, heterotrimeric glycoprotein that is able to bind to the extracellular matrix (ECM) (23). Therefore, laminin-1 functions as a key player in enhancing biological processes such as cell adhesion, homing (24), polarity, migration, proliferation, differentiation (25) and neutrite outgrowth (26). Research has shown that laminin-1 also enhances the invasive phenotype of cancerous cells, promoting tumor metastasis (27). Overexpression of LRP/LR on tumorigenic cell surfaces is a result of its increased interaction with laminin-1 (5). This increased interaction subsequently leads to increased adhesion, followed by activation of Type IV collagenase, leading to degradation of the basement membrane and invasion by the tumorigenic cells (28). Overexpression of LRP/LR, when compared with noninvasive tumorigenic cells, has been observed in various invasive tumors, including pancreatic cancer cells (29); colon, lung, prostate and cervical cancer cells (30); esophageal and breast cancer cells (31); and liver cancer cells (8).

Since the interaction between LRP/LR and laminin-1 is directly proportional to the degree of metastasis, inhibiting this interaction may serve as a crucial mechanism in treating the progression of metastatic cancer. Additionally, it has been found that IgG1-iS18, the full-length anti-LRP/LR–specific antibody, exhibits a significant effect on hampering the LRP/LR-laminin-1 interaction in various cancers, including early-stage colorectal cancer (30).

Thus, by applying the anti-LRP/LR–specific antibody IgG1-iS18 to early- and late-stage colorectal cancer cells, the stage where the antibody is most effective can be determined. Due to the rise in prevalence and mortality rate of colorectal cancer, it is crucial to develop a novel treatment strategy. Hence, the present study aimed to investigate the capacity of IgG1-iS18 to hamper the adhesive and invasive potential in both stages of colorectal cancer, thus allowing IgG1-iS18 to serve as a promising therapeutic tool in the treatment of colorectal cancer.

MATERIALS AND METHODS

Cell Culture and Conditions

Early-stage human colorectal adenocarcinoma (SW 480), late-stage human colorectal adenocarcinoma (DLD-1) and human poorly invasive adenocarcinoma (MCF-7) cell lines were obtained from the American Tissue Culture Collection. Early-stage human colorectal adenocarcinoma (HT-29) was obtained from Fox Chase Cancer Centre. All three colorectal cancer cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM)/Hams F12 (1:1), while the MCF-7 cells were cultured using DMEM high glucose (4.5 g/L). Each medium was supplemented with 10% fetal calf serum and 1% penicillin/streptomycin, and the cells were incubated at 37°C in a 5% CO_2 humidified atmosphere.

Reagents and Antibodies

Matrigel™ used for cell invasion assays was derived from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma and was obtained from Corning Inc. Laminin-1 used for adhesion assays was obtained from Sigma-Aldrich. Monoclonal antibody IgG1-HD37 (Sigma-Aldrich) possessing the same format as primary antibody IgG1-iS18 is directed against CD19 on β-lymphocytes in humans. The full-length antibody IgG1-iS18 was recombinantly produced in a mammalian expression system as previously described by Zuber *et al.* (6).

Confocal Microscopy

Confocal microscopy was used for visualization and confirmation of LRP/LR on the cell surface using fluorescent-labeled antibodies. Cells were first fixed in 4% paraformaldehyde (PFA) in 1 × phosphate based saline (PBS) followed by numerous washes with PBS (Gibco). Thereafter, the cells were blocked using 0.5% bovine serum albumin (BSA) in PBS and washed again in PBS wash, and the excess PBS was blotted off. Thereafter, antibody diluted in PBS and 0.5% BSA was added. The slides containing the coverslips were then incubated overnight at 4°C. Following incubation, three PBS/BSA washes were performed, after which fluorescein isothiocynate (FITC)-coupled secondary antibody (Abcam), which was diluted in PBS and 0.5% BSA, was added. This was followed by 1 h incubation in the dark. Thereafter, three more PBS/BSA washes took place, followed by the addition of Hoescht stain (Sigma-Aldrich). This was followed by a 5–10 min incubation and then rinsing in PBS. GelMount (Sigma-Aldrich) was used for mounting onto a new slide.

Fluorescence-Activated Cell Sorting

For evaluation and quantification of cell surface LRP/LR levels, flow cytometry was used. Cells were first detached using 1 × Trypsin/Versene, centrifuged at 150 × *g* for 10 min and resuspended and fixed with 1 mL of 4% PFA for 10 min. Thereafter, three cell suspensions were prepared by resuspending in 100 µL of PBS. One cell suspension was incubated with the primary antibody IgG1-iS18 (30 µg/mL), the second was incubated with the control antibody

IgG1-HD37 (30 µg/mL) and the third was resuspended in 1 × PBS only, serving as the negative control. This was followed by incubation overnight at 4°C. Thereafter, the cell pellets were washed three times in 300 µl of PBS through centrifugation at 2700 × *g* for 5 min. Following this, all cell pellets were resuspended in PBS and goat antihuman phycoerythrin (PE)-coupled secondary antibody (1:1000) (Abcam), before incubating both cell suspensions for 1 h in the dark. The cell suspensions were then washed three more times in PBS and evaluated using the BD Accuri C6 flow cytometer and software. Three biological replicates of the experiment were performed with different cell cultures.

SDS-PAGE and Western Blotting

Western blotting was used to detect total LRP/LR levels and compare LRP/LR levels at each stage of colorectal cancer. An amount of 11 µg of total protein was subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). The resulting separated proteins were transferred onto a polyvinylidine fluoride membrane (Pall) with 1 × transfer buffer (20% methanol in 25 mM Tris and 19.2 mM glycine) at 450 mV for 45 min. The membrane was then blocked with blocking buffer (1 × PBS-Tween [0.1% Tween in 1XPBS] with 3% BSA) for 1 h to prevent nonspecific binding of primary and secondary antibodies. The membrane was then probed with the primary anti-LRP/LR-specific antibody IgG1-iS18 (1:10000) for 1 h. Thereafter, the membrane was washed three times with 1 × PBS-Tween before incubating with goat antihuman horseradish peroxidase (HRP) secondary antibody (1:5000) (Abcam). As a loading control, 42-kDa β-actin was used, and was detected by means of the monoclonal anti-β-actin-peroxidase. Every experiment was performed in triplicate with different cell cultures.

Adhesion Assay

To evaluate the potential of colorectal cancer cell lines to adhere to the basement membrane, an *in vitro* adhesion assay was performed in 96-well plates coated with laminin-1 (10 µg/mL), with uncoated wells as negative controls. After coating the wells with laminin-1 for 1 h, the 96-well plate was incubated with 100 µl blocking solution containing 0.5% BSA in DMEM for 1 h. Thereafter, cells were suspended in serum-free culture media and treated with IgG1-iS18 (0.2 mg/mL), along with IgG1-HD37 (0.2 mg/mL) antibodies serving as negative control, to analyze the adhesive potential. The cells were then added to wells at a cell density of 4×10^5 cells/mL and incubated for 1 h. Following incubation, cells that did not attach were washed and removed with PBS, while attached cells were fixed in 50 µL of 4% PFA for 10 min, prior to staining with 100 µL of 0.1% crystal violet for 10 min. The dye was then extracted with 100 µL of 2% SDS and the absorbance was measured at 550 nm using an ELISA plate reader. This absorbance was an indicator of cells that were attached to laminin-1. Experiments were performed in triplicate with different cell cultures.

Invasion Assay

To evaluate the potential of the colorectal cancer cell lines to invade the basement membrane, an *in vitro* invasion assay was performed. Matrigel™ was diluted with cold serum-free cell culture medium (Thermo Scientific) and placed onto the upper chamber of a 24-well transwell plate (BD Falcon, 8 µm pore size). The gel was then incubated at 37°C for 4 h, until it was solidified. The cells were harvested and washed with culture medium containing 1% fetal calf serum at a cell density of 1×10^6 cells/mL. Following this, the cells were incubated with the IgG1-iS18 (0.2 mg/mL) antibody or the control IgG1-HD37 (0.2 mg/mL) antibody for 10 min. The cells were then loaded onto the transwell inserts, and the lower chamber was filled with 500 µl serum-free culture medium for the control and 500 µl culture medium containing 10% fetal calf serum for the experimental cell lines. After incubation at 37°C for 18 h, both the upper and lower media were removed. The noninvasive cells were then removed with a cotton swab and the invasive cells were fixed with 100 µL of 4% PFA and rinsed with 100 µL of PBS, followed by staining with 30 µL of 0.5% Toluidine blue for 2 min. The dye was then extracted with 100 µl of 1% SDS for 30–45 min. The absorbance of the resultant solutions was then obtained at 620 nm using an ELISA plate reader. Three biological repeats were performed.

Statistical Analysis

To analyze the data accurately, the two-tailed Student *t* test with a confidence interval of 95% was used, with *p* values greater than 0.05 considered not significant ($^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$). Error bars represent standard deviation. To measure the degree of association between LRP/LR levels and adhesive/invasive potential, Pearson's correlation coefficient was calculated. A positive coefficient shows direct proportionality between the two variables, while a negative coefficient implies inverse proportionality.

All supplementary materials are available online at www.molmed.org.

RESULTS

Early- and Late-Stage Colorectal Cancer Cells Exhibit LRP/LR on the Cell Surface

Research has shown that the interaction between laminin-1 and the 37kDa/67kDa laminin receptor precursor (LRP/LR) is essential for metastasis to occur (24,25). Thus, visualization of LRP/LR on the cell surface was needed to confirm that these tumorigenic cells exhibited LRP/LR on their cell surfaces. In Figure 1, it can be seen that all three colorectal cancer cell lines, as well as the poorly invasive breast cancer (MCF-7) cells posing as control, contained LRP/LR on the cell surface. All cells were nonpermeabilized, thus the green fluorescence depicted below indicates cell surface LRP/LR.

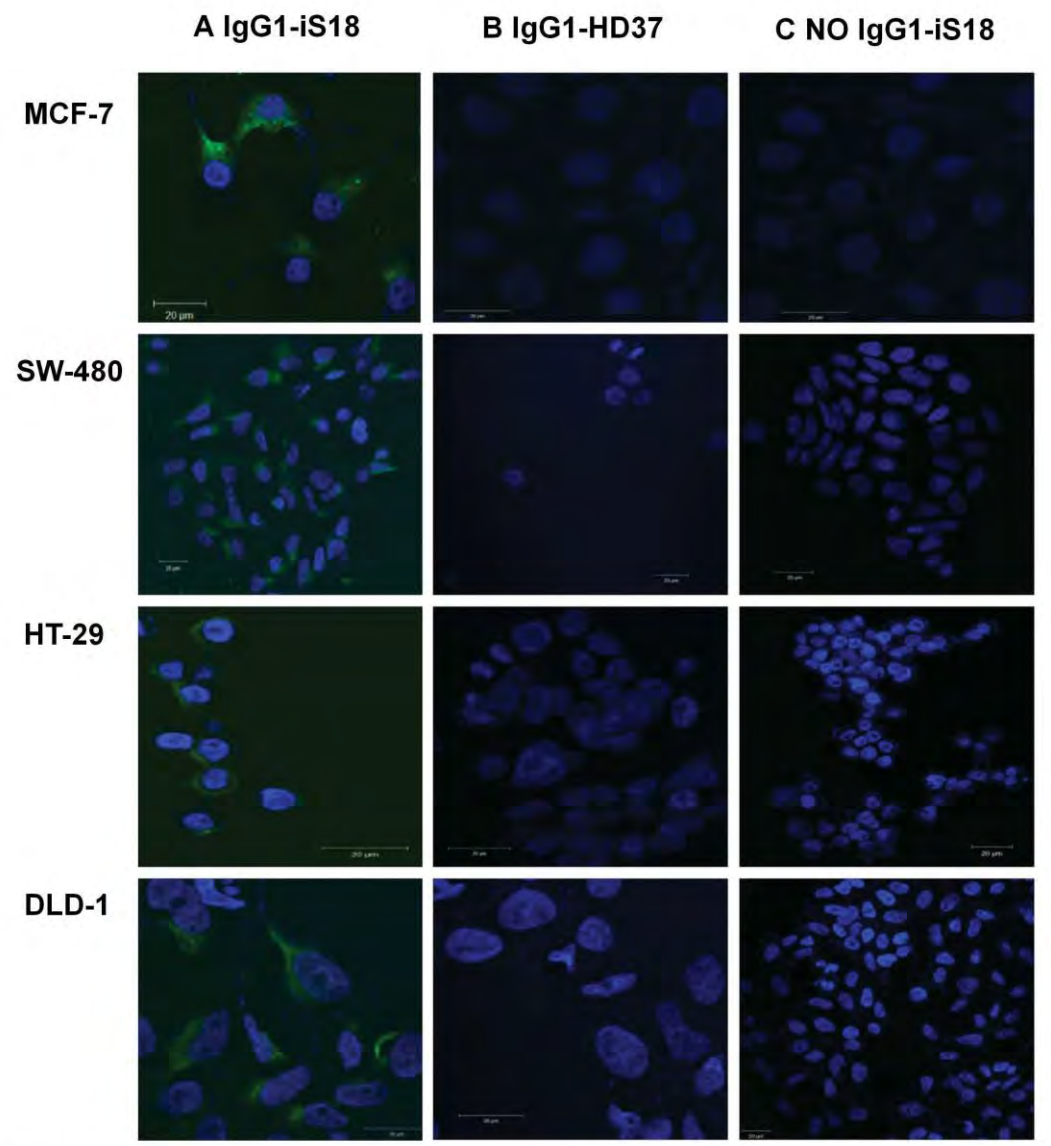

Figure 1. Visualization of LRP/LR on the surface of early-stage (SW-480 and HT-29) and late-stage (DLD-1) colorectal cancer and poorly invasive (MCF-7) tumorigenic cells. (A) Cells labeled with primary antibody IgG1-iS18 and FITC-coupled secondary antibody specific for IgG1-iS18 and found to fluoresce green. (B) Cells labeled with IgG1-HD37 antibody serving as negative control. (C) Cells labeled with only the FITC-coupled secondary antibody to confirm that there was no nonspecific binding coming from the secondary antibody.

Early- and Late-Stage Colorectal Cancer Cells Exhibit High Percentages Of LRP/LR and Significantly Greater LRP/LR Levels on the Cell Surface Compared to Poorly Invasive MCF-7 Controls

Despite confocal microscopy data confirming that LRP/LR is present on the cell surface of colorectal cancer cells at each stage, additional quantification of LRP/LR's being expressed was needed. This was done by flow cytometric analysis. All tumorigenic cell lines were treated alike to enable comparison of cell surface LRP/LR levels. In Figure 2, it can be seen that all three colorectal cancer cell lines, as well as the MCF-7 control cell line, exhibit high percentages of cells that possess LRP/LR on the cell surface within a particular population. This is indicated by the shift between the the black and blue peaks in each graph, depicting changes in fluorescent intensity, which is a result of staining the cell surfaces with anti-LRP/LR–specific antibody IgG1-iS18. As shown in Figure 2, SW-480, HT-29 and DLD-1 colorectal cancer cells display higher percentages (97.69%, 91.25% and 89.28%, respectively) of cells within a specific population that express LRP/LR on the cell surface, compared with the MCF-7 control cells (87.88%), with SW-480 displaying the highest amount.

Once the percentage of cells expressing LRP/LR on the cell surface was analyzed, further quantification of the definite amount of cell surface LRP/LR within a particular population of cells was done with flow cytometry. Each tumorigenic cell line was treated alike; that is, the same amount of cells (20,000) within specific populations were labeled with primary and secondary antibodies containing the same concentration (30 µg/mL) over the same duration. In Figure 3, it can be seen that both stages of colorectal cancer display significantly

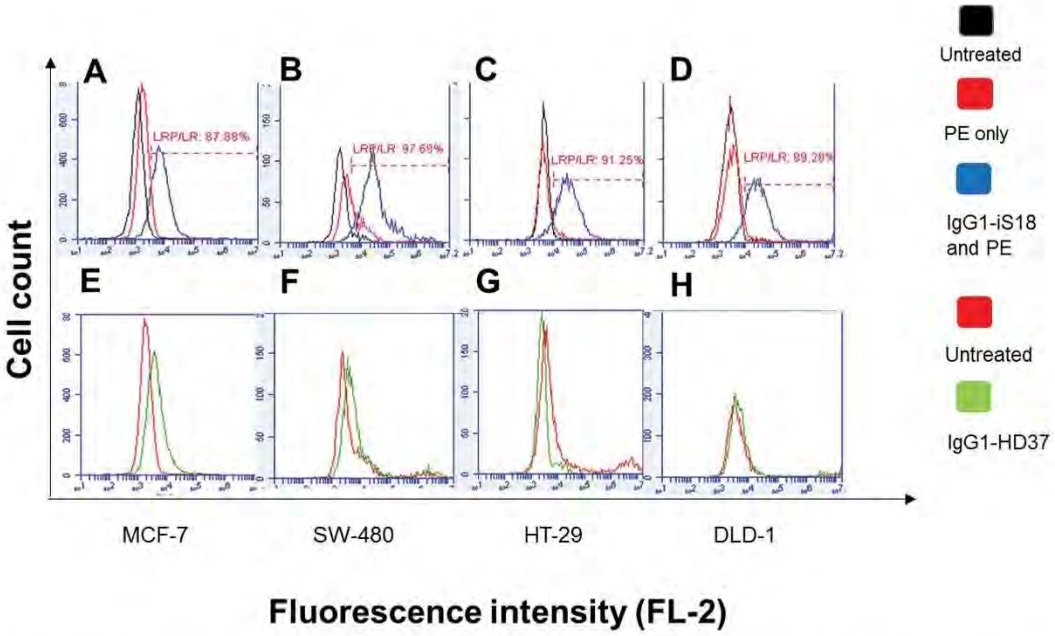

Figure 2. Detection of 37 kDa/67 kDa LRP/LR levels on the surfaces of tumorigenic cell lines. Cells were treated with IgG1-iS18 and IgG1-HD37 (control antibody). (A and E) represent MCF-7, (B and F) represent SW-480, (C and G) represent HT-29, and (D and H) represent DLD-1. In graphs (A, B, C and D) the black peak represents untreated cells, the red peak indicates cells labeled with goat antihuman phycoerythrin–coupled secondary antibody only and the blue peak represents cells labeled with anti-LRP–specific antibody IgG1-iS18 together with secondary antibody, both at a concentration of 30 µg/mL. In graphs (E, F, G and H) the red peak indicates untreated control to show no nonspecific binding from the secondary antibody; the green peak represents control antibody IgG1-HD37 together with the secondary antibody. These data represent experiments performed in triplicate using different cell cultures, with 20,000 cells counted per sample.

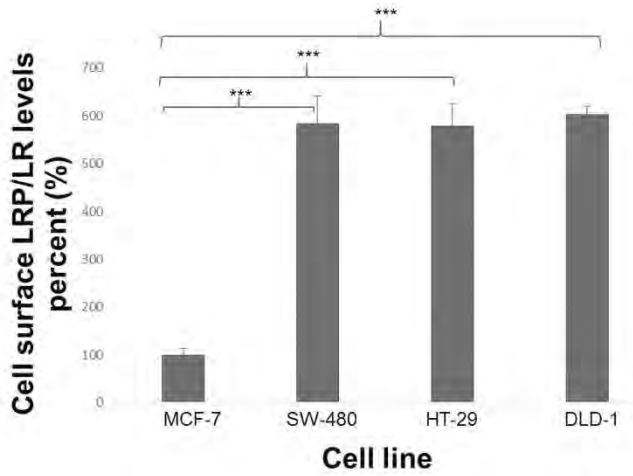

Figure 3. Quantification of cell surface LRP/LR levels on tumorigenic cell lines. Cells were labeled with IgG1-iS18 primary antibody and antihuman phycoerythrin–coupled secondary antibody. Thereafter, 20,000 cells from each tumorigenic cell line were analyzed and fluorescence intensities were used to indicate cell surface LRP/LR levels. These data represent experiments performed in triplicate using different cell cultures.

higher amounts of LRP/LR within a cell population compared with the MCF-7 cells. However, when comparing the colorectal cancer stages to one another, no significant difference was observed (see Supplementary Figure S1).

Late-Stage Colorectal Cancer Cells Exhibit Significantly Higher Total LPR/LR Levels Compared to MCF-7 Control Cells and Early-Stage Colorectal Cancer Cells

Due to the fact that LRP/LR is expressed in the nucleus and cytosol as well as on the cell surface, Western blotting was performed to investigate total LRP/LR levels. It must be noted that only detection of the 37kDa LRP could be achieved through use of the anti-LRP/LR–specific antibody IgG1-iS18. As seen in Figure 4, all tumorigenic cell lines could be seen to express LRP/LR. In addition, late-stage colorectal cancer cells revealed a significant three-and-a-half-fold increase in total LRP/LR levels compared with the MCF-7 cells. Furthermore, there was a significant two-fold increase in total LRP/LR levels between the two early stages when compared with the late stage. There was no significant difference between the two early-stage colorectal cancer cell lines, as expected.

IgG1-iS18 Significantly Impedes the Adhesive Potential of Early- and Late-Stage Colorectal Cancer Cells

Before tumorigenic cells can invade, resulting in metastasis, adhesion to the basement membrane by means of the laminin-1-LRP/LR interaction has to occur. It is this interaction that causes other important cellular interactions, facilitating degradation of the basement membrane. Hence, each cell line's adhesive potential was evaluated through the use of laminin-1–coated plates. Each tumorigenic cell line was incubated with IgG1-iS18 and IgG1-HD37 antibodies; absorbance readings of the resulting solutions depicted the quantity of cells attached to the plates. In Figure 5, it can be seen that all three colorectal cancer cell lines showed more adhesive potential than the poorly invasive MCF-7 control cells. It can also be seen that application of IgG1-iS18 to the cells resulted in significant hampering of the adhesive potential of all three cell lines. However, no reduction in the poorly invasive MCF-7 control was seen. Late-stage colorectal cancer showed the highest adhesive potential as well as the highest decrease in adhesive potential.

IgG1-iS18 Significantly Impedes Invasion of Matrigel™ by Early- and Late-Stage Colorectal Cancer Cells

Before a tumorigenic cell line becomes metastatic, it must undergo a vital process, invasion through the basement membrane. Thus, invasion assays were performed to evaluate the invasive potential of each tumorigenic cell line using the Matrigel™ invasion assay. The gel is known to mimic the basement membrane, since it contains vital components of the ECM, including laminin-1. All cell lines were treated with IgG1-iS18 and IgG1-HD37. In Figure 6, it can be seen that all three colorectal cancer cell lines were significantly more invasive compared with the poorly invasive MCF-7, with late-stage being the most invasive. In addition, once IgG1-iS18 was applied, the invasive potential of all three colorectal cancer cell lines was successfully impeded.

DISCUSSION

Previous studies have proven that numerous cancerous cell lines display overexpressed levels of 37kDa/67kDa LRP/LR (23–25). This suggests that the interaction between LRP/LR and laminin-1 may be essential for tumorigenic cells to transform into malignant cancers. Thus, by inhibiting this interaction, this may in turn inhibit two crucial events necessary for metastasis to occur, adhesion and invasion. Therefore, the purpose of this study was to investigate the role of LRP/LR in adhesion and invasion of early-stage (SW-480 and HT-29) and late-stage (DLD-1) colorectal cancer cells, and whether applying

Appendix C

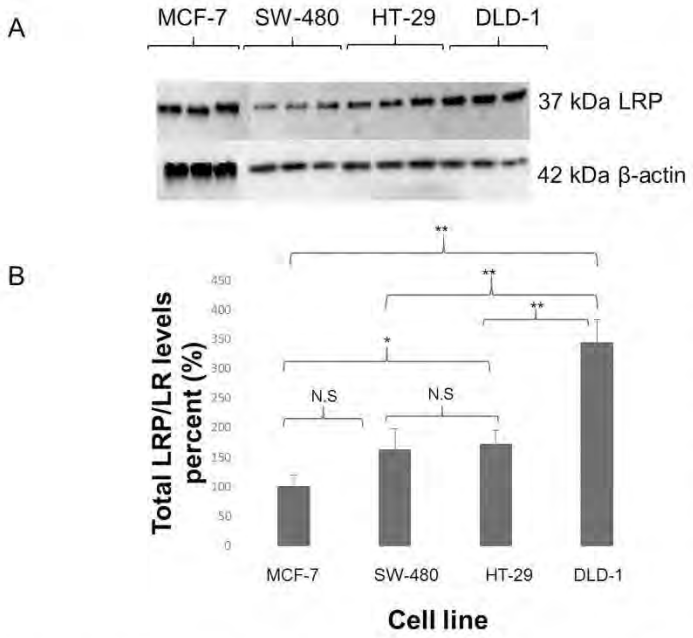

Figure 4. (A) Detection of total 37 kDa LRP levels in protein lysates of MCF-7, SW-480, HT-29 and DLD-1 cells. IgG1-iS18 was used as the primary antibody, with HRP-coupled secondary antibody; 42kDa β-actin was used as a loading control. (B) Densitometric analysis of total LRP/LR levels on SW-480, HT-29 and DLD-1 cell lines revealed that, compared to MCF-7, DLD-1 contained the most total LRP/LR, displaying a 3.5-fold increase. A significant two-fold increase in total LRP/LR levels between early- and late-stage colorectal cancer was also found. These data represent experiments performed in triplicate using different cell cultures.

IgG1-iS18, the anti-LRP/LR-specific antibody, would significantly impede their adhesive and invasive potential.

Confocal microscopy confirmed that all cell lines did display LRP/LR on their cell surfaces (Figure 1). However, this technique is only qualitative and does not reveal the levels of LRP/LR on the cell surface, thus further analysis using flow cytometry was performed.

Flow cytometry revealed that all three colorectal cancer cell lines displayed higher percentages of cells within a specific population that express cell surface LRP/LR compared with the MCF-7 control cells. Further analysis of each cell line's median fluorescence intensity revealed that all three displayed significantly higher amounts of LRP/LR within a cell population, compared with the control MCF-7 cells (Table 1). As mentioned above, LRP/LR is vital for processes such as cell adhesion, invasion, proliferation and migration, and therefore, since all three cell lines are known to be invasive (23), these high levels of cell surface LRP/LR may be contributing to the invasive potential of these cell lines.

Further analysis was done with Western blotting to evaluate total LRP/LR levels, which includes LRP/LR found in the nucleus and cytosol of cancerous cell lines. Analysis of the Western blots (Figure 4) confirmed that each tumorigenic cell line expressed 37kDa LRP. Densitometric analysis of the blots (Figure 4B) revealed a significant three-and-a-half-fold increase in total LRP/LR levels in late-stage colorectal cancer, while both early stages showed no significant difference in the total levels when compared with the poorly invasive MCF-7 cells. It was also revealed that when the stages of colorectal cancer were compared with one another, there was a significant two-fold increase in total LRP/LR levels between early stage and late stage (Figure 4B). Besides contributing to the key steps of metastasis, it is important to note that LRP/LR also plays crucial roles in the nucleus and cytosol. This includes nuclear structure maintenance and translational processes (10). Hence, with increased levels of total LRP/LR, cells acquire heightened levels of protein synthesis, which is necessary for processes that are involved in cancer progression and metastasis, such as tumor angiogenesis. As a result, the increased levels of total LRP/LR observed in DLD-1 compared with the other cell lines may contribute to its invasiveness, aiding in metastasis. The significant differences seen in cell surface and total LRP/LR levels reveal important roles in the progression of both stages of colorectal cancer, compared with the poorly invasive MCF-7 cells.

Analysis of the results obtained in this study suggests that IgG1-iS18 was able to cause a considerable decrease in the adhesive potential of all three colorectal cancer cell lines on laminin-1 as well as impede their invasive potential on Matrigel™. These results suggest that differences in the adhesive and invasive potential of these cell lines may be a consequence of differences in total LRP/LR levels as opposed to cell surface LRP/LR levels. Although there are significant differences in cell surface LRP/LR of these cell lines when compared with the MCF-7 cells, which are poorly

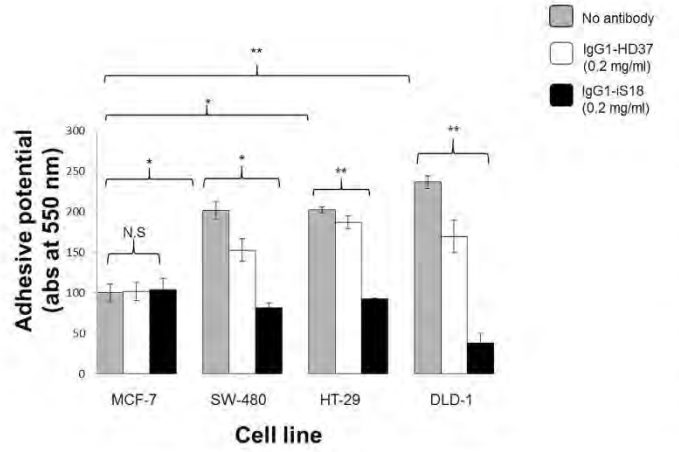

Figure 5. Adhesion of tumorigenic cells to laminin-1 investigated through adhesion assays. Each cell line's untreated cells were compared with MCF-7 untreated control cells to determine adhesive potential. *P* values show all three cell lines had increased adhesive potential compared to MCF-7. After treatment with the anti-LRP/LR–specific antibody IgG1-iS18, *P* values showed significantly decreased adhesive potential compared to MCF-7 control cells; SW-480 by 59%, HT-29 by 56% and DLD-1 by 84%. The control antibody IgG1-HD37 shows a small effect on each colorectal cancer cell line; however, statistical analysis indicates that IgG1-iS18 has a significantly greater effect (****p* = 0.001106 in SW-480, ****p* = 2.97188E-05 for HT-29 and ****p* = 0.000644 in DLD-1).

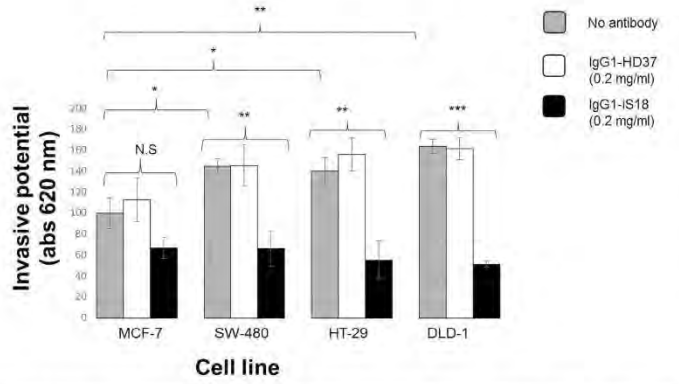

Figure 6. Invasion of ECM-like Matrigel™ by tumorigenic cell lines. Each cell line's untreated cells were compared with MCF-7 untreated control cells to determine invasive potential. *P* values show that all three colorectal cancer cell lines had an increase in invasive potential compared to MCF-7. After treatment with the anti-LRP/LR-specific antibody, *P* values showed significantly decreased adhesive potential compared to MCF-7 control cells: SW-480 by 54%, HT-29 by 60% and DLD-1 by 69%. The control antibody IgG1-HD37 did not affect the invasive potential of the tumorigenic cell lines as expected.

invasive, there is no difference when the colorectal cancer cell lines are compared with one another. However, Figure 4 shows that with total LRP/LR levels, there is a significant two-fold increase between early-stage (SW-480 and HT-29) and late-stage (DLD-1) colorectal cancer cells. Hence, the lower total LRP/LR levels observed for SW-480 and HT-29 (Figure 5) could account for the lower adhesive and invasive potential for these cells lines, while high levels of total LRP/LR leads to the higher adhesive and invasive potential of DLD-1 (Figure 6). This could also account for IgG1-iS18 being most effective in late-stage colorectal cancer, since this stage was observed to contain the highest levels of LRP/LR, which the antibody is targeted against. Evaluation of the correlation between cell surface and total LRP/LR levels and the adhesive and invasive potential of early- and late-stage colorectal cancer cells showed a substantially high correlation (Tables 2 and 3). This indicates a relationship between the two factors that is positive and directly proportional. The high correlation coefficients found for total and cell surface LRP/LR levels to the adhesive and invasive potential of each cell line suggests that cancer progression or aggressiveness is enhanced by increased levels of LRP/LR, which correlates with previous studies performed by Omar *et al.* (30).

The significant reduction in invasive potential of both stages of colorectal cancer upon treatment with IgG1-iS18 may be a result of inhibiting adhesion (Figure 5), since adhesion is known to be a prerequisite for invasion to occur, and both steps are needed to induce metastasis. This was affirmed by the high Pearson's correlation coefficients between the adhesive and invasive potential for all three cell lines (Tables 2 and 3). These results coincide with previous studies showing that the interaction between LRP/LR and laminin-1 is necessary for adhesion as well as secretion of important enzymes needed to degrade the basement membrane, consequently promoting invasion (4).

Table 1. Median fluorescence intensity (MFI) values used as an indicator of differential expression of LRP/LR on the surface of MCF-7, SW-480, HT-29 and DLD-1 cells.

Cell line	MFI of unlabelled cells (A)	MFI of cells stained with IgG1-iS18 and PE-coupled secondary antibody (B)	Difference in MFI values: (B) – (A)
MCF-7	3768.83	7308.67	3539.84
SW-480	3592.67	24244	20651.33
HT-29	11894.17	32351.83	20457.67
DLD-1	6713.67	28045.33	21331.67

All MFI values represent an average of experiments performed in triplicate.

Table 2. Pearson's correlation coefficients between cell surface LRP/LR levels and the adhesive and invasive potential of early-stage (SW-480 and HT-29) and late-stage (DLD-1) colorectal cancer cells.

Cell line	LRP/LR level to adhesive potential	LRP/LR level to invasive potential	Adhesive to invasive potential
SW-480	0.99	0.99	0.91
HT-29	0.96	0.85	0.98
DLD-1	0.99	0.89	0.94

Table 3. Pearson's correlation coefficients between total LRP/LR levels and the adhesive and invasive potential of early-stage (SW-480 and HT-29) and late-stage (DLD-1) colorectal cancer cells.

Cell line	LRP/LR level to adhesive potential	LRP/LR level to invasive potential	Adhesive to invasive potential
SW-480	0.99	0.98	0.91
HT-29	0.98	0.99	0.98
DLD-1	0.98	0.94	0.94

Numerous studies have also investigated the pathological potential of the LRP/LR–laminin-1 interaction through the use of various anti-LRP/LR tools such as monoclonal antibodies (26), transdominant negative mutants (27) and pentosan sulphates (5). These strategies all show significant decreases in the adhesive and invasive potential of particular carcinomas through blockage of the LRP/LR–laminin-1 interaction. Along with these findings, it is proposed that this interaction significantly promotes proteolytic cleavage of the basement membrane and consequently aids in invasion and metastasis. According to the findings of this study, the anti-LRP/LR-specific antibody IgG1-iS18 is able to significantly impede critical steps of metastasis, adhesion and invasion of early- and late-stage colorectal cancer *in vitro*. Therefore, this suggests IgG1-iS18 as a promising therapeutic tool in treating early- and late-stage metastatic colorectal cancer. Finally, to deem this antibody as a therapeutic tool against cancer, further *in vivo* analysis must be performed with animal studies. Once this is achieved, further conclusions can be made on whether IgG1-iS18 has the potential to be a therapeutic tool for the treatment of metastatic colorectal cancer.

CONCLUSION

The anti-LRP/LR-specific antibody IgG1 iS18 was shown to have a significant impact on the behavior of SW-480, HT-29 and DLD-1 cancer cells by impeding their adhesive and invasive potential *in vitro*, suggesting that this antibody may act as a possible therapeutic tool for the treatment of metastatic colorectal cancer.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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Appendix C

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siRNA - Mediated LRP/LR knock-down reduces cellular viability of malignant melanoma cells through the activation of apoptotic caspases

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ABSTRACT

The 37 kDa/67 kDa laminin receptor (LRP/LR) is over-expressed in tumor cells and has been implicated in several tumourigenic processes such as metastasis and telomerase activation, however, more importantly the focus of the present study is on the maintenance of cellular viability and the evasion of apoptosis. The aim of the study was to investigate the role of LRP/LR on the cellular viability of early (A375) and late stage (A375SM) malignant melanoma cells. Flow cytometry and western blot analysis revealed that A375SM cells contain more cell-surface and total LRP/LR levels in comparison to the A375 cells, respectively. In order to determine the effect of LRP/LR on cell viability and apoptosis, LRP was down-regulated via siRNA technology. MTT assays revealed that LRP knock-down led to significant reductions in the viability of A375 and A375SM cells. Confocal microscopy indicated nuclear morphological changes suggestive of apoptotic induction in both cell lines and Annexin-V FITC/PI assays confirmed this observation. Additionally, caspase-3 activity assays revealed that apoptosis was induced in both cell lines after siRNA-mediated down-regulation of LRP. Caspase-8 and −9 activity assays suggested that post LRP knock-down; A375 cells undergo apoptosis solely via the extrinsic pathway, while A375SM cells undergo apoptosis via the intrinsic pathway.

Implications: siRNAs mediated LRP knock-down might represent a powerful alternative therapeutic strategy for the treatment of malignant melanoma through the induction of apoptosis.

1. Introduction

Cancer is seen to be one of the leading causes of mortality and morbidity worldwide, accounting for 8.8 million deaths in 2015 [1] and according to the World Cancer Research Fund (WCRF), 14.1 million new cancer cases were reported worldwide in 2012. The estimated number of cancer deaths in 2012 was 8.2 million, and following this pattern of occurrence, it was estimated that the number of reported cancer cases will increase to 24 million by the year 2035 with a corresponding 13 million cancer-related deaths [2]. Malignant melanoma is the most dangerous form of skin cancer as it causes 74% of skin-cancer related deaths worldwide if left untreated. Malignant melanoma is metastatic, and has the ability to invade into the skin, enter the blood stream or lymphatic system and spread to other parts of the body causing death. As the melanoma progresses from stage 0 (non-metastatic) to stage 4 (metastatic), the thickness of the tumor increases and changes occur in the gene expression as the melanoma progresses [3]. Each change brings the expression pattern of the primary melanoma tumours (Stage 1) closer to the expression pattern observed in the metastatic tumours (Stage 4) [3]. The metastatic melanoma cells (late stage malignant melanoma) are seen to have a reduced expression of cell-stromal interactions therefore increasing the migratory- potential of these cells [3]. The transition from non-metastatic expression levels to metastatic expression levels occurs as the melanoma tumours thicken. Several putative tumor oncogenes and suppressor genes have been identified and validated as changing expression during the transition period in which the metastatic phenotype emerges [3]. The global incidence of malignant melanoma continues to increase, with 1 in every 3 cancer type diagnosed being skin cancer [4]. These alarming statistics exemplify the urgent need to develop alternative therapeutic options to combat this disease. Cancer is characterized as the rapid establishment of abnormal cells that grow beyond normal measures thus invading and spreading to distant organs in the body due to the diminished levels of apoptosis or programmed cell death [5,6]. Transformation of normal cells into the tumorigenic state is a multistep process [1] and the most fundamental alterations are termed the Hallmarks of Cancer by Hanahan and Weinberg [6]. The six essential alterations include: cancer cells sustaining proliferative signalling,

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evading growth suppressors, enabling replicative immortality, activating tissue adhesion and invasion (metastasis), induction of tumor angiogenesis and evasion of apoptosis [6]. A prominent feature of tumor cells, amongst others, is the overexpression of the 37 kDa/67 kDa laminin receptor precursor/ high affinity laminin receptor (LRP/LR) which contributes to the process of cellular transformation [7]. The 37 kDa/67 kDa laminin receptor (LRP/LR) is a non-integrin cell surface glycoprotein that interacts with several ECM proteins. More importantly, LRP/LR binds to its primary ligand laminin-1, with high affinity [8,9]. LRP/LR is a transmembrane receptor with a length of 295 amino acids and consists of three domains; the N-terminal intracellular cytosolic domain, the transmembrane domain and the C-terminal extracellular domain which contains binding sites for laminin-1, carbohydrates, elastin, prion proteins and IgG antibodies [7]. The 37 kDa LRP is thought to be the precursor of the 67 kDa high-affinity laminin receptor LR, however the exact mechanism by which the precursor forms the receptor is unknown [10]. LRP/LR, as a trans-membrane receptor, is responsible for many physiological functions such as cell migration, adhesion, proliferation, and the maintenance of cellular viability [11]. However, LRP/LR can also be localised in the nucleus and cytosol where it is involved in the maintenance of nuclear structures and translational processes, respectively [12–17]. The receptor is seen to be overexpressed on the surface of tumorigenic cells such as cervical, lung, prostate, colon [17], breast, oesophageal [18], liver cancer cells [19], pancreatic cancer and neuroblastoma cancer cells [21,22] thus contributing to tumor invasion and metastasis. Incubation of the above mentioned metastatic cancer cells with the anti-LRP/LR- specific antibody IgG1-iS18 resulted in significant reductions in adhesion and invasion, the two crucial steps of metastasis [18–20]. LRP/LR has also been shown to play a role in angiogenesis, an important hallmark of cancer. LRP/LR has been implicated as a key contributor to the pathogenesis of viral and bacterial infections [23], prion protein related diseases [24], neurodegenerative diseases such as Alzheimer's disease [25–29] as well as enhancing telomerase activity [30–32]. Cancerous cells strive to avoid cell death and the increased expression of LRP/LR assists cancer cells in this regard by interacting with the Midkine protein- a growth factor that enhances cell migration and promotes cellular proliferation and survival [31]. The Midkine protein assists LRP/LR in binding to the nuclear envelope and chromatin in order to stabilize chromosomes, thereby assisting in maintaining cellular viability [33]. LRP/LR further inhibits apoptosis through its interaction with Focal Adhesion Kinases (FAKs) as a result of the LRP/LR- laminin interaction [7]. This interaction activates survival pathways of PI3-kinase/AKT and MEK/ERK1/2 as well as the up-regulation of the anti-apoptotic Bcl-2 protein which is involved in both intrinsic and extrinsic pathways, thereby inhibiting apoptosis [7,34]. A study involving the use of small interfering RNAs (siRNA) to down-regulate and target the mRNA of the 37 kDa LRP in liver (Hep3b) [35], breast and oesophageal [36], cervical (HeLa) and lung (A549) [37] as well as pancreatic (AsPC-1) and neuroblastoma (IMR-32) cancer cells [21], confirmed the role played by LRP/LR in the induction of apoptosis. Due to the affirmation that LRP/LR plays a critical role in maintaining cellular viability, this receptor can therefore be a target for the development of possible therapeutics for the treatment of cancer. Therefore, this study was aimed at investigating whether down-regulation of the LRP/LR receptor via siRNA-mediated action would reduce cell viability of early (A375) and late (A375SM) stage malignant melanoma cells and to determine if apoptosis is the primary form of cell death. Although similar studies performed in our laboratory have shown successful apoptotic induction via siRNA action in different cell lines, it is important to note that not all cancer types may be responsive to siRNA treatment. Therefore it is crucial to investigate whether or not targeting LRP/LR in several other cancer types with siRNAs is successful in inducing apoptosis and in turn being a potential therapeutic tool for the treatment of a broad spectrum of cancers types.

2. Materials and methods

2.1. Cells and cell culture conditions

Early (A375) and late (A375SM) stage malignant melanoma cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) with 4500 mg/L glucose (GE Life sciences) supplemented with 10% heat inactivated Fetal bovine serum (FBS) and 1% Penicillin/Streptomycin antibiotic. The cells were grown in an incubator with a set temperature of 37 °C and 5% CO_2 in order to mimic in vivo conditions.

Early stage A375 cells were commercially obtained with an ATCC catalogue number ATCC® CRL-1619™ and the late stage A375SM cells were obtained from Foxchase and were derived from a 54 year old female and classified as late stage melanoma with high metastatic and invasive properties due to elevated expression levels of the extracellular matrix (ECM) glycoprotein, Tenascin (TN) [38].

2.2. Flow cytometry

Fluorescence Activated Cell Scanning (FACS) was used to determine cell surface LRP/LR levels. The cells were detached using 1 × Trypsin/EDTA and centrifuged for 10 min at 1200 rpm. The resulting pellet was fixed in 4% paraformaldehyde for 10 min at 4 °C and after fixation, two cell suspensions were prepared by re-suspension of the pellets in PBS - one half of the cell suspension was labeled with the primary IgG1-iS18 antibody (30 μg/ml) in PBS while the other half of the cell suspension was unlabeled (serving as a control). The cells were incubated overnight at 4 °C and then washed three times with PBS by centrifugation at 5000 rpm for 5 min. Both cell pellets were re-suspended in PBS and were incubated with the PE- coupled secondary antibody in the dark for 1 h. The cell suspensions were washed and resuspended in PBS before flow cytometric analysis. The same procedure was followed for the negative control Anti-chloramphenicol acetyltransferase (CAT) antibody specific for the CAT bacterial protein.

2.3. siRNA- mediated down-regulation of LRP

Both cell lines were seeded onto 6-well plates and left to grow overnight to a confluency of approximately 40–55% before transfection with Dharmacon Human RPSA and the alternative Mission esiRNA-RPSA siRNA to down-regulate LRP. Mission esiRNA-RLUC was used as the negative control. The target siRNA's and the negative control siRNA were diluted in specified amounts of serum-free Opti-MEM® (as per Supplementary Data), prior to being added to the cells. The cells were further supplemented with the respective transfection reagent in order to facilitate the transfection procedure. The plates were then incubated at 37 °C for 72 h to allow for the transfection to take place, cells were then lysed and the lysates were analysed by western blotting.

2.4. SDS PAGE and western blotting

In order to determine total LRP levels western blotting was performed. The BCA™ protein assay was used in order to successfully determine protein concentration. Approximately 10 μg of total protein was loaded and separated on a 12% gel at 200 V for 30–45 min using the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) system. The separated proteins were transferred to a poly vinylidine fluoride (PVDF) membrane using a 1× transfer buffer (20% methanol in 192 mM glycine and 25 mM Tris) for 50 min at 300 V. The membranes were then blocked for 1 h using blocking buffer (3% BSA in 1×PBS Tween) to prevent non-specific binding of the primary IgG1-iS18 antibody. After a 24 h incubation of the membranes in 2 μl (1:10 000) primary antibody diluted in blocking buffer, the membranes were washed three times in wash buffer (0.1% Tween in 1× PBS) and then incubated in 1.5 μl anti-human IgG-HRP conjugated secondary antibody (1:5 000) for 1 h and 30 min. The membranes were washed again in PBS

Tween and 1 ml of the BIORAD chemiluminescent substrate. Thereafter, the luminescence was detected using the BIORAD Chemidoc apparatus in order visualize the proteins.

2.5. MTT assay

In order to determine the effect on cell viability the MTT assay was performed. A375 and A375SM cancer cells were seeded at a density of 1×10^5 cells/ml in a 24-well plate prior to siRNA transfection. The cells were transfected and incubated at 37 °C for 72 h. To each well, 500 µl of a 1 mg/ml solution of MTT dissolved in 1 × PBS was added, followed by a 2 h incubation period at 37 °C. Post incubation, the MTT- containing medium was discarded and the remaining formazan crystals were dissolved in 500 µl of DMSO. The absorbance of the resulting formazan solution was measured at 570 nm using an ELISA plate reader. Cells treated with 8 mM PCA (protocatechuic acid) were used as the positive control.

2.6. Confocal microscopy and Airyscan processing

Confocal microscopy was performed on a Zeiss LSM 780 confocal microscope in order to assess the nuclear morphological changes that occured upon down-regulation of the LRP/LR receptor via siRNA action. In addition, the Airyscan detector was employed as it allows for efficient light collection and thus improved of sensitivity, speed and resolution (approx. 140 nm).

A375 and A375SM cells were seeded at a density of 1×10^5 cells/ml onto coverslips prior to siRNA transfection. Thereafter, cells were washed with 500 µl 1× PBS for 1 min and fixed in 1 ml 4% paraformaldehyde for 15 min. This was followed by three 1× PBS washes and thereafter the cells were permeabilized by adding 2 ml 0.1%Triton-X into each well for 20 min, followed by two washes with 1 × PBS. The cells were then incubated with 2 µl of DAPI nuclear stain diluted in PBS (0.2 µl DAPI in 4 ml PBS) for 8–10 min in the dark. The coverslips containing the DAPI-treated cells were washed 8 times in PBS and thereafter, coverslips were mounted (with the cells facing down) onto a clean microscope slide using 40 µl of mounting fluid (Sigma-Aldrich) overnight.

2.7. Annexin V-FITC/PI assay

A375 and A375SM cancer cells were seeded in a 6-well plate to an approximate density of 1.8×10^6 cells/ml and transfected and allowed to incubate at 37 °C for 72 h. Cells were harvested using 1× Trypsin/EDTA and then washed thrice with ice-cold PBS. Thereafter, the cell suspensions were centrifuged at 1500 rpm for 10 min. The supernatant was discarded and the pellet re-suspended in 100 µl 1× annexin-binding buffer. Subsequently, 5 µl of Annexin V-FITC solution and 5 µl of PI viability dye was added to the prepared cell suspensions and incubated on ice for 15 min in the dark. The samples were then supplemented with 400 µl of ice-cold 1 × annexin binding buffer prior to flow cytometric analysis which was performed within 30 min. PCA treated cells were used as a positive control. The cell populations were analysed using the BD Accuri C6 Flow Cytometer and BD Accuri C6 software.

2.8. Caspase-3, −8 and −9 assays

A375 and A375SM cancer cells were grown in 6-well plates to an approximate density of 1.2×10^6 cells/ml and transfected with the respective siRNAs. Thereafter, 1.2×10^6 cells were pelleted by centrifugation at 1500 rpm for 10 min and re-suspended in 100 µl ice-cold 1 × cell lysis buffer and incubated on ice for 10 min. The cells were then centrifuged for 5 min at 10,000 ×g and the supernatant was placed on ice. Following this, the protein concentration of the supernatant was determined by use of a BCA™ assay and subsequently, 200 µg of protein was prepared according to manufacturer's instructions (Supplementary Data). The prepared assay mixture for each sample was then added to appropriate wells of a 96- well plate and incubated for 2 h at 37 °C. The absorbance was measured at 405 nm using an ELISA plate reader. In addition to untreated cells, esiRNA-RLUC treated cells and PCA treated cells were used as negative and positive controls, respectively.

2.9. Statistical analysis

The two-tailed student's *t*-test with a confidence interval of 95% was used as a means of analysing the data, with *p*-values of less than 0.05 being considered significant. The statistical analysis was performed using the Microsoft® Excel statistical programme. Pearson's correlation coefficient was used to measure correlation between LRP/LR levels and cell viability as well as apoptotic induction. A positive coefficient is an indication of direct proportionality between the two variables and a negative coefficient implies inverse proportionality. Measured correlation values close to 1 signifies high positive correlation.

3. Results

3.1. Early and late stage malignant melanoma cells exhibit high LRP/LR levels on the cell surface

LRP/LR over-expression has been observed in several cancerous cell lines and it is seen to play a pivotal role in mediating the maintenance of cellular viability [39]. Therefore, cell surface LRP/LR levels of the early and late stage malignant melanoma cells were determined in order to confirm its presence on the cell surface and to quantify the percentage of cells displaying cell surface LRP/LR within a specific cell population. This was achieved through flow cytometry. Fig. 1A indicates a distinct shift between the peaks, representing the characteristic change in median fluorescence intensity occurring as a result of the treatment of the cancer cells with the anti-LRP/LR specific IgG1-iS18 antibody thus indicating that both malignant melanoma cell lines exhibit a high percentage of cells within the cell population that contain LRP/LR on the cell surface. In particular, 97.40% of the early stage (A375) and 98.47% of the late stage (A375SM) malignant melanoma cells display LRP/LR on the cell surface. The anti-CAT antibody which targets the chloramphenicol acetyltransferase (CAT) protein was used as the negative control as it is absent on the surface of mammalian cells. Both cell lines showed a minimal to no shift in fluorescence intensity, thus indicating that no non-specific binding occurred (Fig. 1B). The levels of cell surface LRP/LR within the cell population was also analysed, in addition to the percentage of cells within the given cell population that exhibit cell surface LRP/LR. Therefore, the differential expression of cell surface LRP/LR was indicated through the median fluorescence intensity (MFI) as seen in Table S1 (Supplementary Data). The late stage (A375SM) malignant melanoma cells display significantly higher cell surface LRP/LR levels (86%) in comparison to the early stage (A375) malignant melanoma cells (*p = 0.017) as seen in Fig. 1C.

3.2. Late stage malignant melanoma cells display significantly increased total LRP levels in comparison to the early stage malignant melanoma cells

LRP/LR is not only expressed on the cell surface but it is also found in the nucleus, cytosol and perinuclear region. Therefore in order to determine total LRP/LR levels in both malignant cell lines, western blotting as well as densitometric analysis was performed. A western blot representing both LRP/LR and β-actin (Fig. 2A) is shown with the latter being used as a loading control. The anti-LRP/LR specific antibody IgG1-iS18 was used to successfully detect the 37 kDa laminin receptor precursor (LRP) form. The western blots revealed that both A375 and A375SM malignant melanoma cells express LRP, however upon densitometric quantification the A375SM cells were seen to display 62.5% (*p = 0.04) more total LRP in comparison to the A375 cells (Fig. 2B).

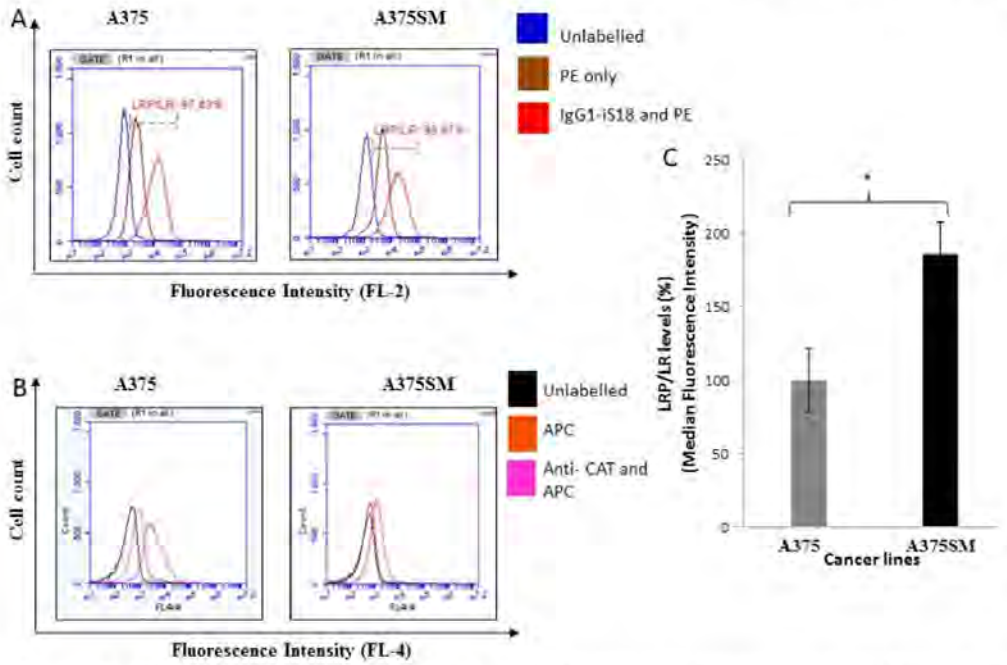

Fig. 1. Detection of the 37 kDa/67 kDa laminin receptor LRP/LR levels on the surface of early (A375) and late (A375SM) stage malignant melanoma cells. A) The blue peak represents the cells that have not been labeled with antibodies, while the orange peak is representative of the cells stained with the goat anti-human phycoerythrin (PE)-coupled secondary antibody only and the red peak is indicative of the cells that have been stained with both the anti-LRP/LR specific antibody IgG1-iS18 and the afore-mentioned secondary antibody. The unlabeled control is included in order to confirm that the secondary antibody does not bind non-specifically, hence a minimal shift in fluorescence intensity is observed between the blue and orange peaks. The shift obtained between the blue and red peaks indicates the presence of LRP/LR on the cell surface of both cell lines. B) Determination of chloramphenicol acetyltransferase protein levels on the surface of early (A375) and late (A375SM) stage malignant melanoma cells. The black peak represents the unlabeled cells, while the orange peak is representative of the cells stained with the goat anti-rabbit allophycocyanin (APC)-coupled secondary antibody only and the pink peak is indicative of the cells that have been stained with both the anti-CAT primary antibody and the afore-mentioned secondary antibody. No distinct shift was observed between the black and orange peaks, thus indicating that the secondary antibody did not bind non-specifically. Furthermore, a minimal shift was observed between the black unlabeled peak and the peak representing the cells labeled with both primary and secondary antibodies. C) Quantification of LRP/LR levels on the surface of early (A375) and late (A375SM) stage malignant melanoma cells by flow cytometric analysis. The median fluorescence intensity (MFI) was used as an indicator of differential cell surface LRP/LR expression for both cell lines. The MFI values presented in the last column of Table S1 were used in the construction of the above graph. The MFI values for A375 was set to 100% and the A375SM cells were found to have 86% more cell surface LRP/LR than the A375 cells.
The above plots are indicative of an average of experiments performed in triplicate, with 20,000 cells counted per sample and the cells were analysed using filter 2 and 4 (FL-2 and −4) which is specific for the PE-fluorochrome and APC- fluorochrome respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. siRNA technology leads to successful down-regulation of LRP expression in early and late stage malignant melanoma cells

In order to determine the role of LRP/LR expression on the cellular viability of both cell lines and to confirm no off target effects occured, Dharmacon ON-TARGETplus Human RPSA and an alternative Mission esiRNA-RPSA were used. Both siRNAs target different regions of LRP (refer to Supplementary data for siRNA sequence). Post transfection of the early (A375) and late (A375SM) stage malignant melanoma cells with the ON-TARGETplus Dharmacon Human RPSA siRNA (targeting the mRNA of the 37 kDa LRP through the action of 4 siRNAs), western blot analysis and densitometric quantification revealed that LRP was successfully down-regulated by 77% and 72%, respectively (Fig. 3A and B). In addition, western blot analysis revealed that post transfection with the Mission esiRNA-RPSA, significant down-regulation in LRP expression of 55% and 50% in early (A375) and late (A375SM) stage malignant melanoma cells occured, respectively (Fig. 4A and B). The observed down-regulation of LRP and the effect thereof on cellular viability was analysed by MTT assays. Post-transfection of the A375 and A375SM cells with the Dharmacon Human RPSA (*$p = 0.04$ and **$p = 0.0064$) and Mission esiRNA-RPSA (*$p = 0.0097$ and *$p = 0.025$), resulted in significantly reduced cellular viability in comparison to the non-transfected cells (Figs. 3C and 4C). Protocatechuic Acid (PCA) is a known apoptotic inducer and was therefore used as a positive control throughout experimentation.

Only western blotting and MTT assays were performed using two siRNAs. Both siRNAs indicated similar effects on cellular viability (Figs. 3 and 4). This therefore confirms that the specific targeting and down-regulation of LRP, reduces the cellular viability in the malignant melanoma cells being investigated. All subsequent experiments were performed with the Dharmacon Human RPSA.

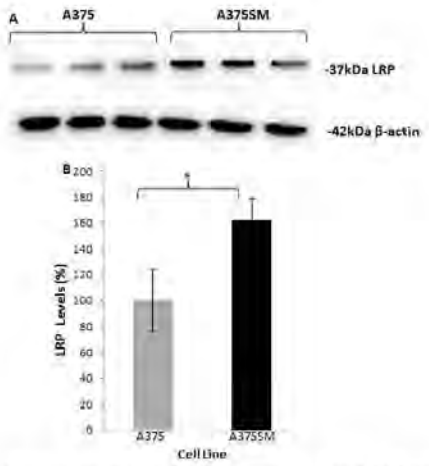

Fig. 2. Western blot analysis of the relative expression of total 37 kDa laminin receptor precursor (LRP) levels in early (A375) and late (A375SM) stage malignant melanoma cells. A) Anti-LRP/LR specific primary antibody IgG1-iS18 and HRP-coupled secondary antibody was used to detect and visualize total 37 kDa LRP levels in both malignant cell lines. β-actin was used as the loading control. B) Densitometric analysis performed on these blots revealed that A375SM cells possess 62.5% more total LRP in comparison to A375 cells. The values obtained from quantification of LRP were an average of triplicate repeats. The resultant values were used in the construction of this graph and the values for A375 were set to 100%. *$p < 0.05$, **$p < 0.01$ ***$p < 0.0001$.

3.4. siRNA-mediated down-regulation of LRP expression results in nuclear morphological changes suggesting the induction of apoptosis in early and late stage malignant melanoma

In order to examine the mode of cell death and possible induction of apoptosis post Human RPSA siRNA treatment, confocal microscopy with the addition of Airyscan processing (resolution: 140 nm) was performed to detect potential nuclear morphological changes. When comparing the nuclei of the non-transfected cells (Fig. 5) to the Human RPSA siRNA treated early stage (A375) malignant melanoma cells, the treated cells displayed distinct nuclear morphological changes such as the formation of apoptotic bodies, nuclear shrinkage and membrane blebbing (Fig. 5). Similarly, the comparison of nuclei of Human RPSA siRNA treated late stage (A375SM) malignant melanoma cells (Fig. 5C) to the nuclei of the non-transfected cells (Fig. 5) revealed the presence of nuclear morphological changes such as nuclear shrinkage and membrane blebbing. In order to confirm that apoptotic induction has indeed occurred in both of the malignant cell lines, Annexin V-FITC/PI assays were performed and upon transfection of A375 and A375SM cells with Human RPSA siRNA (Fig. 6(1)), 91.4% and 97.4% of the cells underwent total apoptotic induction when compared to the untreated cells, respectively. The level of apoptotic induction occurring after treatment of early (A375) and late (A375SM) stage malignant melanoma cells with Human RPSA siRNA was quantified by combining the percentage values obtained for live cells, early and late stage apoptosis as well as necrotic cells for each treatment condition (Fig. 6(2)) in triplicate. This was used as an indication of total apoptotic levels.

3.5. siRNA-mediated down-regulation of LRP expression leads to caspase activation in both early and late stage malignant melanoma cells

In order to further confirm that LRP down-regulation results in the induction of apoptosis, caspase-3 activity assays were performed. It was observed that post transfection with Dharmacon Human RPSA, A375 and A375SM cells displayed a significant 4-fold and 3-fold increase in caspase-3 activity, respectively when compared to the non-transfected cells (Fig. 7A). Caspase-8 activity assays were performed in order to gain further insight into whether or not the caspase-8 mediated extrinsic pathway is activated to induce apoptosis post transfection with Dharmacon Human RPSA. Post transfection of both cell lines with Human RPSA, A375 cells showed a significant 66% (*$p = 0.03$) increase in caspase-8 activity when compared to the non-transfected cells (Fig. 7B). No significant difference was observed in caspase-8 activity in A375SM cells treated with Human RPSA when compared to the non-transfected cells. Caspase-9 activity assays were performed in order to establish whether the caspase-9 mediated intrinsic pathway was activated in early (A375) and late (A375SM) stage malignant melanoma cells after siRNA-mediated LRP down-regulation. A375SM cells show a significant 3-fold (*$p = 0.016$) increase in caspase-9 activity in comparison to the non-transfected cells (Fig. 7C). A non-significant difference was observed in caspase-9 activity in A375 cells treated with Human RPSA by comparison to non-transfected cells.

4. Discussion

Flow cytometric analysis revealed that significantly high percentages of both early (A375) and late (A375SM) stage malignant melanoma cells display LRP/LR on the cell surface. The level of LRP/LR expression present on the surface of both malignant melanoma cell lines was then further investigated and quantified using median fluorescent intensity (Table S1-Supplementary Data) and it was revealed that A375SM cells display significantly more cell surface LRP/LR in comparison to A375 cells (Fig. 1C). As indicated by Munien et al. (2017) A375SM cells have a higher adhesive and invasive potential in comparison to A375 cells and are therefore more dependent on LRP/LR to carry out certain cellular processes [40]. It could then be speculated that A375SM cells rely more on LRP/LR to assist in other processes such as maintenance of cellular viability and evasion of apoptosis- two of several essential functions the LRP/LR trans-membrane receptor may be responsible for.

Total LRP levels in the two malignant melanoma cell lines were quantified and it was observed that this receptor is present in the cytosol, nucleus, perinuclear region and also on the cell surface. Western blot analysis revealed that A375SM cells contain more total LRP in comparison to the early (A375) stage malignant melanoma cells (Fig. 2A and B). The role of LRP/LR in the nucleus is to maintain nuclear structures and to complete translational processes in the cytosol [12–17], therefore the increased total LRP levels present in A375SM cells may indicate that, in comparison to A375 cells, this cell line could be highly dependent on LRP/LR to perform the aforementioned roles internal of the cell. The quantified cell surface LRP/LR levels can be responsible for the observed total LRP levels due to a high positive correlation (Table 1). This is crucial as LRP/LR present on the cell surface is predominantly implicated in the maintenance of cellular viability and apoptotic evasion [34], therefore due to the high correlation, total LRP levels can be used to assess cell viability and the induction of apoptosis.

In order to assess the effect of this receptor on cellular viability, LRP was down-regulated via RNA interference using the Dharmacon ON-TARGET Human RPSA siRNA. The Human RPSA siRNA targets the mRNA of the 37 kDa laminin receptor precursor (LRP) form, targeting both sense and antisense strands of the human LRP gene via the ON-TARGETplus modification pattern to reduce off-target effects (sequence provided in Supplementary Data). Western blotting detected the down-regulated LRP levels with the use of the anti-LRP/LR specific antibody IgG1-iS18. Treatment of A375 and A375SM cells with Dharmacon Human RPSA showed significant reductions in LRP expression (Fig. 3A and B) when compared to the non-transfected cells- thereby proving the efficiency of Dharmacon Human RPSA on LRP expression.

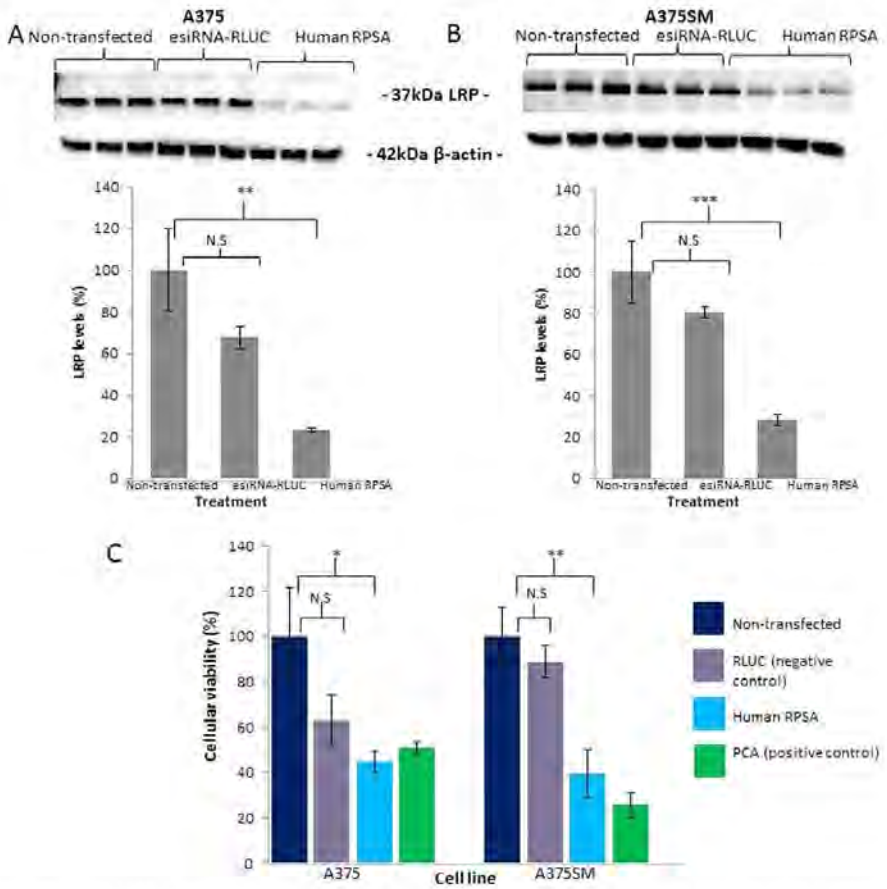

Fig. 3. : Down -regulation of LRP expression in early (A375) and late (A375SM) stage malignant melanoma cells post siRNA transfection. A and B) Transfection of both A375 and A375SM cells with Dharmacon Human- RPSA resulted in the significant down-regulation of LRP by 77% and 72%, respectively when compared to the non-transfected cells (set to 100%). Densitometric analysis also revealed a non-significant difference between the non-transfected and esiRNA-RLUC transfected cells. C) **The effect of siRNA-mediated down-regulation on the cellular viability of early (A375) and late (A375SM) stage malignant melanoma cells.** MTT assays were used to analyse the effect of siRNA transfections with the Human RPSA on the cellular viability of both cell lines. The A375 and A375SM cells treated with the Human RPSA siRNA displayed a significant reduction of 47% and 61% in cellular viability, respectively. Cells treated with the negative control siRNA-RLUC showed no significant difference in cellular viability when compared to the non-transfected cells of both cell lines. PCA was used as a positive control and the value for the non-transfected cells was set to 100% for both cell lines. The graph is representative of an average of experiments performed in triplicate. *$p < 0.05$, **$p < 0.01$ ***$p < 0.0001$, N.S.: $p > 0.05$ non-significant.

Furthermore, A375 cells showed a greater reduction in LRP expression after Human RPSA transfection in comparison to the A375SM cells and this may be due to the fact that the A375SM cells had higher total LRP levels than A375 cells before siRNA treatment (Fig. 2). A high correlation was observed between total LRP levels prior to and post Human RPSA transfection (Table 1), therefore suggesting that high total LRP levels before siRNA transfection leads to less LRP down-regulation post siRNA treatment and vice versa.

Human RPSA siRNA is specific for LRP in relation to the negative control esiRNA-RLUC which had no effect on LRP expression. In order to confirm that this LRP down-regulation wasn't due to off-target effects, an alternative siRNA (Mission esiRNA-RPSA) was used to target the mRNA of the 37 kDa LRP, in a similar manner to that of the Dharmacon Human RPSA. The Dharmacon Human RPSA siRNA is designed to specifically target four sequences within the 37 kDa LRP mRNA, while esiRNA-RPSA targets nucleotides 521–929 of the mRNA sequence (Supplementary Data). Treatment of A375 and A375SM cells with esiRNA-RPSA resulted in significant reductions in LRP expression (Fig. 4A and B). High correlations were obtained between total LRP levels prior to and post esiRNA-RPSA treatment (Table 1), therefore further confirming that higher total LRP levels before siRNA treatment results in less LRP down-regulation post siRNA transfection.

The treatment of A375 and A375SM cells with Dharmacon Human RPSA resulted in significant reductions in cellular viability (Fig. 3C). In addition, high correlation was observed (Table 1). This further confirms the essential role of LRP/LR in the maintenance of cellular viability as the down-regulation of this receptor results in significantly lowered cellular viability. Similarly transfection of A375 and A375SM cells with

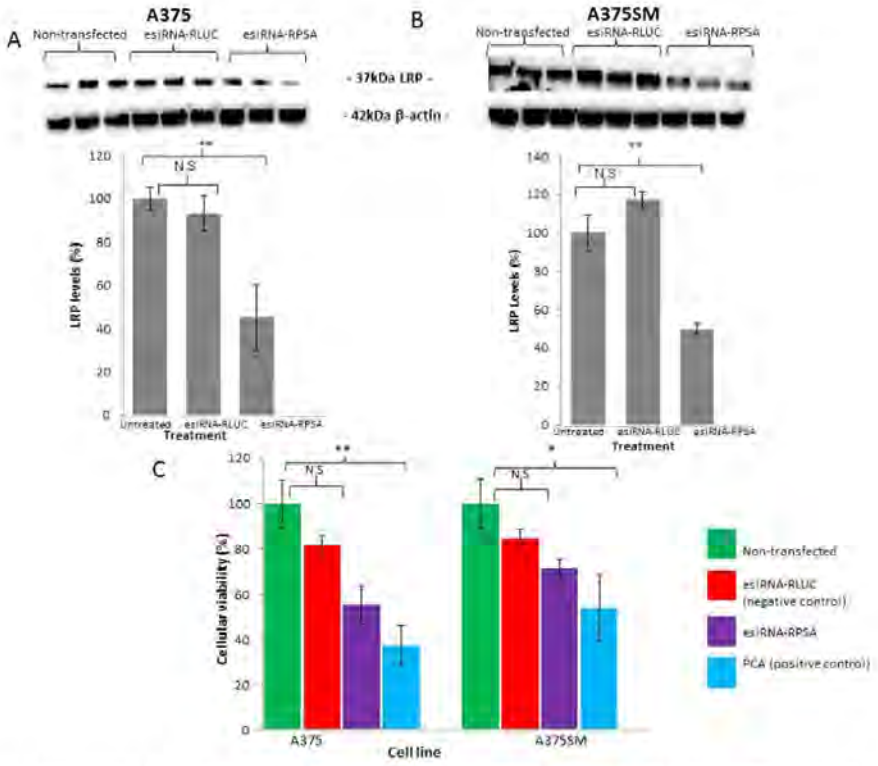

Fig. 4. Detection of LRP expression in early (A375) and late (A375SM) malignant melanoma cells after transfection with an alternative siRNA. A and B) Western blotting and densitometric quantification revealed that after transfection of the cells with esiRNA-RPSA, there was a significant reduction of 55% in LRP expression in the early stage malignant melanoma cells and a 50% reduction in LRP expression observed in the late stage malignant melanoma cells when compared to the non-transfected cell. In addition, there was no significant difference seen in LRP expression between the cells treated with the esiRNA-RLUC (negative control siRNA) and non-transfected cells. β-actin was used as the loading control and the graphs are representative of an average of experiments performed in triplicate. C) The effect of esiRNA-RPSA - mediated LRP down-regulation on the cellular viability of early (A375) and late (A375SM) stage malignant melanoma cells. After treatment of the cells with esiRNA-RPSA, MTT assays were performed to assess the effect on cellular viability. A375 and A375SM cells showed reductions of 45% and 29% in cellular viability, respectively after being treated with esiRNA-RPSA. Non-significant differences were observed in the viability of cells post treatment with the negative control siRNA (esiRNA-RLUC) when compared to the non-transfected cells for both cell lines (set to 100%). PCA was used as the positive control and the graph is representative of an average of three repeats. $^{*}p < 0.05$, $^{**}p < 0.01$ $^{***}p < 0.0001$, N.S.: $p > 0.05$ non-significant.

the alternative esiRNA-RPSA resulted in significant reductions in cellular viability, when compared to the non-transfected cells (Fig. 4C). This observation coincides with that of the cells transfected with the Human RPSA siRNA, therefore providing evidence for the critical role of LRP/LR in the maintenance of cellular viability. High correlation coefficients were observed between esiRNA-RPSA-mediated LRP down-regulation in A375 and A375SM cells and reductions in cellular viability induced upon transfection.

Tumor progression is facilitated through the evasion of apoptosis and this occurs via the over expression of LRP/LR and the important role it expresses in the maintenance of cellular viability [39]. A proposed mechanism by which LRP/LR maintains cellular viability is through the high binding interactions with the multifunctional heparin binding protein known as Midkine [41]. The Midkine protein is classified as a growth factor and it assists LRP/LR in binding to the nuclear envelope and chromatin in order to stabilize the chromosomes and in turn maintain cellular viability [33]. Moreover, the Midkine protein is seen to be expressed at high levels in human carcinomas and therefore it is associated with several cellular processes such as cellular migration and proliferation as well as angiogenesis [41]. *Khumalo* et al., *2015* [33] and *Chetty* et al., *2017* [42] indicated that down-regulation of LRP/LR in breast and oesophageal, as well as pancreatic cancer and neuroblastoma cells respectively, resulted in reductions in cellular viability and in turn the induction of apoptosis. It can therefore be said that siRNA-mediated LRP down-regulation may result in reduced LRP/LR-Midkine interactions thus being responsible for the observed reductions in cellular viability. In addition, *Lu* et al., *2016* [43] showed that siRNA-mediated down-regulation of the 67LR in colon cancer cells resulted in an upregulation of the pro-apoptotic Bax protein and a reduction in the anti-apoptotic Bcl-2 protein which is favourable for anti-cancer treatment as the ability of the cancer cells to survive is decreased. Knowing this, it can be said that siRNA-mediated LRP down-regulation in early and late stage malignant melanoma cells results in an up-regulation of specific pro-apoptotic proteins and a down-regulation in anti-apoptotic proteins.

In order to confirm that apoptosis was the form of cell death responsible for the observed reductions in cellular viability of A375 and A375SM cells post LRP down-regulation, confocal microscopy with the

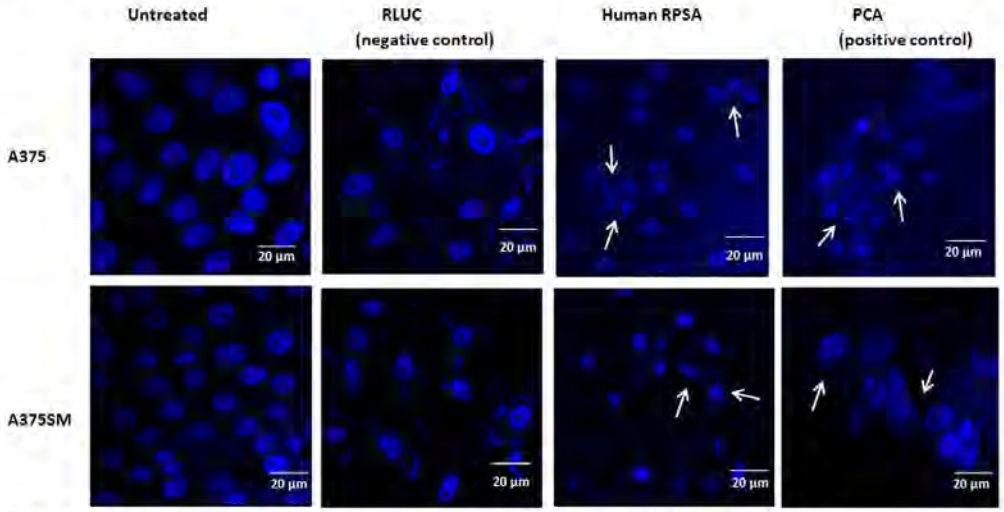

Fig. 5. The effect of siRNA-mediated LRP down-regulation on nuclear morphology of early stage (A375) and late (A375SM) stage malignant melanoma cells. Non-transfected cells with normal rounded nuclei with uncompromised nuclear integrity. Cells treated with the negative control esiRNA-RLUC showed nuclei with similar characteristics to the non-transfected cells. DHARMACON Human RPSA siRNA transfected cells displayed decreased nuclear integrity, membrane blebbing and the formation of apoptotic bodies (as shown by the white arrows) thus indicating the induction of apoptosis. 8 mM PCA treated cells exhibited nuclei that have undergone membrane blebbing and apoptotic body formation (indicated by the white arrows). All images were obtained at a magnification of 63× with the addition of the Aryscan processing (140 nm).

addition of Airyscan processing was performed in order to confirm that the cell death observed occurred via apoptosis. Nuclear morphological alterations were highly evident in the two malignant melanoma cell lines post Human RPSA transfection- this included reduced membrane integrity in the form of apoptotic bodies, nuclear membrane blebbing as well as nuclear shrinkage, in comparison to the non-transfected cells (Fig. 5). LRP/LR is a component of the nuclear machinery of the cell and therefore due to the strong association of nuclear and perinuclear LRP/LR to histones- nuclear structures are maintained [44]. Therefore down-regulation of LRP/LR results in reduced nuclear integrity and altered nuclear morphology (Fig. 5). These observable Human RPSA siRNA- induced changes to the nuclear integrity and morphology of A375 and A375SM cells are indicative of apoptotic induction [45,46]. Furthermore, disruptions in the structure of cellular DNA is characteristic in cells undergoing apoptotic induction [46], therefore using the DAPI stain was an effective method in determining the presence of apoptotic induction in the two malignant melanoma cell lines as it is able to pass through intact cell membranes as well as bind to double stranded DNA.

Further confirmation as well as quantification of apoptotic induction was acquired by performing the Annexin-V-FITC/PI assays. Induction of apoptosis results in reductions in cell membrane integrity as well as a membrane flip- reaction which impacts the phospholipid asymmetry of the cell membrane. This occurs by exposing the intracellularly -located phosphatidylserine (PS) on the outer cell membrane leaflet, allowing the binding of Annexin-V to the negatively charged PS when calcium is present [47]. The Annexin-V protein is bound to the FITC fluorochrome therefore allowing for the PS to be detected though the binding of Annexin-V. However, the binding of Annexin-V does not allow for the differentiation between early and late stage apoptosis thus the Propidium Iodide (PI) viability dye is additionally used to achieve this. The PI stain cannot pass through intact cell membranes and therefore it is only able to stain cells in the late apoptotic/necrotic phase due to the cells in this phase undergoing membrane alterations. This therefore allows PI to stain the double stranded DNA located in the nucleus, excluding early apoptotic cells which lack the disrupted cell membrane.

The non-transfected cells and the cells treated with negative control esiRNA-RLUC for both malignant melanoma cell lines displayed negative staining for Annexin-V, as majority of the cells were situated in the lower left quadrant of the Annexin-V-FITC/PI plots, which is representative of living cells (Fig. 6(1)). Subsequently, Human RPSA-treated and PCA- treated (8 mM) A375 and A375SM cells portrayed positive staining for Annexin-V (Fig. 6(1)), therefore appearing in the lower and upper right quadrants of the plots. The distinct shift from negative to positive Annexin-V staining indicates that Human RPSA siRNA-mediated LRP down-regulation in both malignant melanoma cell lines triggers membrane integrity disruption, but in particular cell membrane blebbing, which is typical of cells undergoing apoptosis. After transfection of both malignant melanoma cell lines with Human RPSA, majority of the A375 cells experienced late apoptosis, whilst majority of the A375SM cells experienced early apoptosis (Fig. 6(2)). The early stage malignant melanoma cells predominantly underwent late apoptosis as they express less LRP, allowing more down-regulation to occur. This in turn results in more cell death, and a higher number of cells entering the late stage of apoptosis. The late stage malignant melanoma cells primarily underwent early apoptosis and this is due to more LRP present in this cell line, therefore less down-regulation and cell death occurring. This signifies the extended rate of apoptotic induction in the A375SM cells as well as reduced toxicity brought about by siRNA-mediated down-regulation in this cell line in comparison to the A375 cells.

Caspase-3 assays were performed to further confirm the apoptosis inducting effects of siRNA-mediated LRP down-regulation. The effector caspase-3 has increased activity in cells that are actively undergoing apoptosis [48]. Consequently, post transfection of A375 and A375SM cells with Human RPSA, there was a significant increase in caspase-3 activity in both cell lines in comparison to the non-transfected cells

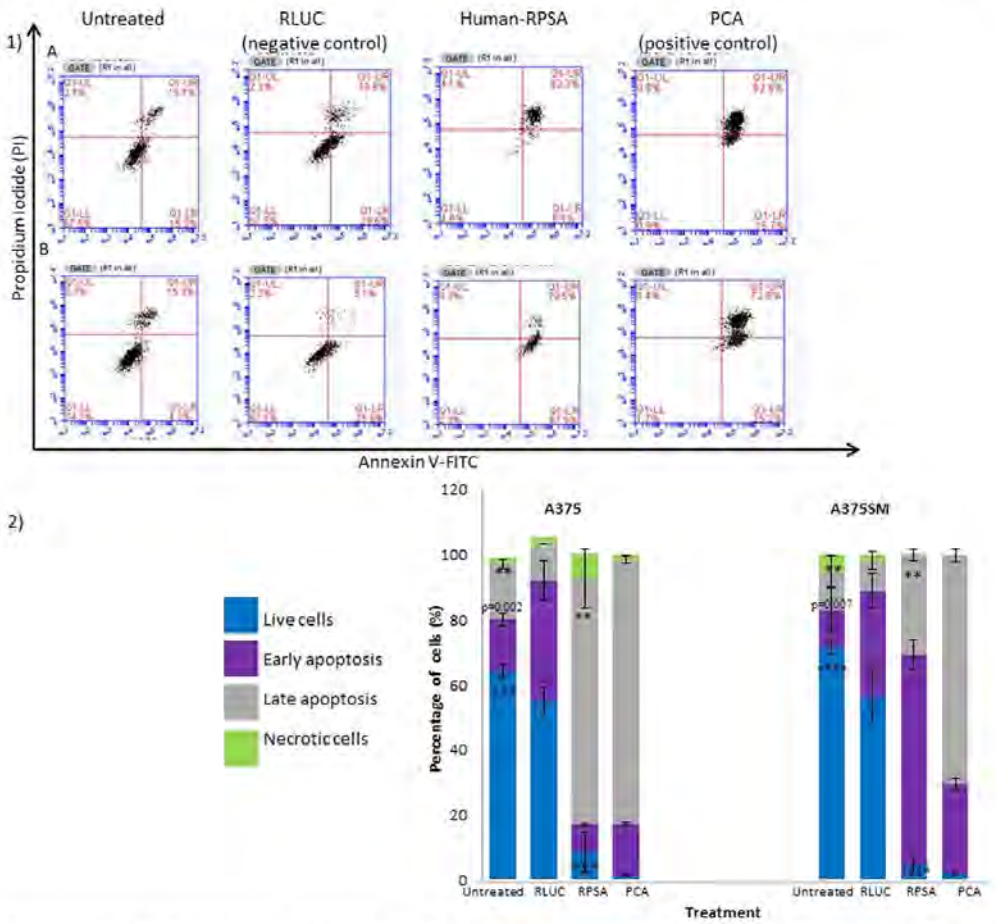

Fig. 6. Induction of apoptosis in early stage (A375) and late (A375SM) stage malignant melanoma cells after Human RPSA siRNA transfection. 1) The non-transfected/untreated plot shows a majority of cells in the lower left quadrant (Q1-LL) as it is representative of normal living cells. Treatment of the cells with the negative control esiRNA-RLUC also resulted in the majority of the cells appearing in the lower left quadrant. Transfection of the A375 cells with Human RPSA siRNA led to 8.1% of cells undergoing early apoptosis, as shown by the cells in the lower right quadrant (Q1-LR), and 83.3% of cells undergoing late apoptosis- therefore a total of 91.4% of cells underwent apoptosis after Human RPSA transfection. 67.9% of A375SM cells transfected with Human RPSA siRNA appeared to be undergoing early apoptosis as shown in the lower, while 29.5% of transfected cells were observed as undergoing late apoptosis (upper right quadrant) - therefore a total of 97.4% of Human RPSA siRNA transfected cells underwent apoptosis. PCA was used as the positive control and resulted in majority of the cells appearing in the lower right quadrant and the upper right quadrant (Q1-UR), which is representative of cells undergoing late apoptosis. The upper left quadrant (Q1-UL) represents cells undergoing necrosis. 20,000 cells were counted per sample. 2) Total levels of apoptotic induction in early (A375) and late (A375SM) stage malignant melanoma cells after siRNA-mediated LRP down-regulation. Post transfection of cells with Human RPSA siRNA, 7% and 76% of A375 cells underwent early and late apoptosis, respectively. 65% and 31% of A375SM cells underwent early and late apoptosis, respectively. A375 and A375SM cells underwent 61% (***$p = 0.00025$) and 78% (****$p = 0.00002$) more apoptosis in comparison to the non-transfected cells, respectively. Total apoptotic levels post siRNA transfection was obtained from the Annexin V-FITC/PI assay plots and the resultant values were used in the construction of this graph. The graph represents an average of experiments performed in triplicate. *$p < 0.05$, **$p < 0.01$ ***$p < 0.0001$.

(Fig. 7A). These results clearly indicate that siRNA-mediated LRP down-regulation induces apoptosis in the two malignant melanoma cell lines, possibly as a result of the inhibition of the above-mention LRP/LR-midkine interaction. The repeated observation of apoptosis induction as a result of LRP- down-regulation via siRNA action may also be as a result of the LRP/LR- focal adhesion Kinase (FAK) interaction. A study conducted by Sun, L. et al. showed that the binding of LRP/LR to laminin, allows for the interaction between LRP/LR and FAK to occur. FAK carries out protein-protein interaction adaptor functions at sites of cell adhesion, and therefore it is seen to play a direct role in tumor growth and survival by activating survival pathways PI3K//AKT and MAPK/ERK as well as up-regulating Bcl-2 and anti-apoptotic proteins [49]. Thereby it can be stated that siRNA-mediated LRP down-regulation may reduce the LRP/LR-FAK interaction, resulting in apoptotic induction. LRP/LR is known to play a role in the biogenesis of ribosomes by facilitating the maturation process of the 21S pre-rRNA into

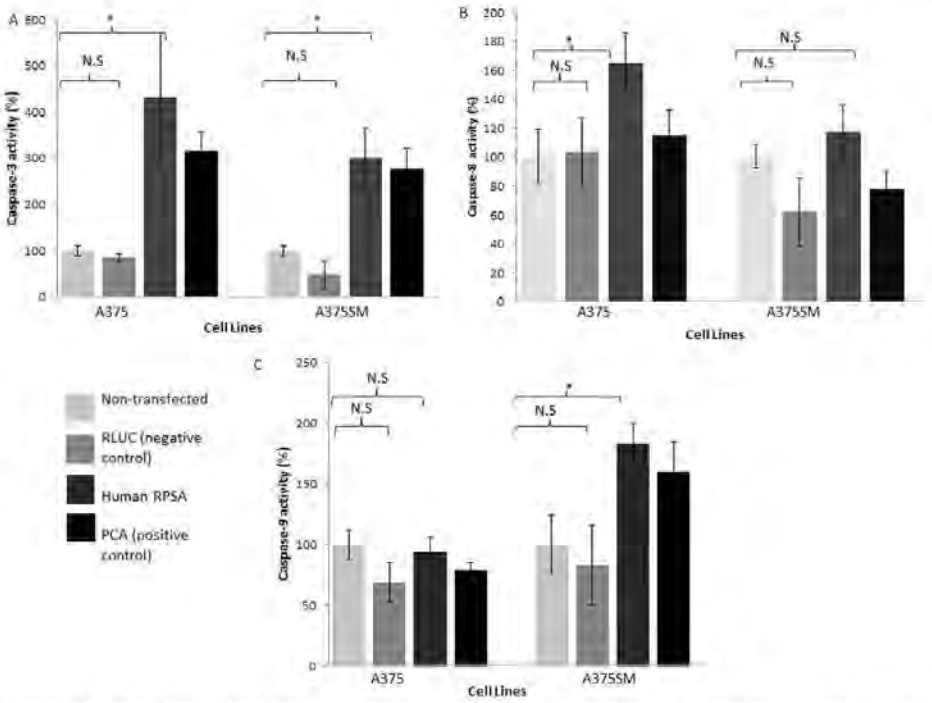

Fig. 7. A) Analysis of the effect of siRNA-mediated LRP down-regulation on caspase-3 activity in early (A375) and late (A375SM) stage malignant melanoma cells. Caspase-3 activity assay indicated that post transfection of cells with Human RPSA siRNA, A375 and A375SM cells showed a significant 4-fold and 3-fold increase in caspase-3 activity, respectively when compared to the non-transfected cells (which was set to 100%). B)The effect of siRNA-mediated down-regulation of LRP expression on caspase-8 activity in early (A375) and late stage (A375SM) malignant melanoma cells. DHARMACON Human RPSA transfected A375 cells showed a significant 66% increase in caspase-8 activity, when compared to non-transfected cells (set to 100%). Treatment of A375SM cells with DHARMACON Human RPSA showed no difference in caspase-8 activity by comparison to non-transfected cells. C) The effect of siRNA-mediated down-regulation of LRP expression on caspase-9 activity in early (A375) and late (A375SM) stage malignant melanoma cells. DHARMACON Human RPSA transfected A375SM cells showed a significant 3-fold increase in caspase-9 activity when compared to non-transfected cells (set to 100%). Treatment of A375 cells with DHARMACON Human RPSA showed no difference in caspase-9 activity by comparison to non-transfected cells. Both tumourigenic cell lines displayed no significant difference in caspase-3, −8 and −9 activity after treatment of cells with the negative control esiRNA-RLUC, when compared to non-transfected cells. PCA was used as a positive control. The graphs are indicative of an average of experiments performed in triplicate. *$p < 0.05$, **$p < 0.01$ ***$p < 0.0001$, N.S.: $p > 0.05$ non-significant.

the 18S rRNA, while associating with and acting as a component of the 40S ribosomal small unit and assisting in protein translation and synthesis [40,48–50]. Therefore the down-regulation of LRP/LR could have an impact on ribosomal formation and in turn translation of proteins crucial in the proper functioning and survival of cells- thereby causing cell death.

Furthermore, A375 cells exhibited a greater increase in caspase-3 activity, in comparison to the A375SM cells after Human RPSA transfection due to the early stage malignant melanoma cells having lower total LRP/LR expression levels therefore a higher chance of subsequent down-regulation and in turn induction of apoptosis. Furthermore, this could be as a result of additional DNA damage occurring in the A375 cells upon Human RPSA transfection, in comparison to the A375SM cells.

Caspase-8 and caspase-9 activity assays were performed in order to evaluate which apoptotic pathways A375 and A375SM cells use in order to undergo apoptosis after Human RPSA-mediated down-regulation of LRP expression. It was observed that early stage malignant melanoma cells displayed a significant increase in caspase-8 activity after treatment with Human RPSA, in comparison to the non-transfected cells (Fig. 7B). As previously described, caspase-8 is involved in the extrinsic death receptor- mediated pathway, therefore it can be said that A375 cells undergo apoptosis via the extrinsic pathway post Human RPSA siRNA LRP down-regulation. In addition, it was observed that Human RPSA treated A375SM cells exhibited a significant increase in caspase-9 activity, by comparison to the non-transfected cells (Fig. 7C). Caspase-9 is highly involved in the mitochondrial-dependent intrinsic pathway, thus suggesting that Human RPSA-mediated LRP down-regulation in A375SM cells induces apoptosis via the intrinsic pathway. Both malignant melanoma cell lines undergo apoptosis via different apoptotic pathways, thus being suggestive of the importance of the differential interactions between LRP/LR expression and the ECM components, particularly laminin. In addition, as the malignant melanoma cells transition from early stage (less invasive) to the late stage (more invasive), there may also be a change in the expression levels of specific apoptotic proteins involved in the extrinsic and intrinsic apoptotic pathways, such that as the transition occurs, the proteins involved in the extrinsic pathway decrease and the proteins in the intrinsic pathway increase. This may occur as apoptotic proteins can be subject to the extent of caspase-mediated cleavage, with the cleavage of caspase substrates being linked to signature alterations of apoptotic cells [51]. However, to obtain more insight in this regard, further

Table 1
Pearson's correlation between total LRP levels prior to and post siRNA transfection and the level of apoptotic induction through reductions in cellular viability (r-value).

Cell line	Correlation between cell surface LRP and total LRP levels prior to siRNA transfection (r-value)	Correlation between total LRP levels prior to and post Human RPSA transfection (r-value)	Correlation between total LRP levels prior to and post esiRNA-RPSA transfection (r-value)	Correlation between Human RPSA-mediated LRP down-regulation and reductions in cellular viability (r-value)	Correlation between esiRNA-RPSA-mediated LRP down-regulation and reductions in cellular viability (r-value)	Correlation between total LRP levels post Human RPSA transfection and total levels of apoptosis (r-value)	Correlation between total LRP levels post Human RPSA transfection and increases in caspase-3 activity after Human RPSA treatment (r-value)
A375	0.98	0.95	0.99	0.6	0.7	0.7	0.63
A375SM	0.65	0.97	0.82	0.88	0.9	0.8	0.9

analysis of the apoptotic proteins involved in the regulation of caspase-8 (extrinsic pathway) and caspase-9 (intrinsic pathway) should be investigated.

Therefore, the specific siRNAs known to target LRP expression may be considered a possible therapeutic tool for the treatment of both early and late stage malignant melanoma cells.

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Author's contributions

Stefan F.T. Weiss conceptualized the project. Thalia M. Rebelo performed the experiments. Thalia M. Rebelo analysed the data. Leila Vania assisted in the experimental merit of the research. Thalia Rebelo wrote the paper. Eloise Ferreira interpreted data and edited the manuscript.

All authors participated in preparation and critical review of the final manuscript.

Competing interests

The authors declare that they have no competing interests in the manuscript.

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Appendix A. Supporting information

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