Investigating the effect of LRP/LR down-regulation on malignant melanoma cells and assessing the effect of LRP/LR antibodies on metastatic malignant melanoma mouse models

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ABSTRACT

On a cellular level cancer is described as abnormal cellular growth resulting from uncontrolled cellular proliferation and reduced apoptosis. Cancer is seen as a non-discriminative disease, therefore exhibiting high incidence and mortality rates. Additionally, cancer is classified as a global burden in both economically developed and developing countries. The 37kDa/67kDa laminin receptor (LRP/LR) is seen to be over-expressed in tumor cells when compared to their normal cell counterparts. This receptor has been implicated in several tumourigenic processes such as cell migration and adhesion but importantly for the present study, the maintenance of cellular viability and the evasion of apoptosis. The aim of the present 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 revealed that both malignant melanoma cell lines exhibit high cell surface LRP/LR levels and with further analysis using median fluorescence intensity, it was observed that A375SM cells contain approximately 86% more cell-surface LRP/LR than A375 cells. In addition, western blotting and densitometric analysis suggested that A375SM cells contain 60% more total LRP/LR levels than A375 cells. Furthermore, western blot analysis revealed that targeting the mRNA of the 37kDa LRP using a LRP-specific siRNA (Dharmacon ON-TARGET Human RPSA) in A375 and A375SM cells led to significant down-regulation of 77% and 72% in LRP expression, respectively. Consequently, MTT assays showed that LRP down-regulation led to significant reductions of 47% and 61% in the viability of A375 and A375SM cells, respectively. An alternative LRP-specific siRNA (Mission esiRNA-RPSA) was used in order to confirm specificity and to exclude any off-target effects of Dharmacon ON-TARGET Human RPSA for LRP. Confocal microscopy with the addition of the Airyscan processing tool indicated nuclear morphological changes suggestive of apoptotic induction in the form of cell death occurring in both malignant melanoma cell lines post LRP down-regulation. Annexin-V/PI assays confirmed this observation, by revealing that A375 and A375SM cells underwent apoptotic induction post LRP down-regulation in comparison to the untreated cells. Additionally, caspase-3 activity assays revealed that both cell lines experienced apoptotic induction after siRNA-mediated down-regulation of LRP. Caspase-8 and -9 activity assays suggested that post LRP down-regulation; A375 cells undergo apoptosis solely via the extrinsic pathway, while A375SM cells are thought to undergo apoptosis via the intrinsic pathway. According to *Munien et al, 2017* [137], application of the anti-LRP/LR specific antibody IgG1-

iS18 has led to a significant reduction in metastatic potential of A375 and A375SM malignant melanoma cells *in vitro*. Therefore the aim of the present *in vivo* study was to further investigate the significance of blocking LRP/LR with the IgG1-iS18 antibody and how this will be effective in the treatment of malignant melanoma *in vivo* using a MF-1 nude mice model. The mice were divided into three groups; with three mice each. This was followed by treatment of group 1 with 0.885mg/ml of the IgG1-iS18 antibody intraperitoneally, twice a week, group 2 was administered with the Phosphate Buffered Saline (PBS) vehicle control solution and the last group remained untreated. The results of this pilot study indicated that three of the nine mice developed external tumour formation; one of the mice being from the untreated group with a tumour volume larger than the other two mice which were from the IgG1-iS18 treatment group. When comparing the tumour formation between the untreated and treated group, there was a large reduction in tumour weight and volume. To conclude, LRP/LR plays a critical role in the maintenance of tumor cellular viability and metastasis, recommending this receptor as a promising therapeutic target and proposing the potential use of siRNA technology as well as the IgG1-iSi8 antibody for the treatment of malignant melanoma.

Formal Declaration

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Dedicated to my Beautiful Father, Beto Rebelo

(19.06.1965- 02.07.2015)

Thank you for all your love, support and motivation throughout the years we had together

I love you forever

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  doi 10.1186/s12885-016-2953-2

- C. Munien, T.M. Rebelo, E. Ferreira and S.F.T Weiss. IgG1-iS18 impedes the adhesive and invasive potential of early and late stage malignant melanoma cells. 2017. *Experimental Cell Research*, 351, 135-141, doi: 10.1016/j.yexcr.2017.01.009.

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List of abbreviations

AIF	Apoptosis-inducing factor
Apaf-1	Apoptotic protease activating factor-1
APC	Allophycocyanin
ATCC	American type culture collection
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
CAT	Chloramphenicol acetyltransferase
CO_2	Carbon dioxide
DD	Death domain
DED	Death effector domain
DISC	Death induced signalling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
ECM	Extracellular matrix
FACS	Fluorescene Activated Cell Scanning
FADD	Fas-associated death domain
FISH	Fluorescent *in situ* hybridization
FITC	Fluorescein isothiocyanate
FCS	Fetal calf serum

HRP	Horseradish peroxidase
IAP	Inhibitor of apoptosis
IgG	Immunoglobulin G
kDa	Kilodaltons
LRP/LR	Laminin receptor precursor/ high affinity laminin receptor
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PAGE	Polyacrylamide gel electrophoresis
PE	Phycoerythrin
PI3K	Phosphoinositide 3-kinase
PBS	Phosphate buffered saline
PCA	Protocatechuic acid
PI	Propidium Iodide
PS	Phosphatidyl serine
PVDF	Polyvinylidene fluoride
RISC	RNA-induced silencing complex
Rpm	Revolutions per minute
RLUC	Renilla luciferase
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
SDS	Sodium dodecyl sulfate

siRNA Small interfering RNA

TEMED Tetramethylethylenediamine

TNFR Tumour necrosis factor receptor

VEGF Vascular endothelial growth factor

List of Symbols

α	Alpha
β	Beta
γ	Gamma
µl	Microlitre
ml	Millilitre
µg	Microgram
mg	Milligram
M	Molar
mM	Micromolar
min	Minutes
nm	Nanometre
ng	Nanogram
^{o}C	Degrees Celcius
A	Amps
V	Volts
%	Percentage

Chapter 1: Introduction

1. Cancer

1.1 Epidemiology, diagnosis and treatment

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 [1]. 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, of which 8 million occurred in economically developing countries (82% of the world's population). 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].

Furthermore, cancer incidence rates are expected to increase by 70% over the next 2 decades, especially in economically developed countries where it is implicated as the primary cause of death. Similarly, in economically developing countries, cancer is identified as the second leading cause of death [1]. Transformation of normal cells into the tumourigenic state is a multistep process [1]. These changes occur as a result of the interaction between genetic factors and external factors such as physical carcinogens which include ultraviolet radiation and ionizing radiation, chemical carcinogens such as tobacco smoke, biological components such as viral or bacterial infections as well as the aging and growth of the world population [1].

The most commonly diagnosed cancers worldwide are breast cancer in females and lung cancer in males [1] and in 2002, 63,800 new cases of cancer were diagnosed in South Africa and 44,200 cancer deaths reported [3].

The present study focuses on early (A375) and late (A375SM) stage malignant melanoma which was ranked 19th worldwide in 2012 contributing to 1.6% of the total number of cases diagnosed (Fig.1) [4]. Malignant melanoma is the most dangerous and deadly form of skin cancer, accounting for approximately 74% of skin cancer related deaths [4]. According to the National Cancer Registry, in South Africa in 2010, 551 males and 509 females were diagnosed with

melanoma. These statistics exemplify the urgent need to develop novel therapeutic strategies to combat this deadly form of skin-cancer.

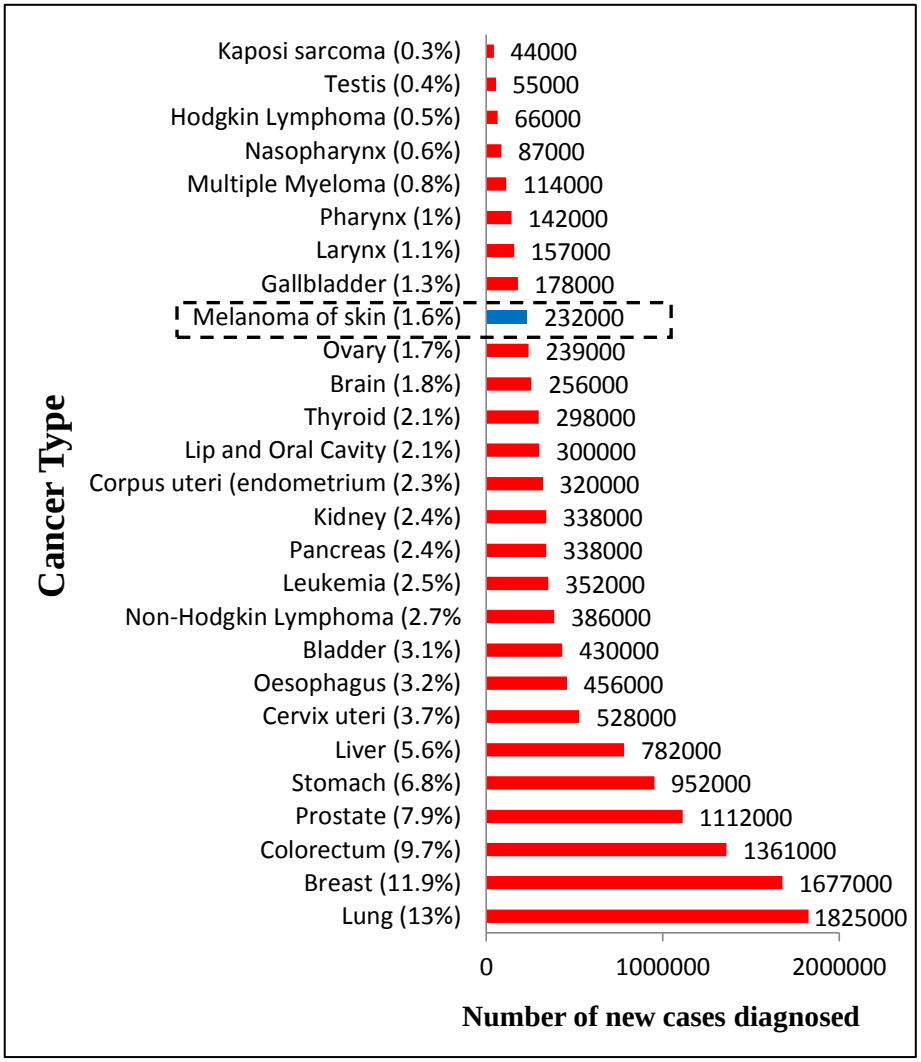


Figure 1: The number of worldwide cancer cases diagnosed in 2012. (http://www.wcrf.org/int/cancer-facts-figures/worldwide-data).

A significant proportion of the worldwide cancer burden is lifestyle related and deaths occurring thereof may be prevented through the application of existing cancer control knowledge, by implementing programs for tobacco control, vaccinations as well as public health campaigns encouraging physical activity and healthier lifestyle behavior [1]. Early diagnosis of cancer is also pivotal to one's health, thus allowing for a higher prospect of recovery and the prevention of metastasis and resultant formation of secondary, tertiary and quaternary tumors at distant sites in

the body [5]. Imaging techniques provide an image of a visible tumor and it is the most common method for cancer detection and subsequent diagnosis. These include Magnetic Resonance Imaging (MRI), X-rays, CT scans and ultrasound [6]. Cancer diagnosis can also be performed via the use of biopsies, genetic testing as well as fluorescent in *situ* hybridisation (FISH) [7].

Tumor formation is not exclusive to a particular site and the course of treatment depends on its location, the type of cancer and the state of advancement [6]. Surgery is considered the first treatment for the removal of solid, benign tumors in early stage cancers [6]. Other treatments include radiation, which kills cancer cells by damaging the cell's DNA, thereby preventing its replication [6]. Chemotherapy is another treatment option; targeting rapidly growing cells i.e. cancer cells [6]. More advanced therapeutics include gene therapy, viral therapy and immunotherapy [7].

1.2 Malignant melanoma

1.2.1 Characterization

Malignant melanoma has a lower incidence rate than other cancer types but is the most dangerous form of skin cancer as it causes the greatest number 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 such as the lungs, liver, bones and brain thus causing death. The global incidence of malignant melanoma continues to increase, with 1 in every 3 cancer type diagnosed being skin cancer [8]. Malignant melanoma occurs within the skin, an organ consisting two layers; the epidermis (outer layer) and the dermis (underneath) as seen in Figure 2. However, the cells which become cancerous are known as the melanocytes (pigment-producing cells) and they are located in the bottom layer of the skin's epidermis, more specifically in the stratum basale. Neoplastic tumors or neoplasms develop from these melanocytes which are cancerous [9]. Alterations in several molecular pathways have been linked to the distinct characteristics of malignant melanoma and have been reported to include: tyrosine kinase receptor (TKR) pathway, Ras/Raf, MEK/ERK pathway, PI3K/Akt/ PTEN/ mTOR pathway, cell cycle regulation pathways, programmed cell death pathways, common apoptosis effectors, protein chaperoning and degradation [10].

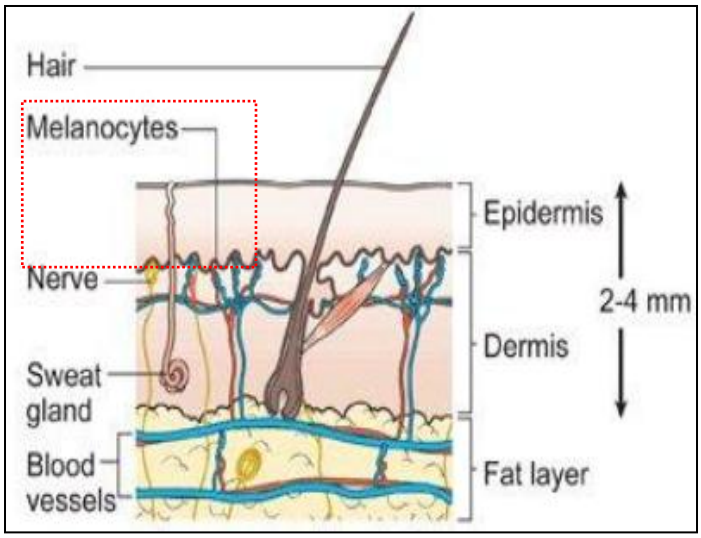


Figure 2: The structure of the skin. The melanocytes are the pigment-producing cells situated in the bottom layer of the skin's epidermis, more specifically in the stratum basale. These cells become cancerous in the case of malignant melanoma (http://www.cancerresearchuk.org/).

1.2.2 Signs, symptoms and risk factors

Malignant melanoma can develop anywhere on the human body, however they most often develop in areas that are exposed to sunlight, such as the legs, arms, face and back. Melanoma can however be found in areas that do not receive as much sun exposure, such as the soles of the feet, palms as well as the fingernail bed and this is most commonly observed in darker skinned individuals. The first signs and symptoms that arise with melanoma are the changes in an existing mole or the developmemt of a new pigment or unusual- looking growth on the skin according to the ABCDEs of melanoma [9] (Fig. 3).

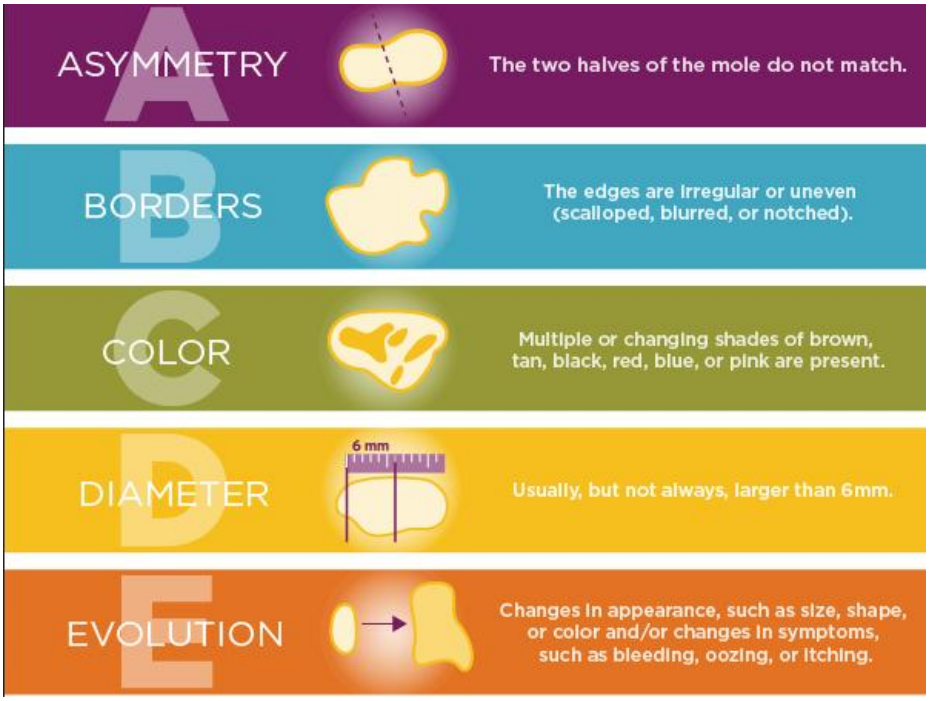


Figure 3: The ABCDEs of melanoma. The signs and symptoms that may assist in the early detection of the disease according to the symmetry, border, colour and diamter of the mole present on the skin. Obtained from (http://www.melanomauk.org.uk/wp-content/uploads/2016/11/ABCDE-Melanoma.png)

One of the main risk factors for malignant melanoma is high exposure to ultraviolet light from direct sunlight or from sunbeds. Other risk factors include: many moles present on the skin, having fair skin that burns easily and does not tan, a history of sunburn especially in childhood or adolescence, previously diagnosed with skin cancer or melanoma, a family history of melanoma or other skin cancers and a weak immune system [11].

1.2.3 Diagnosis and staging

Melanoma can be diagnosed by observing the natural behaviour of the skin but a more efficient method of diagnosis includes a biopsy, in which all or a part of the suspicious mole or growth is removed and analysed. The next step after diagnosis is to determine the extent (stage) of the cancer. The stage of melanoma describes how deeply it has ulcerated into the skin, the thickness of the melanoma and whether it has spread to distant sites in the body [11]. Melanoma can be staged according to four different systems however, in this

particular case the number stages will be used to describe the five main stages of melanoma (Fig. 4). Stage 0 (*in situ*) melanoma describes the location of the tumor being limited to the outer layer of the skin without the ability to spread, at this stage the tumor can be surgically removed. Stage 1 (A and B) is considered early stage melanoma characterized by a growth of less than 2mm thick. Stage 2 (A, B and C) melanoma is charaterised by a thickness of more than 2 mm without any signs of spreading to the lymph nodes or other parts of the body. Stage 3 (A, B and C) melanoma has now spread to nearby lymph nodes and has to be removed. Stage 4 melanoma is the advanced/ late stage and the cancer has now spread to other parts of the body, distant from the primary site of origin and to nearby lymph nodes [11].

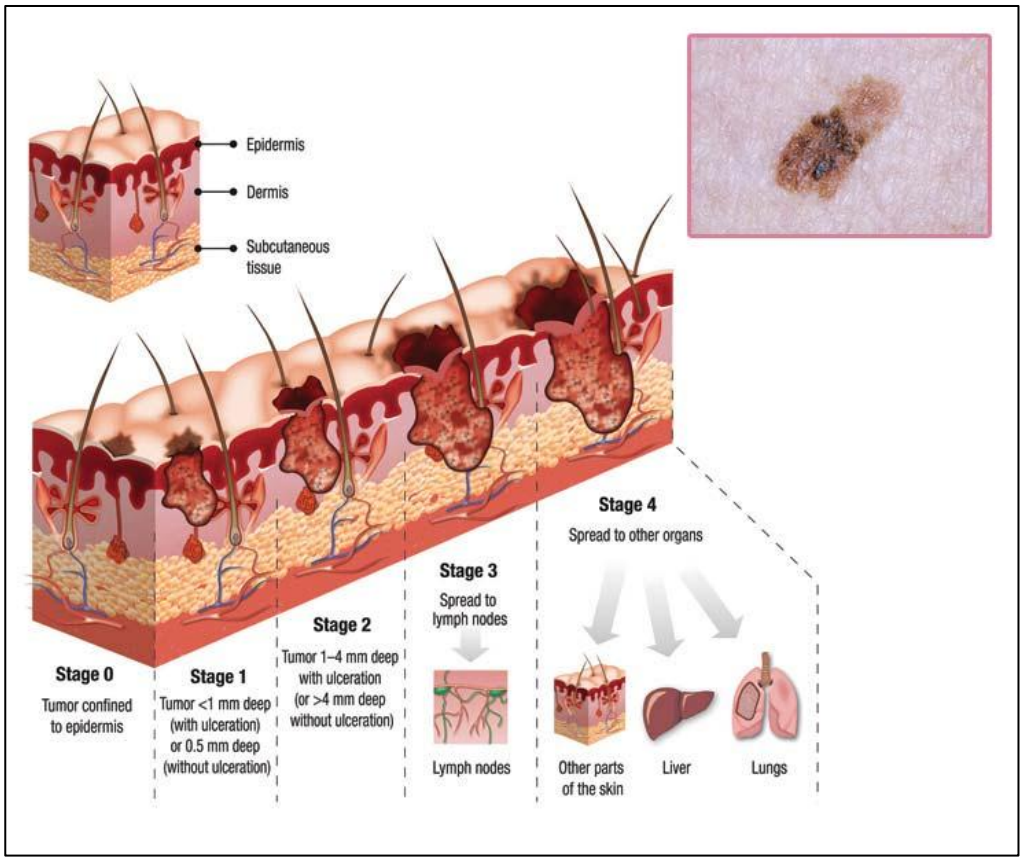


Figure 4: The numerical staging of melanoma. At stage 0, the tumor is confined to the outer layer of the skin (epidermis). At stage 1, the tumor is less than 2mm thick with or without ulceration (breakdown of the skin layer over the site of the melanoma). At stage 2, the tumor is greater than 2 mm thick with or without ulceration. At stage 3 and 4, the tumor

has spread to the lymph nodes and other organs, respectively (http://www.hlifestyle.net/wp-content/uploads/2015/05/Stage-0-4-melanoma-layers-diagram-Medium.jpg).

1.2.4 Treatment

The most effective treatment regime for malignant melanoma will depend on the size and stage of the cancer as well as the state of the individual's health and immune system. The treatment for early stage melanoma includes surgery and therefore removal of the cancerous mole and a section of the surrounding normal skin. No further treatment is usually required at this stage as the melanoma is confined to the epidermis without further ulceration into the skin and has not metastasised. Medium stage melanoma (stage 2 and 3) requires surgical removal, followed by an additional operation referred to as a wide local excision to remove more tissue and possible melanoma cells that may have been left behind in the area. In conjuction to surgery, a sentinel node biopsy test may be performed to determine whether the melanoma cells have metastasised into the nearby lymph nodes [11]. For advanced stage melanoma, a combination of treatment regimes may be employed. The treatments include: chemotherapy and radiotherapy which may help shrink the melanoma and relieve symptoms, biological therapies such as interferon and interleukin 2 which may assist in shrinking and controlling the melanoma for a short period of time [11].

1.3 Cancer

1.3.1 Molecular mechanism of cancer

Cancer is defined as the uncontrolled proliferation of cells due to the diminished levels of apoptosis or programmed cell death, thus resulting in abnormal cellular growth [12, 13]. In the cancerous state, damaged, old or abnormal cells survive whilst new cells divide uncontrollably to form growths referred to as either a malignant tumor (invasive) or a benign tumor (non-invasive). Cancer cells are not as specialized in terms of function in comparison to normal cells and therefore have the ability to divide uncontrollably. The transformation ability of a normal cell to a cancerous state is referred to as tumorigenesis, and it is facilitated by several cellular alterations which can be collectively described as "the hallmarks of cancer" (Fig. 5) [14].

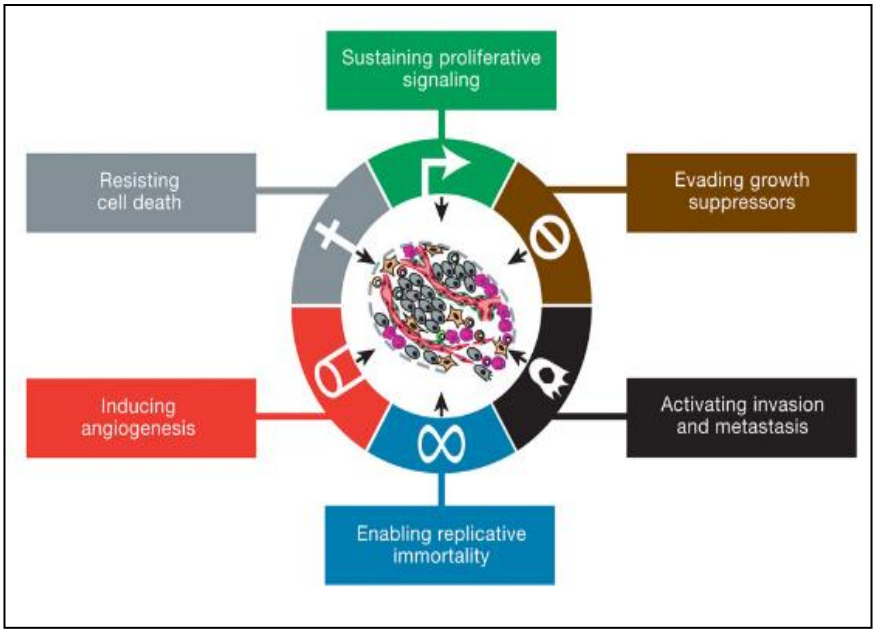


Figure 5: The hallmarks of cancer. The six hallmarks responsible for the transformation of a normal cell into the tumorigenic state [14].

1.3.2 The hallmarks of cancer

In order for a normal cell to be transformed into the neoplastic state, the acquisition of six essential traits are required- as descibed below (Fig. 5).

1.3.2.1 Cancer cells- sustaining proliferative signaling

Normal cells control the production and release of growth-promoting signals that direct entry into and the progression through the cell's growth-and-division cycle, thus ensuring homeostasis of cell number, tissue arichitecture and cellular function [14]. However, cancer cells have the ability to deregulate these signals and sustain chronic proliferation [14]. This may occur via the following mechanisms: cancer cells may produce their own growth-factor ligands, allowing them to respond via the expression of associated receptors, thus resulting in autocrine proliferative stimulation [14]. Alternatively, cancer cells may stimulate the growth of normal cells within the tumor-associated stroma by sending signals which in turn supply the cancerous cells with various growth factors [15, 16]. The levels of receptor protein present on the cancer cell surface may be elevated, rendering the cell hyper responsive to limiting amounts of growth factor ligand or structural alterations in the receptor molecule which render the same outcome [14]. Furthermore,

somatic mutations in the B-Raf protein and Phosphoinositide 3- kinase (PI3-kinase) isoforms may be responsible for the disruption of the Mitogen-activated protein (MAP) - kinase [17] and PI3K signalling pathways [18], respectively.

1.3.2.2 Cancer cells- evading growth suppressors

Cancer cells have the ability of inducing and sustaining growth-stimulatory signals, however in order for them to continuously grow and proliferate they must also be able to evade programs that negatively regulate cell proliferation; many of which depend on the actions of tumor suppressor genes [14]. The two key tumor suppressor genes: RB (retinoblastoma-associated) and p53 operate as central control nodes which govern the decision of cells to proliferate or, alternatively, activate cellular senescence and apoptosis [14].

The essential function of the RB protein is to integrate extracellular and intracellular signals and in turn determine whether or not the cell qualifies to enter through the cell growth and cell division cycles [19-21]. Defective RB proteins therefore allow cancer cells to proliferate uncontrollably due to the lack of effective regulatory functions provided by the RB pathway [14]. p53 on the other hand, receives stress and abnormality signals through the intracellular sensors of the cell, which operate to halt further cell-cycle progression if excessive damage is caused [14]. p53 is a pro-apoptotic member which induces apoptosis in response to excessive damage to the genome and unfavourable cellular conditions. Cancer cells thereby have the ability to evade the action of p53, allowing the cells to avoid apoptosis and continue proliferating [22].

1.3.2.3 Enabling replicative immortality

Normal, non-cancerous cells are able to undergo a limited number of growth and division cycles due to the association with two key barriers to proliferation: senescence, the irreversible entrance into the nonproliferative but viable state, and crisis, which involves cell death [14]. However, tumorigenic cells are able to go through unlimited amounts of growth and division cycles thus exhibiting unlimited replicative potential via the transition of immortilization [14]. According to extensive lines of evidence, telomeres play a key role in immortilization, thus allowing for unlimited proliferation [23]. Telomeres are composed of several tandem repeats and are responsible for the protection of the ends of chromosomal DNA from damage such as end-to-end fusions [23, 24]. Telomerase is a specialised RNA dependent DNA polymerase that adds

telomere repeats to the ends of chromosomes, thereby preventing progressive telomere erosion from occurring [14]. Furthermore, the length of telomeric DNA governs the number of replicative cycles a cell can endure before erosion occurs and the protective function of the telomere has been lost [14]. Therefore, cancerous cells exhibit a significantly increased level of telomerase expression in comparison to the normal cell counterpart, thus reducing telomere shortening and allowing cells to continually proliferate by avoiding senescence and crisis or apoptosis [14].

1.3.2.4 Activating tissue adhesion and invasion - key components of metastasis

Tumor cells have the ability to selectively down-regulate and inactivate key cell-to-cell adhesion molecules (CAMs) such as E-cadherins [14]. This occurs as E-cadherin has the ability to form sheets of epithelial cells which are successfully kept in the quiescent state. Down-regulating the expression of E-cadherin allows cells to advantageously undergo tumorigenic transformation [25]. Specific proteases, such as collagenase are up-regulated in tumor cells, and this up-regulation results in the degradation of the extracellular matrix (ECM), which in turn allows the cancerous cells to migrate to and invade secondary organs via the degradation of the basal lamina [26].

1.3.2.5 Induction of tumor angiogenesis

Tumor cells, like normal cells require a sustained source of nutrients and oxygen as well as the removal of metabolic waste and carbon dioxide in order to grow and survive effectively [14]. During tumor progression, normal quiescent vasculature is continuously producing new vessels in order to sustain the expansion of neoplastic growths, and this occurs as a result of the activated "angiogenic switch" [27]. The angiogenic switch is governed by angiogenic regulators which are signaling proteins that bind to stimulatory or inhibitory cell surface receptors. Therefore, in tumor cells, the expression of the angiogenic inducer known as vascular endothelial growth factor (VEGF) is deregulated allowing for the initiation and promotion of blood vessel formation [28, 29]. The promoted blood vessel formation creates the vasculature required by the tumor for nutrients and oxygen as well as waste disposal [29].

1.3.2.6 Resisting cell death/ apoptosis

Apoptosis is referred to as programmed cell death and it acts as a natural barrier in the prevention and development of cancer. The apoptotic process is triggered in response to cellular stress and DNA damage, thereby maintaining a homeostatic environment within the cell [30]. Tumor cells are able to evade apoptosis and in this way, the tumor is able to extensively proliferate and progress [30]. The loss or the deregulation of the p53 tumor suppressor gene enables cancerous cells to avoid undergoing apoptosis and proliferate irrepressibly to form aggressive tumors [31]. Additionally, in the tumorigenic state the cells up-regulate the expression of anti-apoptotic factors and down-regulate the expression of pro-apoptotic factors in order to evade apoptosis [32, 33]. Evasion of apoptosis by tumor cells has been implicated as the key contributing factor in the aggressiveness of many cancer types [14], therefore it is crucial to target the mechanisms that govern this hallmark with respect to cancer therapeutics- this being the main focus of the present study.

1.3.3 Apoptosis

Apoptosis is a morphologically and biochemically well-ordered process of cell death that occurs via a genetically determined cell suicide programme that serves an essential function in embryonic development and maintenance of tissue homeostasis [34]. In contrast, necrosis is a form of cell death that is considered as non-programmed. Apoptosis has been described by its induced morphological characteristics, including DNA fragmentation and chromatin condensation in early apoptosis and membrane blebbing, diminished membrane integrity and alterations in the structure of cytoplasmic organelles in late apoptosis [34, 35]. The apoptotic process is crucial from infancy throughout adulthood, but defects that arise in the apoptotic pathways are known to contribute to several human diseases, including autoimmune diseases, AIDS, neurodegenerative disorders and malignancy [36]. However, biochemical alterations to the apoptotic cell include activation of caspases, protein and DNA breakdown and phagocytosis as a result of membrane changes [37]. The membrane integrity of the cell is lost as it moves from an early to a late apoptotic phase, thus resulting in the package of the cellular contents into apoptotic bodies, stimulating phagocytosis of dead cells by macrophages without harming other cells (Fig. 6) [37]. Apoptosis can be induced by internal stimuli such as DNA damage and external stimuli such as oxidative stress [13]. The apoptotic machinery is composed of upstream

regulators and downstream effector components [30]. The regulators are divided into two key circuits; one receiving and processing extracellular death-inducing signals (the extrinsic apoptotic pathway) and the other pathway which senses and integrates a variety of signals of intracellular origin (the intrinsic pathway). Each of the above described pathways, result in the activation of the latent Cysteine- aspartic protease (caspase) 8 and 9, respectively [14]. The family of proteases is abundant but in particular to the apoptotic pathways, caspase 8, 9 and 10 serve as initiator caspases and caspase 3, 6 and 7 function as the effector caspases [38]. Once activated, a cascade of proteolysis is initiated involving effector casapases which are responsible for the execution phase of apoptosis [14]. The intrinsic apoptotic pathway is widely implicated as the barrier to cancer pathogenesis [14].

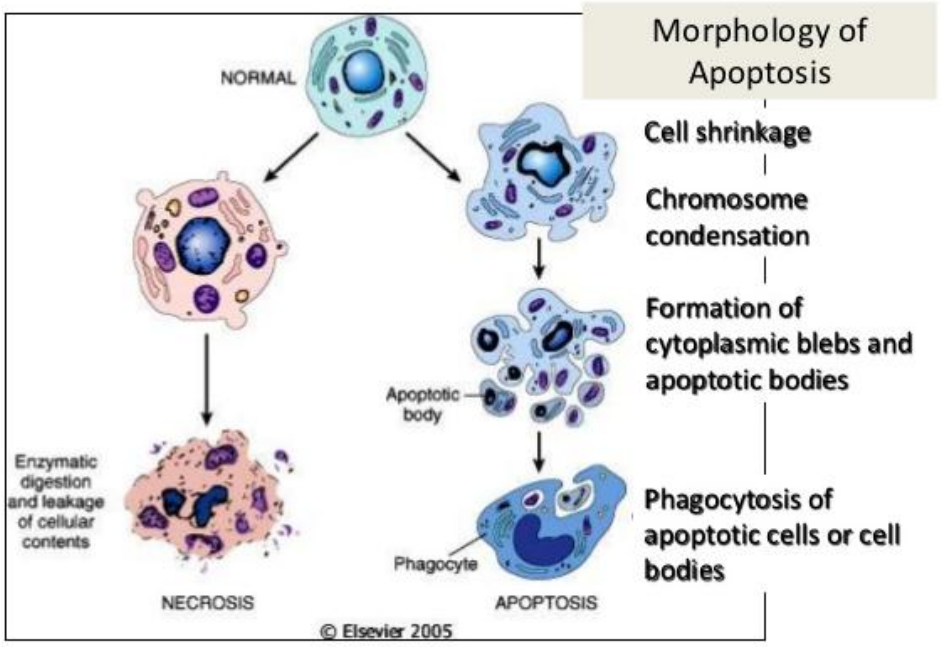


Figure 6: The morphological and biochemical alterations in cells undergoing apoptosis. Once apoptosis is induced, the cell begins to shrink and the DNA is fragmented. Thereafter, membrane blebbing occurs and at a later stage phosphatidylserine (PS) is exposed on the outer surface of the cell membrane. Through this process, dead cells can be recognised by macrophages, allowing phagocytosis of the resultant apoptotic cells or cell bodies (http://image.slidesharecdn.com/apoptosis-121108135829-phpapp01/95/apoptosis-3-638.jpg?cb=1352383200).

1.3.3.1 Death receptor-mediated (extrinsic) pathway

The extrinsic signalling pathway initiates apoptosis via transmembrane receptor-mediated interactions which involve death receptors of the tumor necrosis factor (TNF) receptor gene superfamily [39, 40]. Members of the TNF receptor family have a cytoplasmic domain made up of 80 amino acids and it is referred to as the "death domain" [41]. The death domain plays a role in transmitting the death signal from the cell surface to the intracellular signaling pathways [39]. Some of the well-characterized ligands and corresponding death receptors include Apo3L/DR3, Apo2L/DR4 and DR5, TNF-α/TNFR1 and FasL/FasR [41-45]. However, the FasL/FasR and TNF-α/TNFR1 models best describe the sequence of events that define the extrinsic phase of apoptosis. In these two models, the FasR and TNFR1 receptors group and the homologous trimeric ligand FasL and TNF- α binds, respectively, upon binding, cytoplamic adaptor proteins are recruited exhibiting a corresponding death domain which binds to the receptor [39]. The binding of the Fas ligand to the Fas receptor results in the subsequent binding of the FADD adaptor protein and the binding of the TNF ligand to the TNF receptor resulting in the binding of the TRADD adaptor protein with additional recruitment of FADD and RIP [39]. FADD then associates with procaspase-9 through the dimerization of the death effector domain [39]. A death-inducing signalling complex (DISC) is formed, leading to the activation of procaspase-8 via auto-catalysis (Fig. 7) [39]. Upon activation of caspase-8, the execution phase of apoptosis is triggered.

The extrinsic pathway can also be inhibited by certain factors such as the c-FLIP protein which binds to FADD and caspase-8, rendering them ineffective. Another protein referred to as Toso is seen to inhibit caspase-8 processing, via the Fas-induced apoptotic pathway [39].

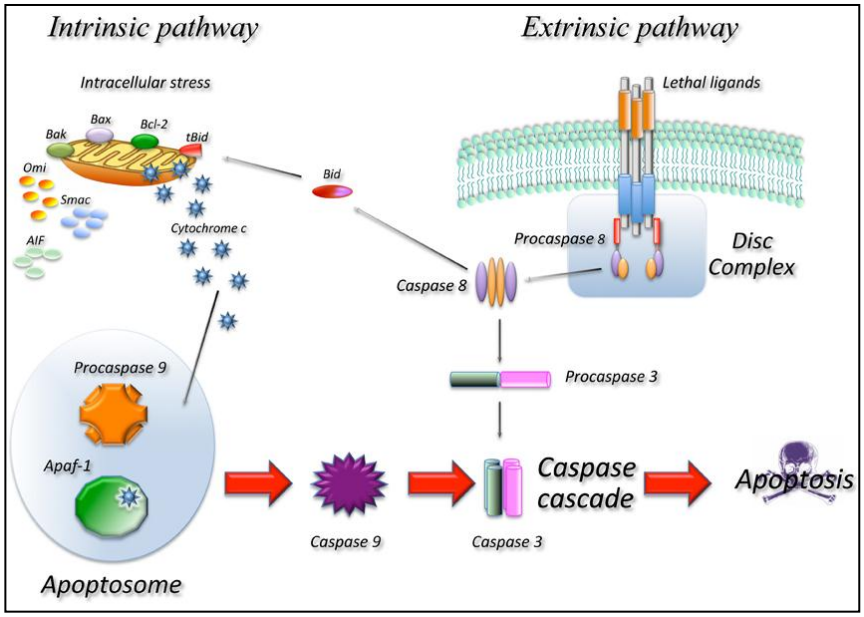


Figure 7: The two main apoptotic pathways by which programmed cell death occurs. (http://www.impactaging.com/papers/v4/n5/full/100459/Figure1.jpg).

1.3.3.2 Mitochondrial-dependent (intrinsic) pathway

The intrinsic signaling pathway initiates apoptosis via a diverse collection of non-receptor-mediated stimuli which produce intracellular signals that act directly on cellular targets [39]. These events are mitochondrial-dependent. The non-receptor-mediated stimuli produced intracellular signals may act in either a negative or positive manner, with the negative signals involving the absence of certain growth factors, hormones and cytokines [39]. This can therefore lead to the failure in suppression of death programs, triggering apoptosis [39]. Several stimuli which act in a positive fashion include radiation, toxins, hypoxia, hyperthermia, free radicals and viral infections [39]. These stimuli result in the change of the inner mitochondrial membrane leading to the opening of the mitochondrial permeability transition (MPT) pore, loss of mitochondrial transmembrane potential and the release of two main groups of pro-apoptotic proteins from the intermembrane space and into the cytosol [39]. The first group of proteins activate the caspase-dependent mitochondrial pathway and they consist of cytochrome c, Smac/DIABLO, and the HtrA2/Omi serine protease [39]. Cytochrome c in particular, binds to and activates Apaf-1 and procaspase-9 forming an apoptosome (Fig. 7) [46, 47]. The procaspase-9 protease forms a cluster, and activates

caspase-9. Smac/DIABLO and HtrA2/Omi promote apoptosis by inhibiting the Inhibitors of apoptosis proteins (IAP) activity [48, 49]. The second group of pro-apoptotic proteins are released from the mitochondria during apoptosis, this occurs as a late event after the cells have died [39]. These groups of proteins include AIF, CAD and endonuclease G [39]. AIF translocates to the nucleas causing DNA fragmentation and condensation of peripheral nuclear chromatin [50]. AIF and endonuclease G function in a caspase-independent manner. Furthermore, CAD is released from the mitochondria where it translocates to the nucleas in order to fragment DNA, after cleavage by caspase-3 [39].

The dysregulation of these apoptotic pathways has been implicated in several pathological conditions such as cancer and neurodegenerative disorders as previously stated. Cancer is seen to show low levels of apoptosis thus allowing abnormal cellular proliferation and tumorigenesis- the main focus of the present study.

1.3.4 Relationship between apoptosis and cellular proliferation

Coordination and balance between apoptosis and cellular proliferation is crucial for tissue homeostasis and normal development in the adult [51]. Therefore apoptosis is responsible for the loss of damaged cells via the reduction in cell number, thus ensuring a balance in cell numbers [52]. Cell proliferation, differentiation and death are fundamental for the functioning of multicellular organisms and therefore several lines of evidence link the process of apoptosis and proliferation. Several genes including oncogenes, are involved in the regulation of the cell cycle as well as being pivotal to the induction of apoptosis and proliferation; such genes include c-fos, c-jun, c-myc and p53 [52]. Important for the association between cell death and cellular proliferation is the tumor suppressor gene- p53, whose essential role is to maintain genomic integrity by regulating cells in the G1-S and G2-M checkpoints of the cell cycle (Fig. 8) [52]. This is done in order to prevent abnormal cells from progressing through the cell cycle, and this is achieved via the detection of unfavourable genomic alterations [53].

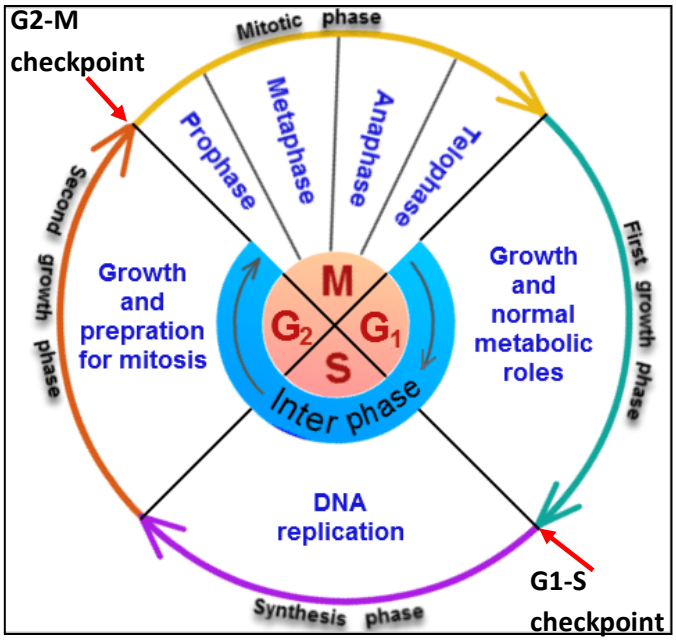


Figure 8: Stages of the cell cycle. Phase 0/Gap 0 is the stage at which the cells are metabolically active but quiescent. In order for inactive quiescent cells to enter into the G1 phase, stimulated entry by growth factors and nutrients must be employed in order to generate extracellular signals. Cells 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. (http://images.tutorvista.com/cms/images/123/cell-cycle-2.PNG)

p53 becomes activated in one of the following ways when an apoptotic promoting signal is detected; 1) activation of ARF or 2) inhibition of MDM2 [54]. Pro-apoptotic genes such as *Bax* and CD95 are activated upon p53 activation. p53 induces apoptosis via mitochondrial interactions that promote the release of cytochrome c which is a pro-apoptotic molecule [55]. When the *Bax* gene is directly activated by p53, the Bcl-2 oncogene becomes inhibited, thus hampering the inhibition of Smac- a pro-apoptotic IAP- neutralising molecule [55]. Hence, apoptosis is induced by the activation of a cascade of caspases, as a result of the interaction that occurs between Smac and cytochrome c (Fig. 9) [55].

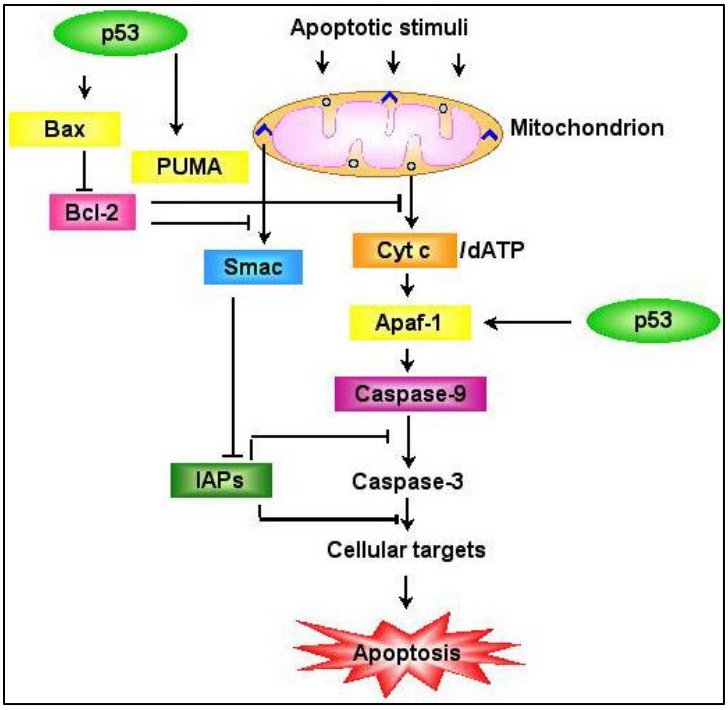


Figure 9: The role of p53 in apoptotic induction. Once an apoptotic signal is detected, p53 becomes activated, subsequently activating *Bax*. This causes an activation of Smac via Bcl-2 inhibition. Consequently, inhibitors of apoptosis (IAPs) become inactivated. The pro-apoptotic, cytochrome c molecule is then released from the mitochondrion upon detection of an apoptotic signal. Smac and cytochrome c interact in order to induce apoptosis via the intrinsic pathway. The apoptosis induction process is prevented if mutations in the p53 gene are present, thereby promoting the tumorigenic phenotype (https://webhome.weizmann.ac.il/home/ligivol/apoptosis_project/apoptosis.jpg).

1.3.5 Relationship between apoptosis and tumorigenesis

As previously mentioned, the occurrence and regulation of apoptosis relies exclusively on genes, thus making the process of cell death highly vulnerable to genetic mutations. The deregulation or suppression of apoptosis therefore contributes to carcinogenesis and the disruption of homeostasis via the aberrant expression of specific genes. This therefore allows for an increase is cellular life span, growth-factor independent cell survival and ultimately permitting uncontrolled cellular proliferation. Three protein families responsible for these processes are discussed below.

1.3.5.1 Bcl-2 family of proteins

This protein family is made up of 15 Bcl-2 family members, which include pro-apoptotic and anti-apoptotic proteins that are crucial for the regulation of apoptosis [56]. According to studies conducted, *bcl-2* does not exhibit the behavioural characteristics of a normal oncogene; instead of disrupting normal proliferation controls, Bcl-2 promotes cell survival by blocking apoptosis [57, 58, 59]. Many cancer types have been characterised by the altered expression of the genes encoding for these proteins [13]. The over-expression of Bcl-2 has been shown to contribute to the evasion of apoptosis by breast, brain [60] and prostate [61] cancers. Further studies have suggested that over-expression of the Bcl-2 proteins by cells, allows for the acquired multi-drug resistant phenotype, thereby aiding cells in tumorigenesis [62].

1.3.5.2 Tumor necrosis factor (TNF) family of proteins

Tumor cells often avoid the death receptor-mediated (extrinsic) pathway that induces apoptosis as a result of resistance to key components that are involved in this cell death pathway. The caspase homolog; c-Flip is an inhibitor of the death receptor pathway and has therefore been implicated in the occurrence of a variety of cancer types [63]. These inhibitors compete with caspases for binding to FADD, thereby inhibiting apoptosis and resulting in the formation of a Fas-resistant tumor [63]. The resultant tumors benefit from this resistance by having the ability to evade apoptosis in the presence of the Fas-ligand [63]. The resistance to Fas enables cells to express the Fas ligand on the cell surface without inflicting any harm on themselves, thus enhancing tumor progression [64].

1.3.5.3 Inhibitor of apoptosis (IAP) family of proteins

The IAP family has been suggested to interact with certain caspases and inhibit their pro-apoptotic function. Proteins that form part of the IAP family include: Survivin, cIAP1, cIAP2 and XIAP [65]. The IAP proteins have been seen to interact with Smac/DIABLO and HtrA2, thus leading to the activation of caspases and the induction of pro-apoptotic events [65]. Little is known about the expression pattern of genes encoding IAPs in tumorigenic cells, however the dysregulation of this expression has been implicated in many cancer types. In particular, the over-expression of Survivin and XIAP have been found in non-small cell lung carcinomas [66]

and the over-expression of the IAPs such as Livin and Apollon have been identified in melanoma cells [67] and gliomas [68], respectively.

1.3.6 The extracellular matrix (ECM)

1.3.6.1 The family of laminins

Laminins are non-collagenous glycoproteins with common as well as specific functions [69]. Laminins play a major role in the formation of the extracellular matrix (ECM), thus assisting in holding cells and tissues together [69]. The ECM is crucial in that it serves the principal function of supplying and co-ordinating signals that regulate efficient cellular functions, direct and control cell migration, ensure that the cells receive the adequate receptor-mediated provision of nutrients and facilitate cell-cell associations [70]. Furthermore, the ECM is composed of two essential domains, namely: the basement membrane and the condensed layer known as the matrix [71]. Other components that form the basement membrane are the proteoglycans, matrix metalloproteinases (MMPs) and a selection of glycoproteins such as integrins, elastin, fibronectins, collagens and specific to this study, the family of laminins [70, 71].

Laminin-1 is composed of three chains namely α-, β- and γ- chains (Fig. 10), which are constituted by three disulfide-linked polypeptides and assembled differentially to form 17 heterotrimeric laminin isoforms [72]. The heterotrimeric laminin isoforms can bind several ECM components such as collagen, entactin, proteoglycans and integrins [72]. One of the most important functions of laminin is to interact with receptors which are anchored in the plasma membrane of cells adjacent to basement membranes [69]. However, laminins have also been identified to play key roles in processes such as adhesion [73], cell migration [74], neurite outgrowth [75], cell differentiation and proliferation [76] and tumorigenic processes such as metastasis and angiogenesis [77].

As previously mentioned, laminins contain receptor- binding sites, one such receptor is the 37-kDa/67-kDa non-integrin laminin receptor precursor/ high-affinity laminin receptor (LRP/LR) [78] - which is of particular importance to this study.

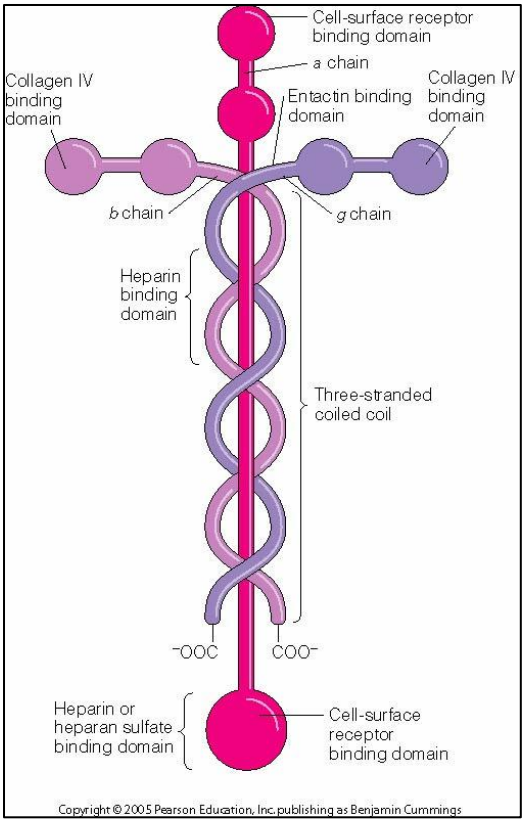


Figure 10: The structure of laminin-1. Laminins are composed of α-, β- and γ-chains which are stabilized by interchain disulphide bonds. Laminins serve to bind several ECM molecules such as collagen IV, heparin and entactin as well as a group of cell surface receptors (https://bshia13.files.wordpress.com/2009/05/laminin.jpg)

1.3.7 The 37-kDa/67-kDa laminin receptor precursor/ high affinity laminin receptor (LR/LR)

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 [79]. 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 (Fig. 11) [80]. 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 [81]. Early studies speculate that this mechanism may entail the process of

homodimerization that occurs via several post-translational modifications [81], however when a yeast two hybrid study showed failure of LRP to interact with itself and dimerize, this study was disproved [82]. Additional studies have suggested the process of fatty acylation by fatty acids such as oleate or stearate as the key modification that results in the maturation of the 37-kDa LRP into the 67-kDa LR form [83]. Recent studies have proposed the mechanism of SUMOylation of LRP as the modification that results in the maturation of the 37-kDa LRP into the LR form. LRP is therefore a target for small ubiquitin-like modifier (SUMO) proteins that have the potential to form oligomeric chains [84, 85].

When LRP/LR is found as a transmembrane receptor, it plays physiological roles in processes such as migration, cell adhesion, proliferation, and the maintenance of cellular viability [78]. 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 [86-91]. The receptor is seen to be overexpressed on the surface of tumorigenic cells such as cervical, lung ,prostate, colon [92], breast, oesophageal [93] and liver cancer cells [94], thus contributing to the process of cellular transformation in terms of 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 [92-94]. LRP/LR has also been shown to play a role in angiogenesis, an important hallmark of cancer. When blood vessels were treated *in vitro* with the anti-LRP/LR- specific antibody W3, blood vessel formation was significantly impeded [95]. LRP/LR has been implicated as a key contributor to the pathogenesis of viral and bacterial infections [96], prion protein related diseases [97], neurodegenerative diseases such as Alzheimer's disease [98-102] as well as enhancing telomerase activity [103] through the overexpression of LRP::FLAG [104] which is seen to play a role in the progression of the above mentioned diseases [105] as well as chemotherapy resistance in some cancers.

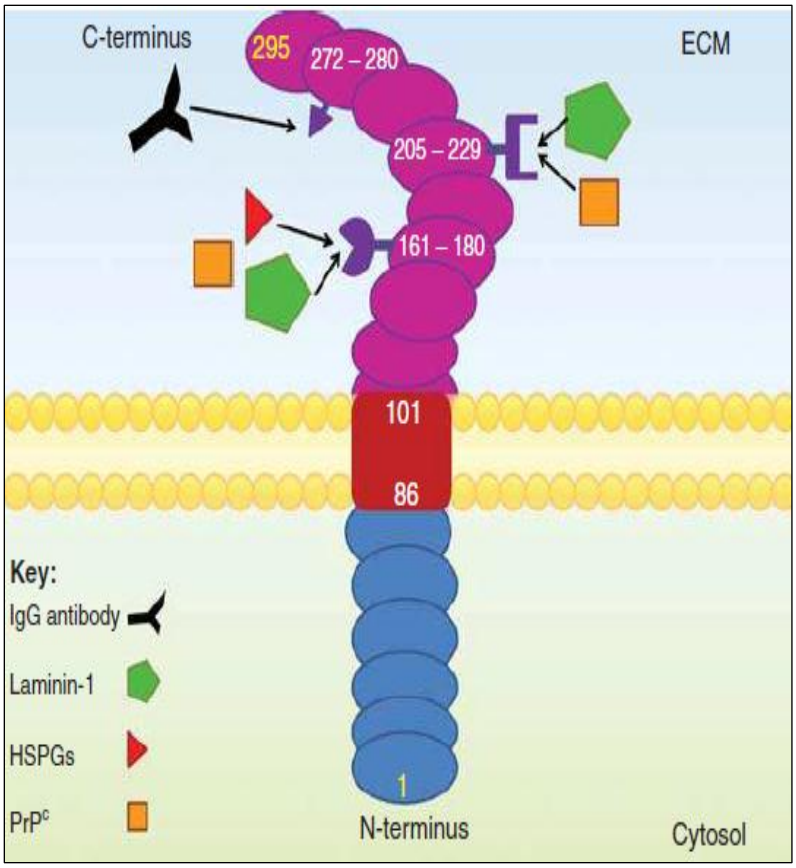


Figure 11: Structural representation of the 37 kDa/ 67 kDa laminin receptor (LRP/LR). This transmembrane receptor consists of 295 amino acids and comprises three domains: the intracellular cytosolic N-terminal domain (blue), transmembrane domain (maroon) and the extracellular C-terminal domain (purple). The amino acid region 161-180 serves as a binding site for laminin-1 and heparin and amino acid region 272-280 is the IgG antibody binding domain [80].

1.3.8 LRP/LR and evasion of apoptosis

LRP/LR plays a role in the maintenance of cell viability, as previously stated. Cancerous cells strive to avoid cell death and the overexpression 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 [106]. The Midkine protein assists LRP/LR in binding to the nuclear envelope and chromatin during interphase of the cell cycle in order to stabilize chromosomes, thereby assisting in maintaining cellular viability [106]. Focal

Adhesion Kinases (FAKs) interact with LRP/LR as a result of the LRP/LR- laminin interaction [80]. 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. This process inhibits apoptosis (Fig. 12B) [80]. LRP/LR up-regulation in cancer cells facilitates tumor enhancement and progression by promoting apoptotic evasion [107]. A study involving the use of small interfering RNAs (siRNA) directed towards the mRNA of the 37- kDa LRP in liver (Hep3b) cancer cells, confirmed the role played by LRP/LR in the inhibition of apoptosis [107]. Recent studies indicated that LRP/LR does indeed play a role in inducing apoptosis and this was observed by the down-regulation of LRP expression using siRNA-LAMR1 in breast and oesophageal [108], cervical (HeLa) and lung (A549) [109] as well as pancreatic (AsPC-1) and neuroblastoma (IMR-32) cancer cells [110] (Fig. 12A(D)).

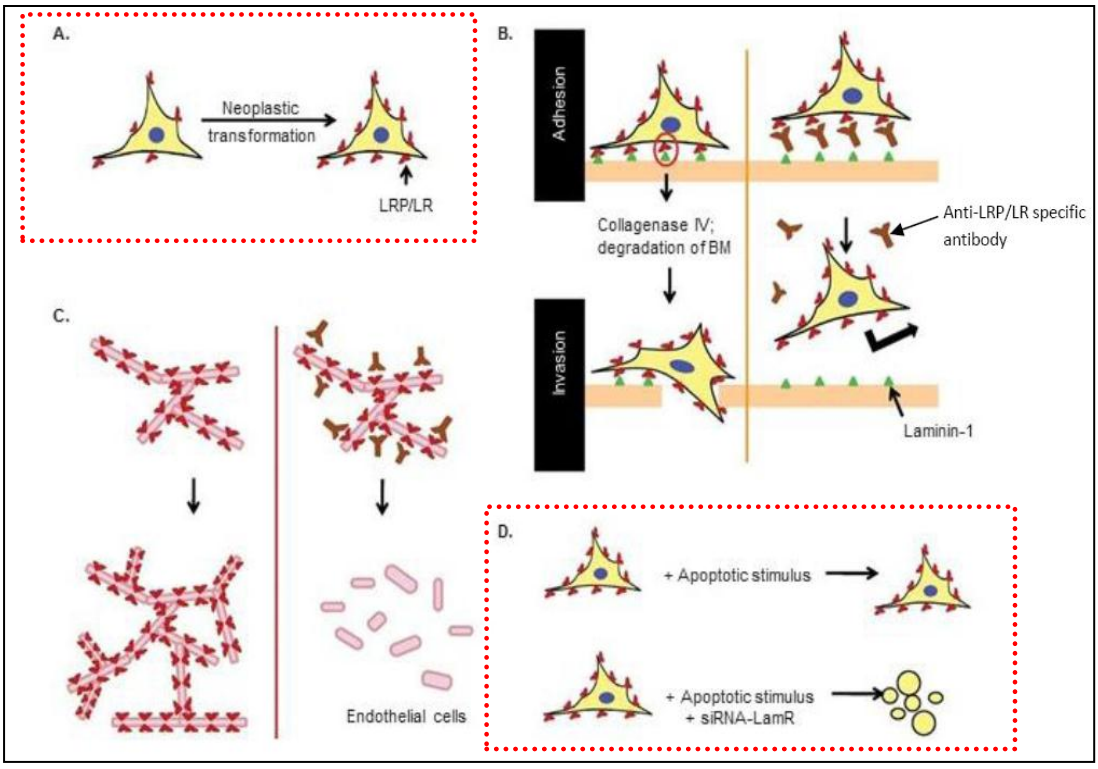


Figure 12A: The role of LRP/LR in tumorigenesis. A) Several cancer cells exhibit an over-expression of LRP/LR in comparison to their normal cell counterparts. B) Up-regulation of LRP/LR in tumor cells facilitates an increased binding of the receptor to laminin-1 in the basement membrane (adhesion), leading to the activation of type IV

collagenase, triggering the degradation of type IV collagen in the basement membrane- therefore allowing tumor cells to invade organs (invasion). The key events in metastasis, adhesion and invasion are impeded upon blockage of LRP/LR with the anti-LRP/LR-specific antibody IgG1-iS18. C) Increased levels of LRP expression have been implicated in the formation of endothelial tubes, thus suggesting that the receptor plays a pivotal role in tumor angiogenesis. D) Of particular importance to this study: Tumor cells survive by evading apoptosis; however the use of siRNA targeted to LRP/LR successfully induced apoptosis in certain cancer types by LRP down-regulation (Adapted from Jovanovic et al, 2015 [80]).

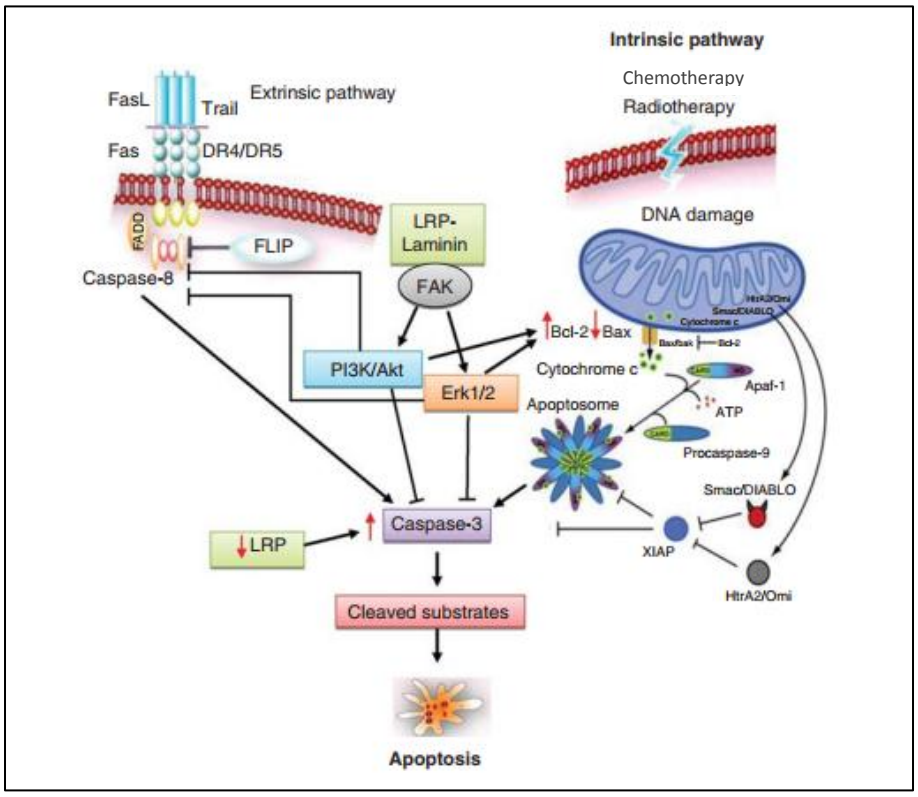


Figure 12B: Schematic representation of the role performed by LRP/LR in extrinsic and intrinsic apoptotic pathways. Specific trigger signals are required by each pathway to initiate an energy-dependent molecular cascade of events. The extrinsic pathway is activated through the binding of ligands to surface receptors and for the intrinsic pathway, internal threats result in the release of intracellular proteins. The executioner caspases are then

activated, resulting in characteristic morphological features within the execution pathway. These include: cell shrinkage, formation of cytoplasmic blebs**,** chromatin condensation and apoptotic bodies. The binding of LRP/LR to laminin, activates the PI3K/Akt and Mek/Erk1/2 survival pathways, increasing Bcl-2 levels and inhibiting caspase-3 and caspase-8. There are several other pathways by which apoptosis can be induced that are caspase dependent, however only the extrinsic and intrinsic pathways have been depicted here. (Adapted from Jovanovic et al, 2015 [80])

Due to the affirmation that LRP/LR plays a critical role in maintaining cellular viability, this receptor can therefore act as a target for the development of possible therapeutics for the treatment of cancer.

1.3.9 RNA interference

RNA interference/ silencing refers to a gene regulatory mechanism in which small, 20- to 30- nucleotide noncoding RNAs and their associated proteins control the expression of genetic information [111]. RNA interference events have been described in the majority of eukaryotic organisms [112]. The gene silencing functions to limit the level of transcription by either suppressing transcription (by a process referred to as transcriptional gene silencing (TGS)) or by activating a sequence- specific process of RNA degradation (by a process referred to as posttranscriptional gene silencing (PTGS) or RNA interference (RNAi)) [112]. This mechanism of regulation is employed by small interfering RNAs (siRNAs), which are double stranded RNA (dsRNA) molecules that aid in the degradation of sequences that are complementary to and present in the target mRNA [113]. During the process of PTGS/RNAi, the dsRNA molecules, cleave the inducer molecules into smaller fragments, degrading the target mRNA molecules by serving as the activators and inducers of this process [112]. The process of RNAi controls many crucial processes such as tissue differentiation, heterochromatin formation, cell proliferation as well as cell growth however, if this process is dysregulated it can be linked to the formation of neurological disorders, cardiovascular disease and several cancer types [114].

In order to assist in the effective functioning of the PTGS/RNAi process, two enzyme complexes, namely Dicer and RISC are of importance (Fig. 13) [115].

Dicer is an enzyme that forms part of the RNase III nuclease family and it is involved in the initiation of the PTGS/RNAi process, while being highly specific for dsRNA [112]. Functionally, the dimeric Dicer folds onto dsRNA, cleaving it into short nucleotide duplexes; known as small interfering ribonucleic acids (siRNAs) of approximately 21-23 bp in length, with 3' overhangs of 2-3 nucleotides as well as 5'-phospate and 3'-hydroxyl termini [116].

Research by Hammond *et al*, (2000) [115] indicated that targeting endogenous genes of *Drosophila S2 cells* in a sequence-specific manner could be achieved by transfecting these cells with dsRNA. Due to the inability of these cells to degrade the target mRNA when treated with Ca^{2+}- dependent nuclease (able to degrade DNA and RNA), indicates that RNA is an essential component of the nuclease activity which leads to the degradation of the target mRNA [115]. This nuclease activity is referred to as the RNA-induced silencing complex (RISC).

1.3.9.1 The mechanism of RNA interference

Several aspects of the PTGS/RNAi machinery are unknown, however the mechanism has become evident and it is incorporated into three essential steps:

Step 1 (RNAi initiating step): the RNA nuclease Dicer binds to a large dsRNA molecule and cleaves it into fragments that are 21-25 nucleotides in length [109]. These fragments are the siRNAs (Fig. 13A).

Step 2 (Effector step): the siRNAs join to the multinuclease complex: RISC. The siRNAs form double-stranded, duplexed structures consisting of 3' two-nucleotide overhangs and 5' phosphate termini, as previously described [117]. In this organisation, siRNA cannot be incorporated into RISC [117]. The siRNAs unwind and incorporate ATP in order to allow for the enzymatic activation of RISC zymogens. The outcome of this is the residing attachment of the most stable siRNA with the RISC complex (Fig. 13B) [118].

Step 3 (Gene silencing step): this step is charaterised by the activated RISC complex accompanied by the bound, stable siRNA strand in cleaving the target mRNA (Fig. 13C) [112]. The target mRNA is understood to be endonucleolytically cleaved at the central and complementary region of siRNA, which is located 10 nucleotides upstream from the paired

nucleotide at the siRNA's 5' end [119]. Target mRNA cleavage can occur in the absence of ATP, but if ATP is present the cleaved products are released with greater efficiency [112].

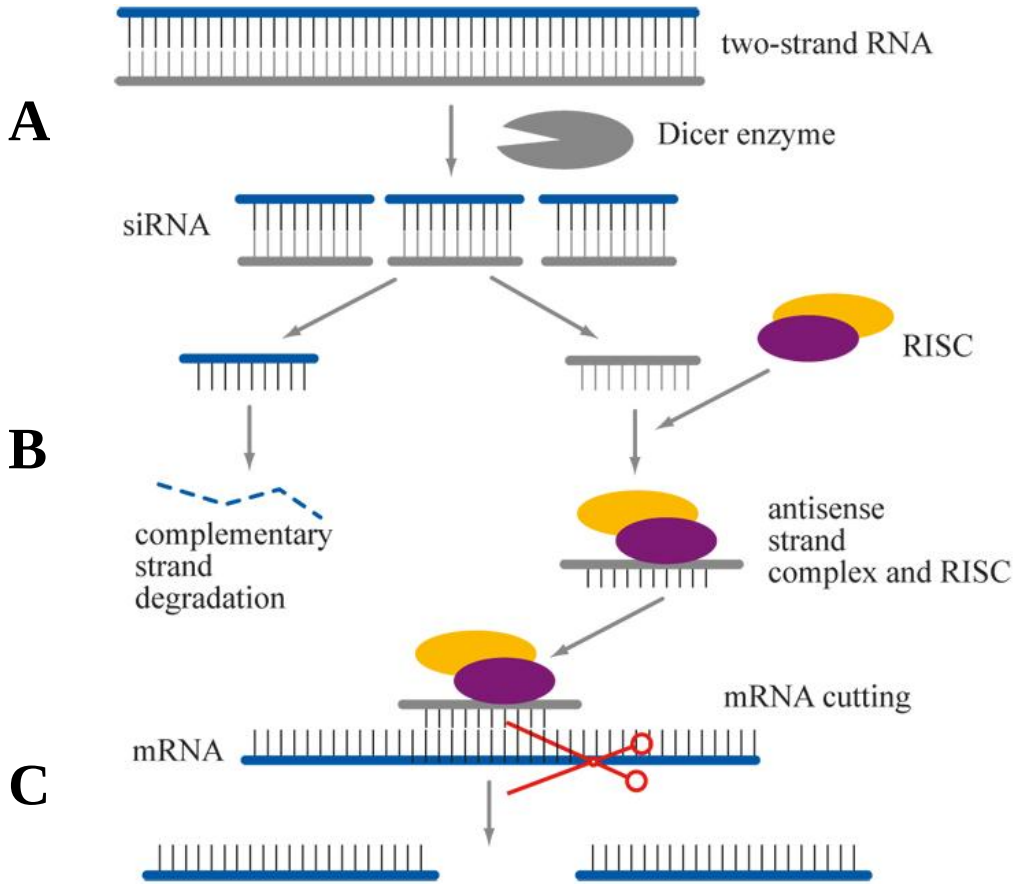


Figure 13: The mechanism of RNA interference. A) Dicer binds to and cleaves dsRNA into siRNA fragments. B) RISC incorporates siRNA and binds to complementary sequences of mRNA. C) After binding, the activated RISC facilitates the cleavage and degradation of target mRNA (http://eng.thesaurus.rusnano.com/upload/iblock/775/RNA-interference_1.jpg).

1.3.9.2 Applications of RNA interference

The process of RNAi has become popular in terms of being a key mechanism for future biological applications that can be easily applied [112]. RNAi technology has been proven to be useful in analysing the functions of several genes in a wide variety of

organisms [112]. In one instance, chromosome I and III of *C.elegans* has been screened by RNAi to identify the genes involved in cell division and embryonic development [120, 121], and whole genome screening by RNAi may become common in the near future. Additionally, RNAi may facilitate drug screening and development by identifying genes that can explain drug resistance or genes whose mutant phenotypes are enhanced by drug treatment, and in particular providing the modes of action of these therapeutic compounds [112]. Due to the gene specific features of RNA interference, it has gained an increasing amount of attention as a possible therapeutic with regards to gene-related ailments [122], such as viral-based infections, genetic and autoimmune diseases and in particular cancer. siRNA gene silencing *in vitro* is easily achievable, however, *in vivo* studies prove to be a continuous challenge mainly due to off-target effects that negatively impact healthy, normal cells. Although challenging, RNA interference-based therapeutics have been developed to treat HIV and the hepatitis virus [120] as well as neurodegenerative disorders and cancer [124].

Chapter 2: Rationale, Hypothesis, Aim and Objectives

2.1 Rationale and research question

The 37-kDa/67-kDa laminin receptor (LRP/LR) has been observed to be over-expressed in several tumor cell types in comparison to their normal cell counterparts. The over-expression of this receptor has been identified as a key factor in tumor progression via the maintenance of tumor cell viability. It is evident that LRP/LR plays a critical role in enhancing tumor cell viability as indicated by the reduction in cellular viability upon down-regulation of this receptor. According to previously published studies, it has been proven that siRNA-mediated down-regulation of LRP/LR led to reductions in the viability of breast (MCF-7), oesophageal (MDA-MB 231 and WHCO1) [108], lung (A549), cervical (HeLa), liver (Hep3B) [109], pancreatic and neuroblastoma [110] cancer cells via apoptotic induction. These findings encouraged the question whether down-regulation of the LRP/LR receptor via siRNA-mediated action will induce apoptosis in early (A375) and late (A375SM) stage malignant melanoma cells through the reduction in their viability, and to determine if apoptosis is the primary form of cell death responsible for the reduction in tumor cell viability.

2.2 Hypothesis

siRNA-mediated down-regulation of the LRP/LR receptor will significantly reduce the viability of early and late stage malignant melanoma cells via the induction of apoptosis.

2.3 Aim

To investigate how siRNA-mediated down-regulation of the 37kDa/67kDa laminin receptor (LRP/LR) influences the cellular viability of early (A375) and late (A375SM) stage malignant melanoma cells and to determine which mode of cell death is induced by this down-regulation.

2.4 Objectives

- To determine cell surface LRP/LR levels in the above-mentioned cell lines by flow cytometry (FACS analysis).
- To determine endogenous LRP/LR levels in the above-mentioned cell lines by performing western blotting and densitometric analysis.

- To successfully down-regulate the expression of LRP/LR in the cell lines mentioned via cell transfection with siRNA targeted to LRP mRNA.
- To detect siRNA-mediated LRP/LR down-regulation by use of western blotting.
- To analyse the effect of siRNA-mediated down-regulation on the cellular viability of the above mentioned cell lines using MTT assays.
- To assess the induction of any nuclear morphological changes upon siRNA treatment targeting LRP/LR in the afore-mentioned cell lines using immunofluorescence microscopy with the addition of the Airyscan processing tool.
- To assess possible apoptosis-inducing effects of siRNA-mediated LRP/LR down-regulation by use of Annexin-V-FITC/PI assays as well as caspase-3 assays.
- To analyse the specific pathways utilised by the above-mentioned cell lines to undergo siRNA-assisted apoptotic induction by performing caspase-8 and caspase-9 assays.

Chapter 3: Materials and Methods

3.1 Cell culture

3.1.1 Cell lines

The following cell lines used were commercially available and established (Ref. W-CJ-140804-1):

- A375 (ATCC® CRL-1619™): Human malignant melanoma- derived from a 54 year old female and classified as early stage melanoma with low metastatic and invasive properties due to decreased levels of the extracellular matrix (ECM) glycoprotein, Tenascin (TN) [122] .
- A375SM (ATCC® CRL-1619™): Human malignant melanoma- 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) [122].

3.1.2 Cell culture media and method of cultivation

The base medium for these two cell lines is Dulbecco's Modified Eagle's Medium (DMEM)/ High Glucose. For complete growth media, the above-mentioned media was supplemented with:

- 10% Fetal bovine serum (FBS) - heat inactivated.
- 1% Penicillin/Streptomycin antibiotic.

Both cell lines were cultivated in the media described above. The cells were cultured on cell culture plates or flasks in an incubator with a set temperature of 37ºC and 5% CO_2 in order to mimic *in vivo* conditions. Cells were sub-cultured when a confluency of 90% was reached or twice a week. Seeding and sub-culturing involved the removal of the cell culture media and washing of the cells with 5 ml phosphate buffered saline (PBS) to remove the excess media. The adherent cells were detached using 1X Trypsin/EDTA and incubated for approximately 10 minutes and re-suspended in fresh media.

3.1.3 Cryopreservation and storage of cells

The process of cryopreservation involved cell detachment using 1X Trypsin/EDTA post washing of the cells with PBS. The cells were re-suspended in the full growth media and centrifuged at 1200 rpm for 10 minutes. The supernatant was discarded and the pellet was re-suspended in fresh warmed cell culture medium. Additionally, 10% FBS and 10% DMSO was added to constitute the specific freezing medium. Following re-suspension, cells in 1 ml aliquots were incubated overnight at -20 ºC prior to being incubated at -70˚C. The cells were then transferred to and stored in liquid nitrogen until required.

3.1.4 Thawing of cells

In the case that a cryopreserved stock of cells was needed, 1 ml of fresh culture media was pre-warmed at 37ºC and used to thaw the 1 ml frozen cell stock. The resulting suspension was centrifuged at 1200 rpm for 10 minutes. The supernatant was discarded and the pellet re-suspended in fresh media containing an additional 10% FBS. The cells were left to attach, differentiate and grow at 37ºC as previously stated.

3.1.5 Quantification of cultured cells

In order to ensure that a constant number of cells were used to obtain a specific cell density for the experimental procedures, cell counting was performed on both cell lines, in order to allow for an effective comparison. The BIORAD TC20 cell counter was used to perform cell quantification and to observe percentage cell viability using the Trypan blue staining solution. Trypan blue in principal stains dead cells blue and the viable cells appear clear.

The cells were washed with PBS, detached using 1X Trypsin/EDTA and then re-suspended in the culture media. An aliquot of 10 µl cell suspension was combined with 10 µl Trypan blue staining solution. Thereafter, 10 µl of the mixture was placed on the cell counting plate. Once cell quantification was complete the cells were diluted to a required density, using warmed cell culture media.

3.2 siRNA-mediated downregulation of the laminin receptor (LRP/LR)

3.2.1 Materials

- 24-well and 6-well plates
- Dharmacon™ ON-TARGETplus SMARTpool Human RPSA (targeted to LRP/LR)*
- esiRNA-RLUC (employed as a negative control)
- DharmaFECT® Transfection Reagent
- MISSION® esiRNA-RPSA (targeted to LRP/LR)*
- MISSION® esiRNA-RLUC (negative control)*
- MISSION® Transfection Reagent
- Serum-free Opti-MEM®

*: These treatments were used following the transfection procedure described in section 3.2.2 below for SDS-PAGE/ western blotting and MTT assays only; as a means of eliminating any off target effects that may arise upon transfection.

3.2.2 Transfection procedure

Cells were seeded onto the appropriate plates at specific densities depending on the experiment or assay performed. The cells were left to grow overnight to a confluency of approximately 40-55% before transfection was performed. The target siRNA's and the negative control siRNA's were diluted in specified amounts of serum-free Opti-MEM® (as per Appendix A), 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 37ºC for 72 hours to allow for the transfection to take place prior to the downstream experimental procedures.

3.3 Protein biochemistry

3.3.1 Preparation of cell lysates

3.3.1.1 Materials

- Cell scrapers
- Phosphate buffered saline (1X PBS)
- 1X RIPA buffer (10mM Tris-Cl (pH 8), 10mM EDTA, 300mM NaCl, 1% sodium deoxycholate (DOC), 1% Triton-X, 0.1% SDS)

3.3.1.2 Methodology

The attached cells were washed with PBS and then incubated in 400 µl of RIPA buffer for 2 minutes, prior to cell detachment and membrane disruption using a cell scraper. The resulting cell suspension solution was incubated at 4ºC for 15 minutes, where after it was centrifuged at 14000 rpm 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.

3.3.2 BCA™ assay for protein quantification

3.3.2.1 Principle of the BCA™ protein assay

The BCA™ protein assay was performed in order to successfully determine the presence of protein in preparation for western blotting. A serial dilution of bovine serum albumin (BSA) standards were prepared and a standard curve constructed in order to determine cellular protein concentration. This assay involves the reduction of Cu^{2+} to Cu^{+} by the peptide bonds present in the proteins thus producing a violet solution of bicinchoninic acid (BCA, 2, 2 –Bichinolin-4,4'-dicarbonicacid) as well as Cu^{+}. The absorbance was measured at 562 nm in order to provide an estimation of the amount of protein present in the sample.

3.3.2.2 Materials

- 96-well plate
- Eppendorf tubes
- Bicinchoninic Acid solution (BCA)

- Copper (II) sulfate solution
- Bovine Serum Albumin (BSA) powder
- ELISA plate reader

3.3.2.3 Methodology

Bovine serum albumin (BSA) standards in the following concentrations: 0, 0.2, 0.4, 0.6, 0.8 and 1 mg/ml were prepared. Thereafter, 25 µl of each standard was loaded into separate wells of a 96-well plate in triplicate. Similarly, 25 µl of cell lysates obtained from section 3.3.1 were diluted (1:5) and added to the 96-well plate in duplicate. Subsequently, 4.9 ml of BCA reagent and 100 µl of copper (II) sulphate solution were combined and 200 µl of this solution was added to the wells containing the BSA standards as well as the cell lysates. The plate was incubated at 37˚C for 30 minutes and thereafter, the absorbance of the resulting solution was measured at 562 nm using an ELISA plate reader. In order to determine the concentration of the proteins, a standard curve was constructed.

3.3.3 Sodium dodecyl sulphate- Polyacrylamide gel electrophoresis (SDS-PAGE)

3.3.3.1 Materials

- 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
- Pre-stained protein molecular weight marker
- 2X loading buffer (100mM Tris/HCl pH 6.8, 4% SDS, 2% β-mercaptoethanol, 20% glycerol, 0.02% bromophenol blue)
- 1X SDS running buffer (10% of 10X running buffer)
- 10X SDS running buffer (250mM Tris, 192mM glycine in distilled water, 10% SDS)
- Gel casting apparatus

3.3.3.2 Methodology

SDS-PAGE was employed in order to separate proteins in a lysate sample. This technique was performed before the detection of LRP/LR levels by use of western blotting. The lysate samples 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 was utilised. After the lysate samples were heated at 95ºC for 5 minutes in the loading buffer, 10µg of the samples were loaded on the gel. The gel was resolved at 200 V for 30-45 minutes.

3.4 Western blotting and immunological detection of proteins

3.4.1 Materials

- Transfer buffer (20% methanol in 192mM glycine and 25mM Tris)
- Blocking buffer (3% BSA in 1X PBS-Tween)
- Wash buffer (0.1% Tween in 1X PBS)
- Polyvinyldifluoride (PVDF) membrane
- Whatman filter papers
- 100% methanol
- Primary antibody (anti-LRP/LR specific antibody IgG1-iS18) solution
- Secondary antibody (anti-human IgG-HRP conjugated antibody) solution
- Conjugated primary antibody (mouse monoclonal anti-β-actin peroxidase) for detection of β-actin loading control
- Chemiluminescent substrate

- Blotting device

3.4.2 Methodology

Endogenous cellular LRP/LR levels and LRP/LR levels in siRNA tansfected cells were determined by employing western blotting. The separated proteins were transferred to a PVDF membrane via electro-blotting. Whatman papers were soaked in transfer buffer for 5 minutes while the PVDF membranes (cut to the dimensions of the SDS-PAGE gel) were soaked in methanol for 2 minutes and in transfer buffer for 5 minutes thereafter. The PVDF membranes were then placed between soaked Whatman papers that were assembled onto the electro-blotting device. The gels were placed on top of the membranes and electro-blotting took place for 50 minutes at 300 V and 350 A. The membranes were then blocked for 1 hour using blocking buffer, followed by a 24 hour incubation of the membranes in 2 µl primary antibody diluted in blocking buffer (1: 10 000). The membranes were washed three times in wash buffer (10 minutes each) and then incubated in 1.5 µl secondary antibody diluted in blocking buffer (1:5000) for 1 hour and 30 minutes. Three washes were performed as previously mentioned and 1 ml of the chemiluminescent substrate was then used to detect the Horseradish peroxidase (HRP) conjugated secondary antibody. Thereafter, the fluorescent light emission was captured and detected using the BIORAD Chemidoc apparatus in order visualise the proteins.

It is important to note that the β-actin antibody, which is used to detect β-actin and thus serves as a loading control, is directly conjugated to HRP and therefore does not require a secondary antibody. Densitometry was performed in order to quantify total protein levels using the ImageJ™ software.

3.5 Flow Cytometry (FACS analysis)

3.5.1 Materials

- 1X Trypsin/EDTA
- 4% paraformaldehyde
- Primary antibody (anti-LRP/LR specific antibody IgG1-iS18)

- Secondary antibody (anti-human phycoerythrin (PE)-coupled)
- Anti-chloramphenicol acetyltransferase antibody (CAT) produced in rabbit- will be used as an effective negative control primary antibody
- Allophycocyanin (APC)-coupled secondary antibody produced in goat and directed towards rabbit IgG- will be used as the secondary antibody for the negative control
- PBS

3.5.2 Methodology

Fluorescence Activated Cell Scanning (FACS) is a quantitative, analytical technique for counting and examining cells by suspending them in a stream of fluid and passing them through an electronic detection apparatus. This technique was used to determine cell surface LRP/LR levels in the afore-mentioned cell lines. FACS employs the principal of immunodetection whereby primary antibodies that are recognised by specific fluorochrome-coupled secondary antibodies are used for the detection of a target protein; in this case the LRP/LR receptor. The flow cytometer is comprised of an argon laser beam that helps in the differentiation between antibody-labelled cells and unlabelled cells, thus allowing for the quantified expression of target LRP/LR on the cell surface.

The cells need to be in suspension in order for flow cytometry to be performed, hence the cells were detached using 1X Trypsin/EDTA and centrifuged for 10 minutes at 1200 rpm. The resulting pellet was fixed in 4% paraformaldehyde for 10 minutes at 4ºC. After fixation, two cell suspensions were prepared by re-suspending the cell 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 4ºC and then washed three times with PBS by centrifugation at 5000 rpm for 5 minutes. Both cell pellets were re-suspended in PBS and the PE- coupled secondary antibody. The cell suspensions were allowed to incubate in the dark for 1 hour, thereafter the cell suspensions were washed 3 times in PBS as previously described and re-suspended in PBS before flow cytometric analysis. The same procedure was followed in order to obtain results for the negative control; CAT bacterial protein, using the negative

control antibodies outlined in section 3.5.1 above. The unlabeled cells prepared were used as a control.

3.6 Assessing cellular viability

3.6.1 MTT assay

3.6.1.1 Principle of the assay

This assay is a colorimetric assay that is used to determine the amount of cell survival in response to external treatments such as drugs or antibodies. The assay relies on the cleavage of mitochondrial tetrazolium salt (MTT), which is water-soluble and yellow in colour, by viable cells. The cleavage of MTT occurs via the mitochondrial succinate dehydrogenase (reductase) enzyme; present in the mitochondria of viable cells to produce water-insoluble purple formazan crystals (Fig. 14 a). In non-viable cells, the mitochondrial succinate dehydrogenase enzyme becomes inactivated, thus the MTT salt is not converted into formazan crystals (Fig. 14 b). The measurement of the absorbance of the resulting formazan crystal solution indicates the percentage of cell survival. Lower absorbance values therefore correspond to enhanced levels of cell death/ non-viable cells due to lower levels of MTT cleavage.

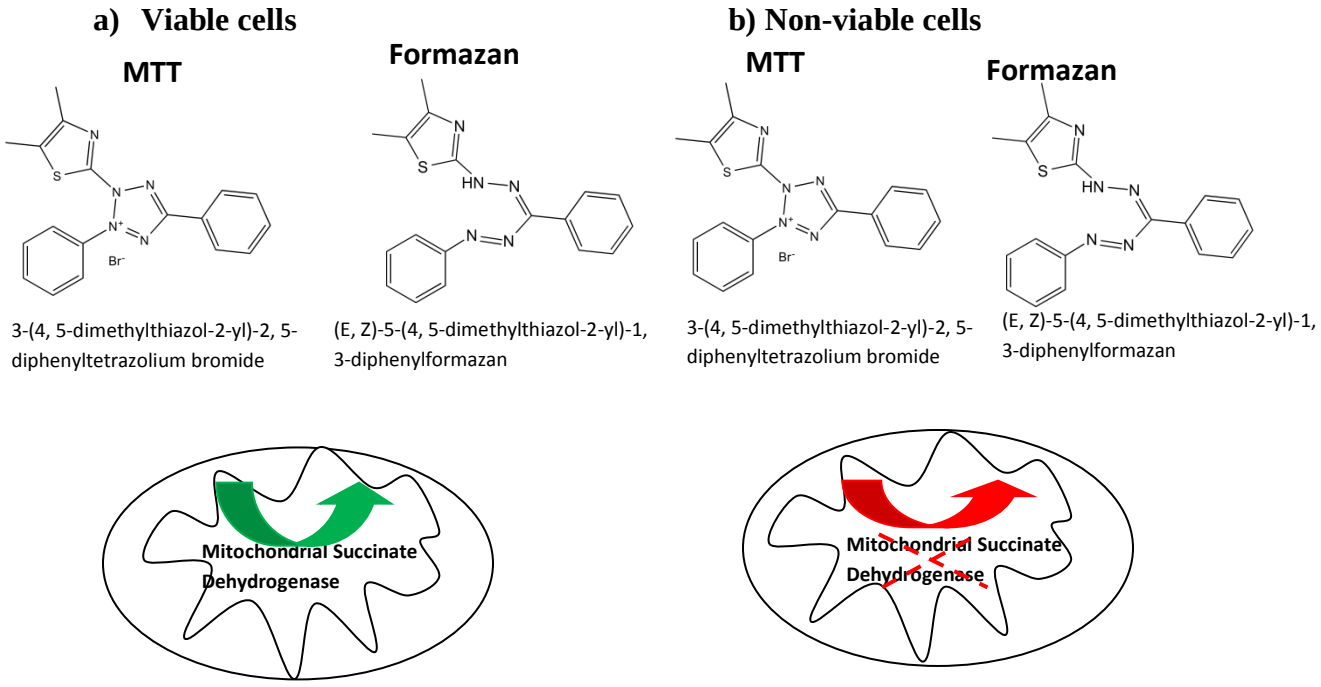


Figure 14: The MTT cell viability assay. a) In viable cells the yellow MTT salt is converted to the purple formazan crystals by the action of mitochondrial succinate dehydrogenase. b) In non-viable cells, the reductase enzyme is inactive; therefore MTT is not converted into formazan. **(**Adopted from https://upload.wikimedia.org/wikipedia/commons/d/de/MTT_reaction.png)

3.6.1.2 Materials

- 24-well plates
- PBS
- MTT (1 mg/ml)
- Dimethyl sulfoxide (DMSO)
- ELISA plate reader

3.6.1.3 Experimental procedure

Both malignant melanoma cell lines (A375 and A375SM) were allowed to grow and reach a density of approximately 1 x 10^5 cells/ml and 100 µl of cells at this density were seeded per well prior to siRNA transfection. After transfecting the cells, they were incubated at 37˚C for 72

hours. Thereafter, a 1 mg/ml solution of MTT was dissolved in 1X PBS and added to each well, followed by a 2 hour incubation period at 37˚C. Post incubation, the MTT- containing medium was discarded from each well 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. Controls were prepared and these included: untreated cells, negative control siRNA treated cells and positive control protocatechuic acid (PCA) treated cells.

3.7 Assessing nuclear morphological changes

3.7.1 Confocal microscopy and Airyscan processing

3.7.1.1 Materials

- 4% paraformaldehyde
- PBS
- 1 µg/ml DAPI stain
- Slides
- Coverslips
- Gelmount mounting fluid (Sigma-Aldrich)

3.7.1.2 Methodology

This technique was performed 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 sensitivity, speed and resolution. Airyscan benefits the confocal laser scanning microscope by increasing its power, thus providing fast read out times and very low background noise.

The cells were grown and allowed to reach a density of approximately $1x10^5$ cells/ml. At this density, 200 µl of cells were seeded onto coverslips prior to siRNA transfection. Thereafter, cells were washed with 500 µl 1X PBS for 1 minute and fixed in 1 ml 4% paraformaldehyde for 15 minutes. This was followed by three 1X PBS washes and thereafter the cells were permeabilized by adding 2 ml 0.1%Triton-X into each well for 20 minutes, followed by two washes with 1X PBS. The cells were then treated with 2 µl of DAPI nuclear stain diluted in PBS (0.2 µl DAPI in 4 ml PBS) for 8 to 10 minutes 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. The cells were stored at 4˚C after setting took place overnight, until visualisation using a confocal microscope with the addition of the Airyscan. The controls were as follows: untreated cells, siRNA-RLUC treated cells (negative control) and PCA (positive control).

3.8 Assessing apoptotic induction

3.8.1 Annexin V-FITC/PI assay

3.8.1.1 Principle of the assay

This assay is dependent on the Annexin V intracellular protein binding to phosphatidylserine (PS) in a calcium-dependent manner. PS is normally only located on the intracellular leaflet of the plasma membrane in healthy cells but during early apoptosis, subsequent asymmetry of the membrane is lost and PS translocates from the intracellular membrane to the external leaflet (Fig. 16). Translocation of PS to the external leaflet enables the fluorochrome-labelled Annexin V to bind to the protein in the presence of calcium; hence Annexin V staining allows for measurement of translocated PS and therefore allows for the detection of apoptosis by flow cytometry. The red-fluorescent dye; Propidium Iodide (PI) binds to DNA by intercalating between the bases with little or no sequence preference and it was employed in this assay to distinguish between early apoptotic and late apoptotic or necrotic cells (Fig. 16). Therefore, apoptotic cells show green fluorescence, dead cells red and green fluorescence, and live cells little or no fluorescence.

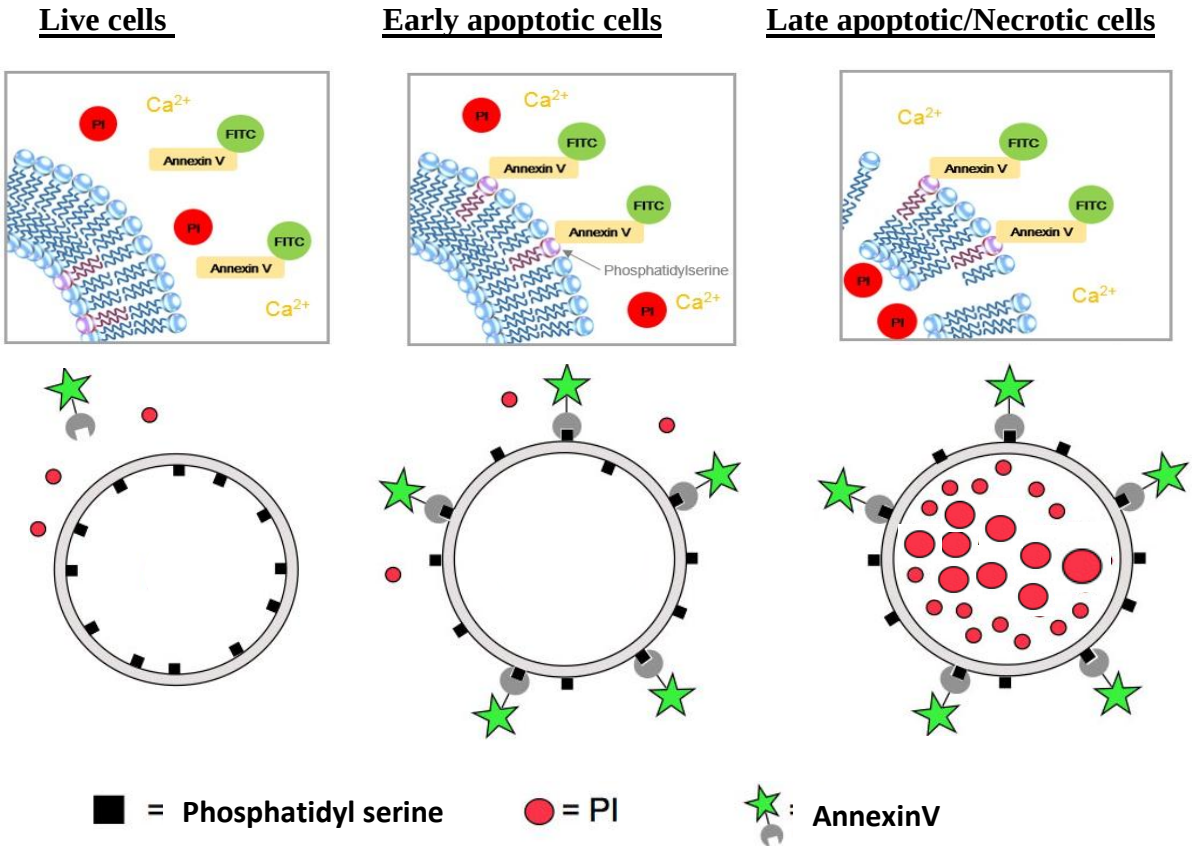


Figure 15: Principle of the Annexin V-FITC/PI assay. Cells undergoing apoptosis experience a membrane-flip reaction that exposes phosphatidylserine (PS) on the external leaflet of the plasma membrane. Annexin V conjugated to FITC binds to the exposed PS, and FITC allows for detection of apoptotic cells. PI is a viability dye that is used to distinguish between early and late apoptotic cells. (Adapted from http://www.lifesci.dundee.ac.uk/sites/www.lifesci.dundee.ac.uk/files/Annexin.pngandhttps://www.google.co.za/search?q=annexin+VFITC/+7AAD+assay&biw=1280&bih=865&source=lnms&tbm=isch&sa=X&ved=0ahUKEwiMmI3R887KAhUBshQKHZuhCvoQ_AUIBigB#tbm=isch&q=+annexin+VFITC%2F+7+AAD+assay+&imgrc=XRgS2Qa44nSqDM%3A)

3.8.1.2 Materials

- PBS
- Trysin/EDTA
- Annexin V-FITC solution (Refer to Appendix A)
- 1X Annexin-binding buffer
- Propidium Iodide

- Flow cytometer (BD Accuri™ C6 Cytometer)

3.8.1.3 Experimental procedure

The cells were grown and seeded to an approximate density of 1.8 x10 ^{6} cells/ml and transfected with the previously mentioned siRNAs. Cells were harvested using 1X Trypsin/EDTA and then washed thrice with ice-cold PBS. Thereafter, the cell suspensions were centrifuged at 1500 rpm for 10 minutes. The supernatant was discarded and the pellet re-suspended in 100 µl 1X 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 minutes in the dark. The samples were then supplemented with 400 µl of ice-cold 1X annexin binding buffer prior to flow cytometric analysis which was performed within 30 minutes. Importantly, it must be noted that untreated cells, esiRNA-RLUC treated cells, and PCA treated cells were used as controls, as with the previously mentioned methods. The FL1 laser detects the Annexin V-FITC staining and FL2 detects PI staining. The compensation and quadrants were set using the following controls: 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.

3.8.2 Caspase-3, -8 and -9 assays

3.8.2.1 Principle of the assay

The caspase-3, -8 and -9 assay is a colorimetric protease assay that relies on caspase-mediated hydrolysis of a chromophore known as para-nitroaniline (*p*-NA) that is coupled to a peptide substrate. These assays are capable of quantifying the enzyme activity of caspases that recognise the peptide substrate sequence Asp-Glu-Val-Asp *p*-nitroaniline (DEVD-*p*-NA) for caspase-3, Ile-Glu-Thr-Asp *p*-nitroaniline (IETD-*p*-NA) for caspase-8 and Leu-Glu-His-Asp *p*-nitroaniline (LEHD-*p*-NA) for caspase-9. The substrates used in the assays are synthetic peptides labelled at their C-termini with para-nitroaniline (*p*-NA) and are designed to measure the activity of these caspases. The caspases cleave the peptide, thereby releasing the *p*-NA chromophore which possesses a high absorbance at 405 nm. The absorption of light by free *p*- NA can therefore be

quantified using microtiter plate reader at 405nm. The absorbance reading of *p*- NA from an apoptotic sample in comparison to an un-induced control allows for the determination of the fold increase in caspase activity and consequently the occurrence of apoptosis.

3.8.2.2 Materials

- 1X Cell Lysis Buffer
- 5X Assay Buffer
- Caspase-3, -8 and -9 Substrate (2.5 mg/ml)* (Refer to Appendix A)
- Eppendorf tubes
- 96-well plates
- Spectrophotometer

3.8.2.3 Experimental procedure

Cells were grown to an approximate density of 1.2 $x10^6$ cells/ml and transfected with the respective siRNA. Thereafter, $1.2x10^6$ cells were pelleted by centrifugation at 1500 rpm for 10 minutes and re-suspended in 100 µl ice-cold 1X cell lysis buffer and incubated on ice for 10 minutes. The cells were then centrifuged for 5 minute at 10 000 x g and the supernatant was placed on ice. Following this, the protein concentration of the supernatant was determined by use of a BCA™ assay as previously described in section 3.3.2 and subsequently, 200µg of protein was prepared according to manufacturer's instructions (appendix section). 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. In addition to untreated cells, esiRNA-RLUC treated cells and PCA treated cells were used as negative and positive controls, respectively.

3.9 Statistical evaluation

3.9.1 Statistical significance using student's *t*-test

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.

3.9.2 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.

Chapter 4: Results

4.1 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 metastasis, tumor angiogenesis and in the maintenance of cellular viability [78]. Therefore, cell surface LRP/LR levels of 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. Figure 16 indicates that both malignant melanoma cell lines exhibit a high percentage of cells within the cell population that contain LRP/LR on the cell surface. The distinct shift between the peaks represents the characteristic changes in median fluorescence intensity occurring as a result of the treatment of the cancer cells with the anti-LRP/LR specific antibody IgG1-iS18 and an appropriate secondary antibody, conjugated to a fluorochrome. 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, thus confirming the presence of this receptor on the surface of both cell lines.

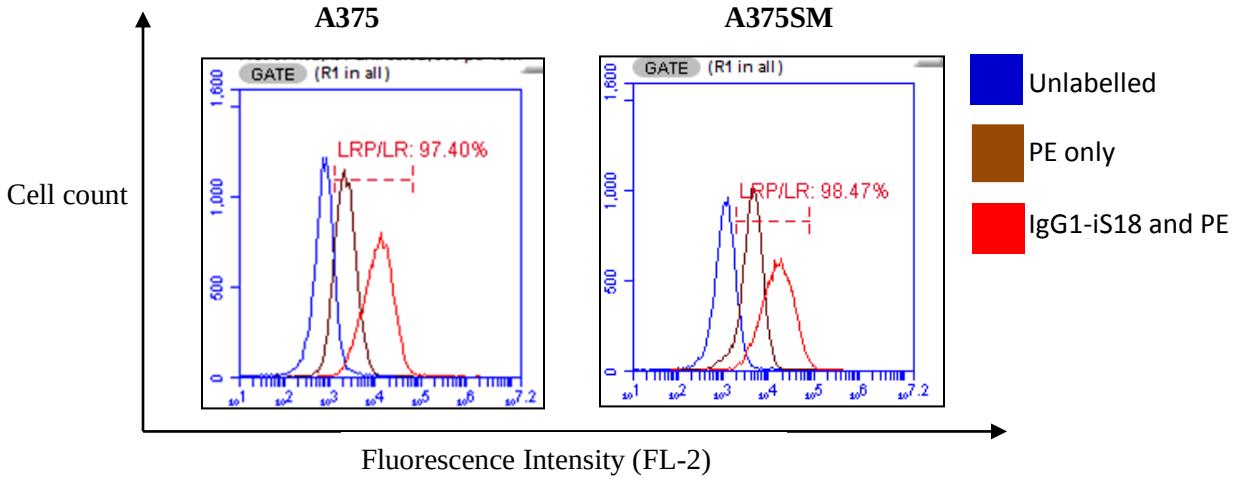


Figure 16: Detection of the 37kDa/67kDa laminin receptor LRP/LR levels on the surface of early (A375) and late (A375SM) stage malignant melanoma cells. The blue peak represents the cells that have not been labeled with antibodies, while the orange peak is representative of the cells stained with the 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. 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 (FL-2) which is specific for the PE-fluorochrome.

The anti-CAT antibody which targets the chloramphenicol acetyltransferase (CAT) protein was used as the negative control. CAT is absent on the surface of mammalian cells. As anticipated, both cell lines showed a minimal to no shift in fluorescence intensity, thus indicating that the CAT protein was indeed absent on the surface of the malignant cell lines thus indicating that no non-specific binding occurred (Fig.17).

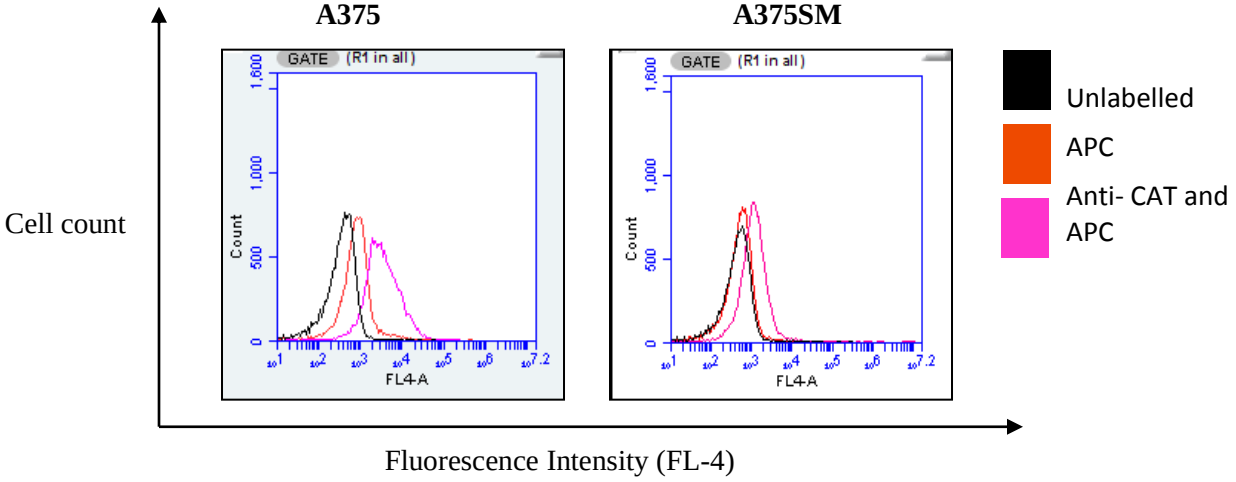


Figure 17: Determination of chloramphenicol acetyltransferase protein levels on the surface of early (A375) and late (A375SM) stage malignant melanoma cells. The black peak represents the unlabeled cells, while the orange peak is representative of the cells stained with the goat anti-rabbit allophycocyanin (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. The above plots are indicative of an average of experiments performed in triplicate, with 20 000 cells counted per sample and the samples analysed using filter 4 (FL-4) which is specific for the APC-fluorochrome.

4.2 Late stage malignant melanoma cells exhibit higher cell surface LRP/LR levels in comparison to the early stage malignant melanoma cells.

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. This was done using flow cytometry, with the same concentration of primary IgG1-iS18 antibody and PE-coupled secondary antibody used to label the same number of cells (20 000) within a specific population of both cell lines over the same period of time. The differential expression of cell surface LRP/LR was indicated through the median fluorescence intensity (MFI) as seen in Table 1. 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 as seen in Figure 18.

Table 1: Median Fluorescence Intensity (MFI) values used to determine differential expression of LRP/LR on the surface of A375 and A375SM cancer cells.

Cell Line	MFI of unlabeled cells (A)	MFI of cells stained with IgG1-iS18 and PE-coupled secondary antibody (B)	Difference in MFI values i.e. (B-A)
A375	2053.67	10135.5	8081.83
A375SM	1819.33	16833.5	15014.17

***All MFI values are representative of an average of experiments carried out in three biological repeats performed in triplicate.**

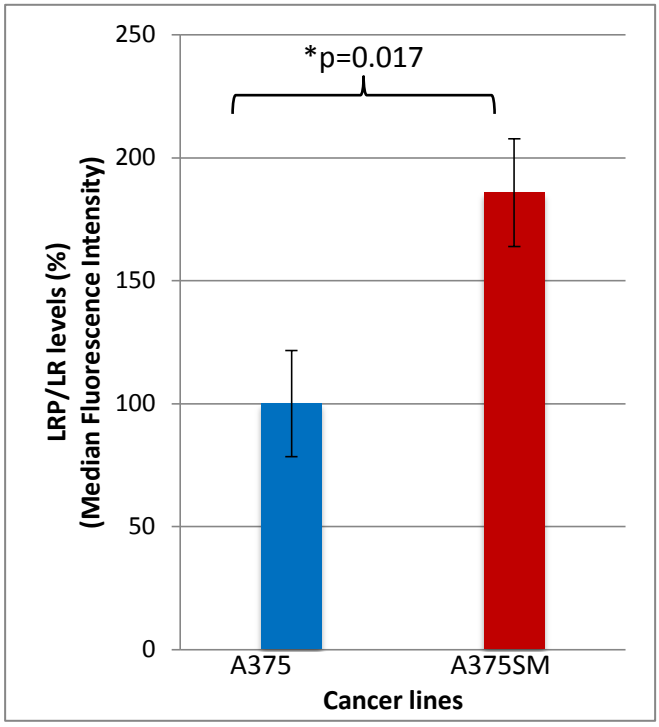


Figure 18: Quantification of LRP/LR levels on the surface of early (A375) and late (A375SM) stage malignant melanoma cells by flow cytometric analysis. The cells were labeled with primary IgG1-iS18 antibody and the anti-human PE-coupled secondary antibody. Analysis was performed on 20 000 cells per sample in triplicate and 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 1 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. *p<0.05, **p< 0.01 ***p<0.0001

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

As previously indicated, 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 under study, western blotting as well as densitometric analysis was performed. A western blot representing both LRP/LR and β-actin (Fig.19A) 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 37kDa 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% more total LRP in comparison to the A375 cells (Fig.19B).

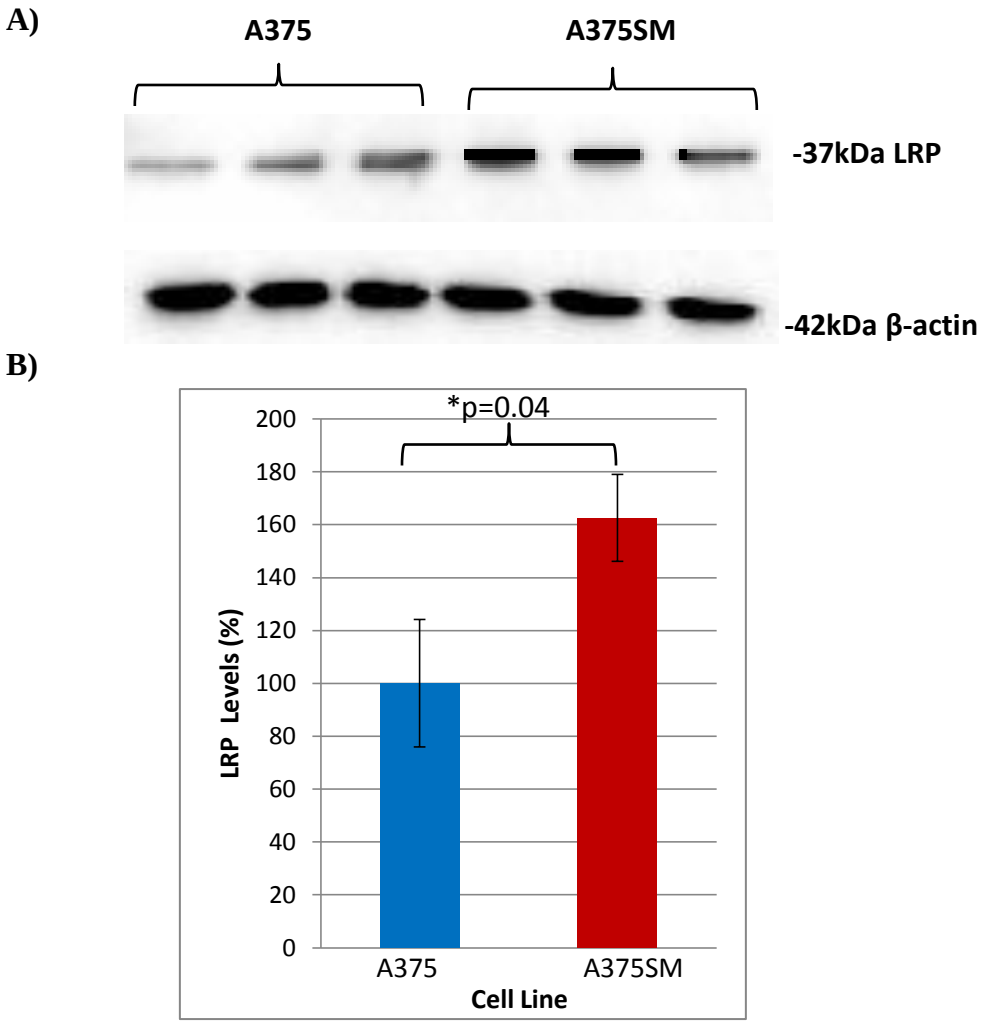


Figure 19: Western blot assessment of the relative expression of total 37kDa laminin receptor precursor (LRP) levels in early (A375) and late (A375SM) stage malignant melanoma cells. A) Anti-LRP/LR specific primary antibody IgG1-iS18 and HRP-coupled secondary antibody was used to detect and visualize total 37kDa 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

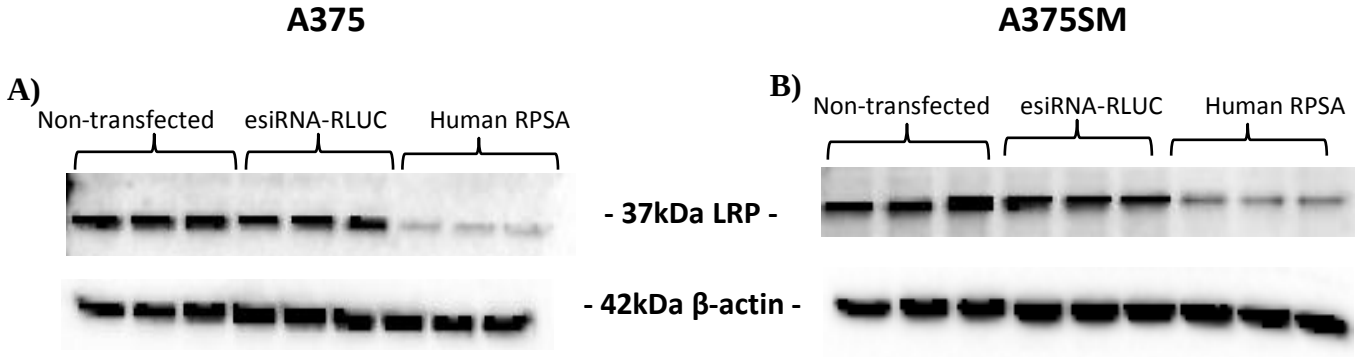
A high, positive correlation was observed between cell surface and total LRP/LR levels prior to siRNA transfection (Table 2), thus indicating that the quantified cell surface LRP/LR levels can be responsible for the observed total LRP levels.

Table 2: Pearson's correlation coefficient (r) between cell surface and total LRP levels prior to siRNA transfection.

Cell Lines	Correlation between cell surface LRP and total LRP levels prior to siRNA transfection (r-value)
A375	0.98
A375SM	0.65

4.4 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 the cell lines under study, siRNA- mediated down-regulation of LRP was performed. 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 37kDa LRP with higher specificity and potency through the action of 4 siRNAs), western blot analysis and densitometric quantification was performed. Densitometric analysis of the western blot signals revealed that LRP was successfully down-regulated in both A375 and A375SM cells (Fig. 20A and 20B) by transfection with the afore-mentioned siRNA. It was observed that siRNA-Human RPSA transfected A375 and A375SM cells exhibited a 77% and 72% reduction in LRP levels, respectively in comparison to the non-transfected cells whose LRP expression levels were set to 100%. In addition, the transfection of A375 and A375SM cells with the Mission esiRNA-RLUC (negative control siRNA) showed no significant down-regulation of LRP when compared to the non-transfected cells (Fig. 20A and 20B). The sequences of the above-mentioned siRNAs are provided in the appendix section.



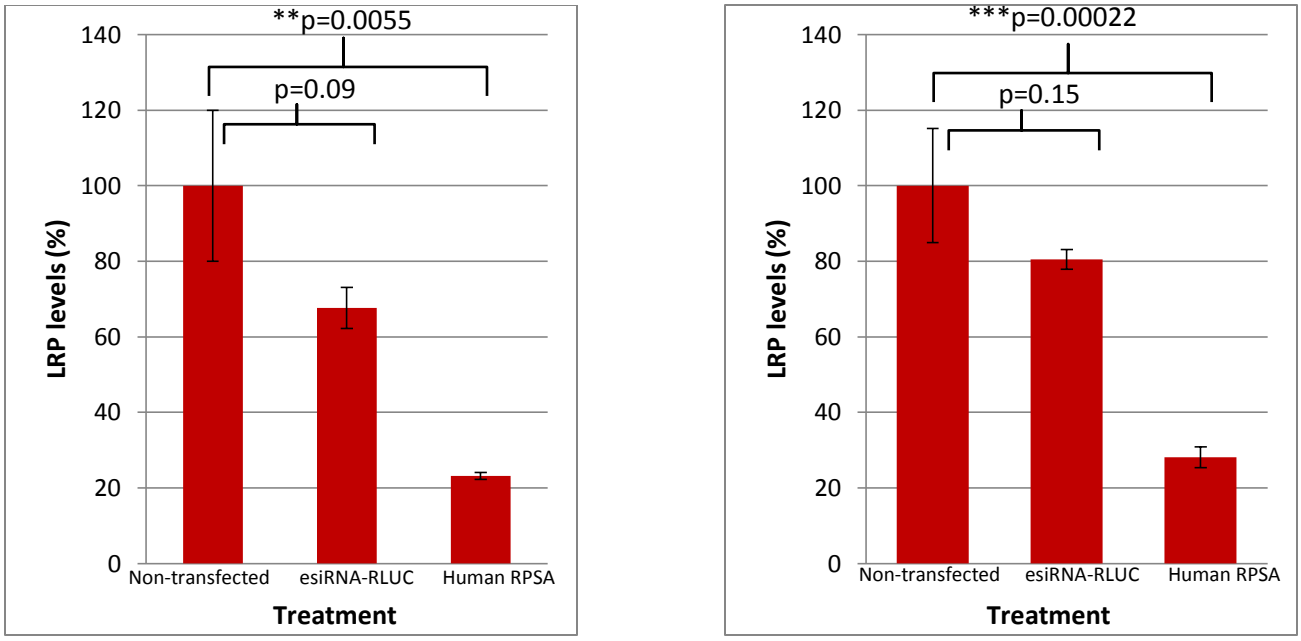


Figure 20: Down -regulation of LRP expression in early (A375) and late (A375SM) stage malignant melanoma cells post siRNA transfection. A and B) Transfection of both A375 and A375SM cells with Dhatmacon Human- RPSA resulted in the significant down-regulation of LRP by 77% and 72%, respectively when compared 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. *p<0.05, **p< 0.01 ***p<0.0001, N.S.: P>0.05 non-significant.

A high correlation was observed between total LRP levels prior to and post Human Dharmacon RPSA transfection (Table 3), this is indicative of the success rate of transfection in both cell lines.

Table 3: Pearson's correlation between total LRP levels prior to and post Human Dharmacon RPSA transfection.

Cell Lines	Correlation between total LRP levels prior to and post Human RPSA transfection (r-value)
A375	0.95
A375SM	0.97

4.5 siRNA-mediated down-regulation of LRP expression results in significantly reduced viability of early and late stage malignant melanoma cells.

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, resulted in a significantly reduced cellular viability in comparison to the non-transfected cells and the cells treated with the afore-mentioned negative control siRNA. A375 and A375SM cells treated with the Human-RPSA showed a 47% and 61% reduction in cellular viability, respectively, compared to the non-transfected cells whose cell viability was set to 100% (Fig.21). Moreover, no significant reduction in cellular viability was observed for both cell lines when treated with the negative control esiRNA-RLUC, compared to the non-transfected cells (Fig. 21). Protochatechuic Acid (PCA) is a known apoptotic inducer and was therefore used as a positive control throughout experimentation.

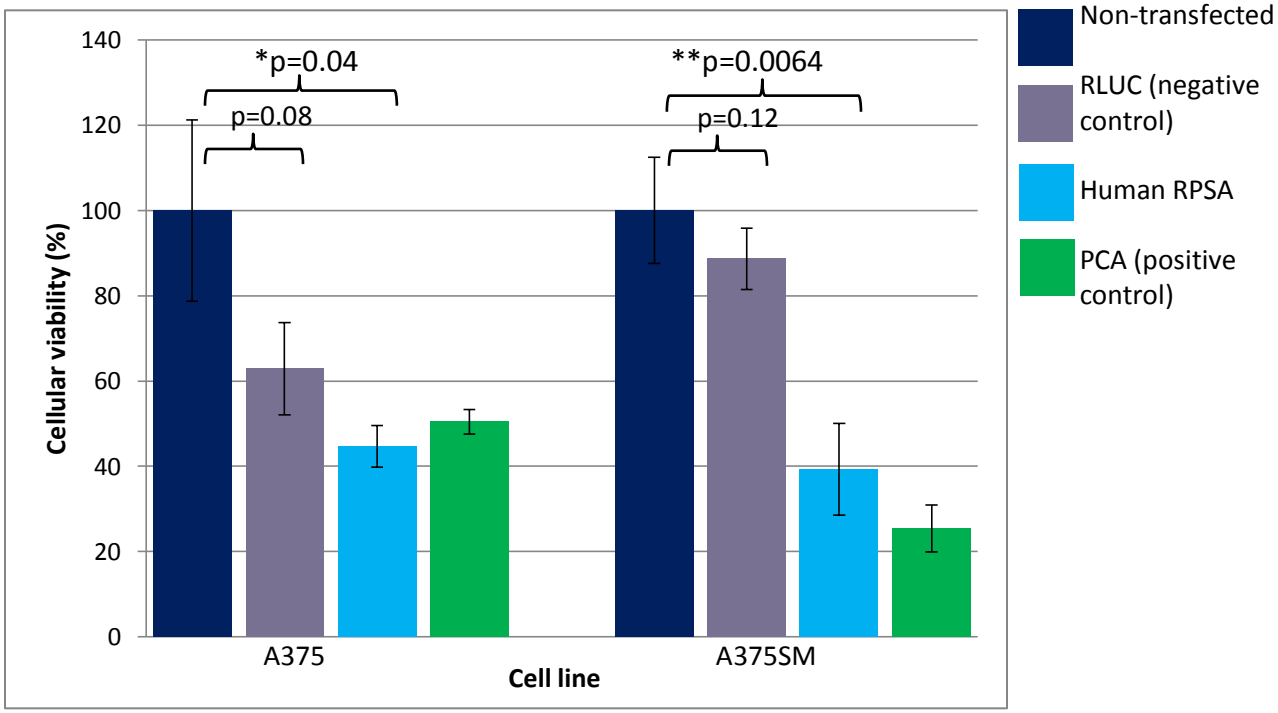


Figure 21: The effect of siRNA-mediated down-regulation on the cellular viability of early (A375) and late (A375SM) stage malignant melanoma cells. MTT assays were used to analyse the effect of siRNA transfections with the Human RPSA on the cellular viability of both cell lines under study. The A375 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.

A high correlation was observed between the level of Human RPSA-mediated LRP down-regulation in A375 and A375SM cells, and the Human RPSA induced decrease in cellular viability in both malignant melanoma cell lines (Table 4).

Table 4: Pearson's correlation analysis between levels of Human RPSA siRNA-mediated LRP down-regulation and reductions in cellular viability of early (A375) and late (A375SM) stage malignant melanoma cells.

Cell Lines	Correlation between Human RPSA-mediated LRP down-regulation and reductions in cellular viability (r-value)
A375	0.6
A375SM	0.88

4.6 Use of an alternative siRNA confirms that LRP down-regulation is responsible for reductions in cellular viability in both early and late stage malignant melanoma cells.

In order to confirm whether the observed reduction in cellular viability occurred as a result of Human RPSA siRNA- mediated down-regulation and not due to due to off-target effects, an alternative siRNA (esiRNA-RPSA) that targets a different region of LRP was utilized (refer to appendix for siRNA sequence). Western blot analysis and densitometric quantification revealed that early stage (A375) malignant melanoma cells showed a significant down-regulation of 55% in LRP expression (Fig. 22A and 22B). The late stage (A375SM) malignant melanoma cells exhibited a significant reduction in LRP expression by 50% in comparison to the non-transfected cells (Fig. 22A and 22B). Treatment of both cell lines, A375 and A375SM with the negative control siRNA (esiRNA-RLUC) displayed no significant difference in LRP expression when compared to the non-transfected cells.

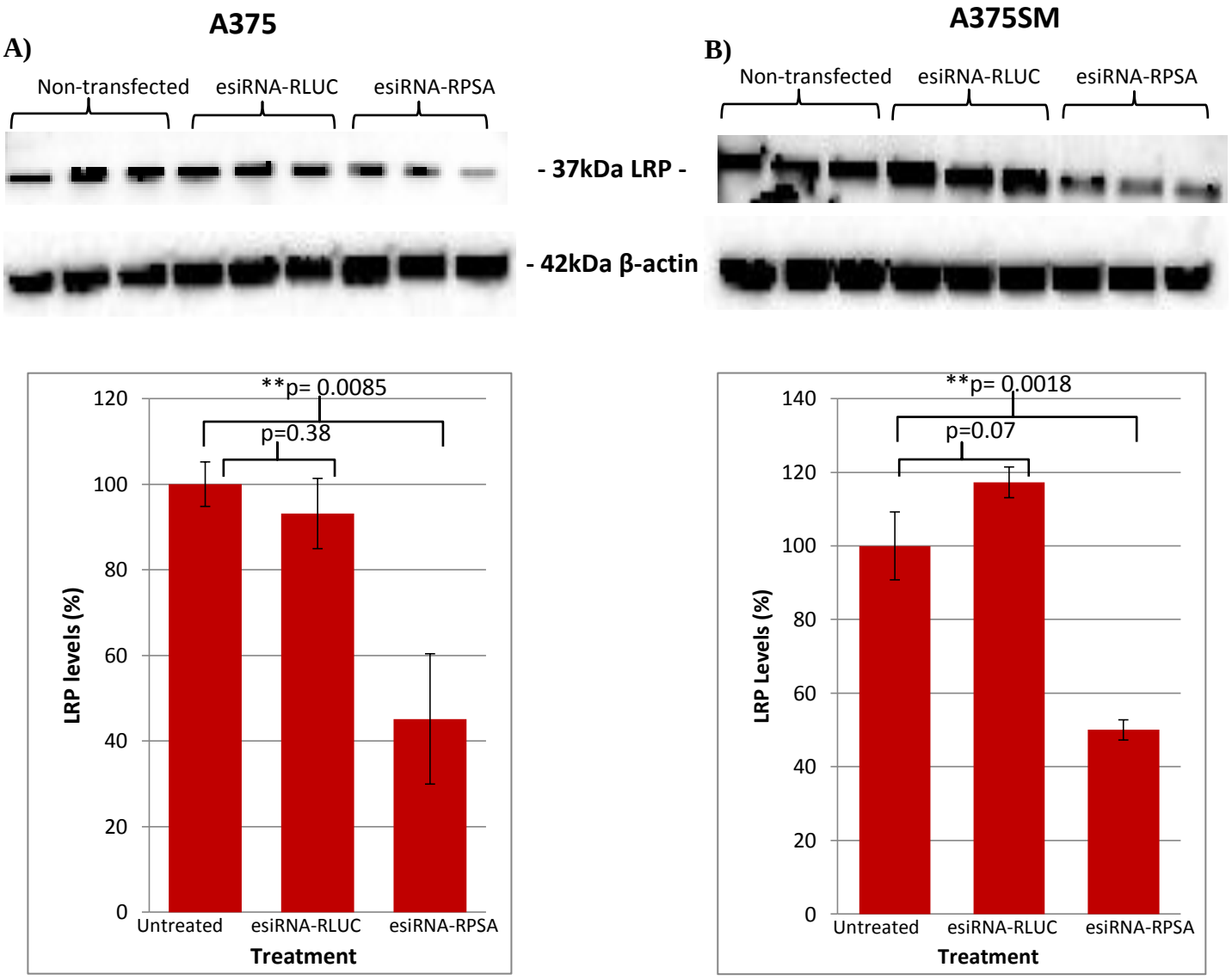


Figure 22: Detection of LRP expression in early (A375) and late (A375SM) malignant melanoma cells after transfection with an alternative siRNA. A and B) Western blotting and densitometric quantification revealed that after transfection of the cells with esiRNA-RPSA, there was a significant reduction 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 cells, which was set to 100% as seen on the graph. 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. *p<0.05, **p< 0.01 ***p<0.0001, N.S.: p>0.05 non-significant.

High correlations were obtained between total LRP levels prior to and post esiRNA-RPSA treatment (Table 5), a similar result to the one observed for the Human RPSA siRNA.

Table 5: Pearson's correlation between total LRP levels prior to and post esiRNA-RPSA transfection.

Cell Lines	Correlation between total LRP levels prior to and post esiRNA-RPSA transfection (r-value)
A375	0.99
A375SM	0.82

In order to assess the effects of esiRNA-RPSA-mediated LRP down-regulation on the cellular viability of A375 and A375SM cells, MTT assays were performed. A significant reduction in cellular viability in both malignant melanoma cell lines was observed post transfection with the alternative siRNA. It was found that A375 cells showed a 45% decrease in cellular viability after siRNA treatment, while the A375SM cells indicated a 29% reduction in comparison to the non-transfected cells (Fig 23). In addition, upon treatment of both cell lines with the negative control siRNA (esiRNA-RLUC), no significant change in cellular viability was observed when compared to the non-transfected cells (Fig. 23). Only western blotting and MTT assays were performed using two siRNAs (DHARMACON Human RPSA and the Mission esiRNA-RPSA) in order to confirm that no off target effects took place. Both siRNAs, DHARMACON Human RPSA and Mission esiRNA-RPSA, indicated the same effects on cellular viability in both the cell lines under study (Fig.21 and Fig. 23). 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 and Mission esiRNA-RLUC.

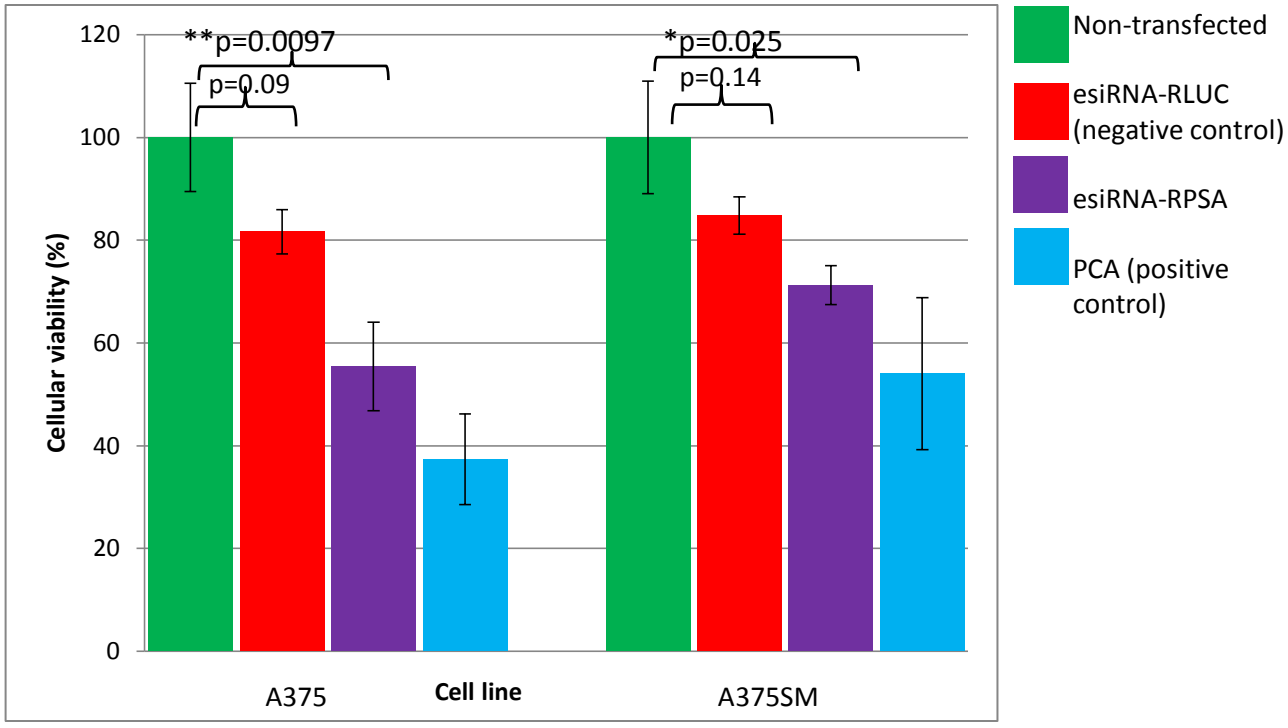


Figure 23: The effect of esiRNA-RPSA - mediated LRP down-regulation on the cellular viability of early (A375) and late (A375SM) stage malignant melanoma cells. After treatment of the cells with esiRNA-RPSA, MTT assays were performed to assess the effect on cellular viability. A375 and A375SM cells showed 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.

High correlations were observed between the level of esiRNA-RPSA-mediated LRP down-regulation in A375 and A375SM cells, and the esiRNA-RPSA induced decrease in cellular viability in both malignant melanoma cell lines (Table 6).

Table 6: Pearson's correlation analysis between levels of esiRNA-RPSA-mediated LRP down-regulation and reductions in cellular viability of early (A375) and late (A375SM) stage malignant melanoma cells.

Cell Lines	Correlation between esiRNA-RPSA-mediated LRP down-regulation and reductions in cellular viability (r-value)
A375	0.7
A375SM	0.9

4.7 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. 25A) 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. 24C). Similarly, the comparison of nuclei of Human RPSA siRNA treated late stage (A375SM) malignant melanoma cells (Fig. 25C) to the nuclei of the non-transfected cells (Fig.25A) revealed the presence of nuclear morphological changes such as nuclear shrinkage and membrane blebbing. In addition, treatment of both cell lines with the negative control esiRNA-RLUC did not result in nuclear morphological changes when compared to the non-transfected cells (Fig. 24B and 25B). PCA is known as the positive control and was used to study the nuclear morphological changes that occur under apoptotic conditions (Fig. 24D and 25D).

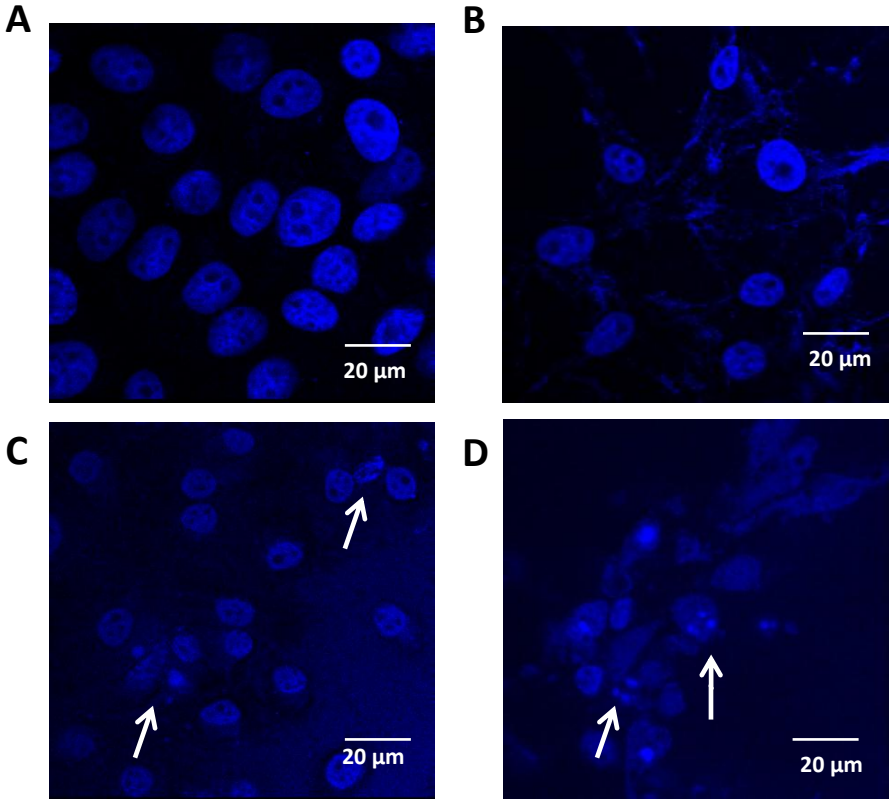


Figure 24: The effect of siRNA-mediated LRP down-regulation on nuclear morphology of early stage (A375) malignant melanoma cells. A) Non-transfected cells with normal rounded nuclei with uncompromised nuclear integrity. B) Cells treated with the negative control esiRNA-RLUC showed nuclei with similar characteristics to the non-transfected cells. C) 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. D) 8mM 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 63X with the addition of the Aryscan processing.

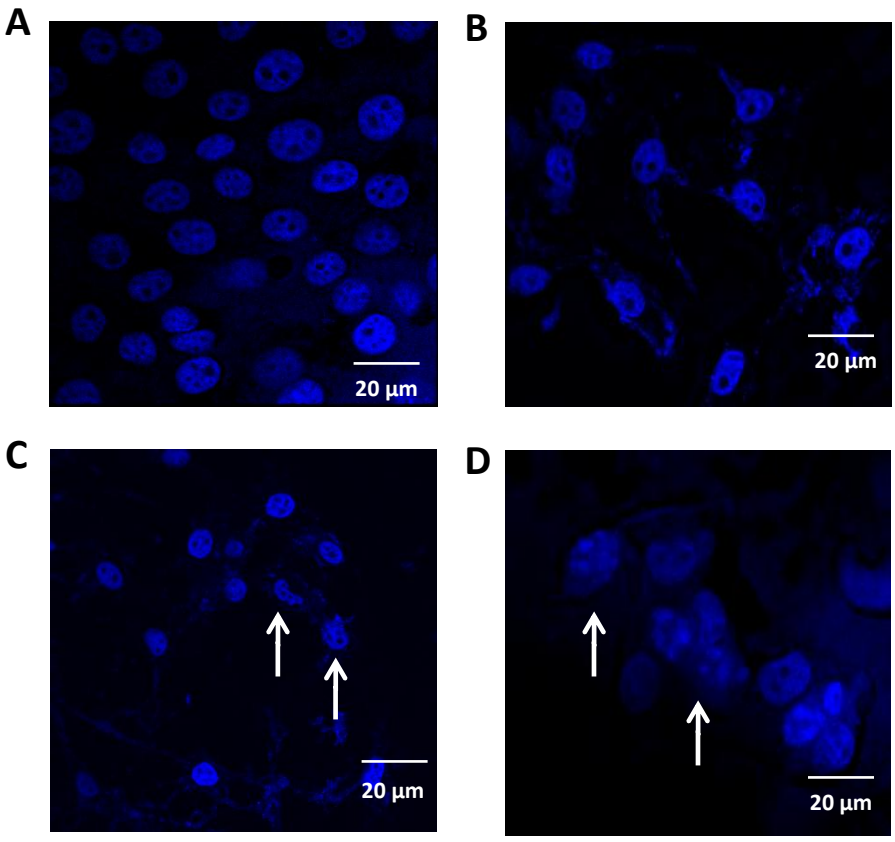


Figure 25: Distinct changes in nuclear morphology in late stage (A375SM) malignant melanoma cells post siRNA-mediated LRP down-regulation. A) The nuclear integrity is maintained in the non-transfected cells as shown by the normal and rounded shape of the nuclei. B) Negative control esiRNA-RLUC treated cells showed no disruptions in nuclear integrity, as expected. C) DHARMACON Human RPSA siRNA transfected cells showed reductions in membrane integrity, nuclear shrinkage and membrane blebbing (indicated by the white arrows).D) Positive control PCA treated cells showed reductions in membrane integrity and

apoptotic body formation (shown by the white arrows). All images were obtained at a magnification of 63X with the addition of the Airyscan processing.

4.8 Annexin V-FITC/PI assays indicated that siRNA-mediated down-regulation of LRP expression leads to apoptotic induction in both the early and late stage malignant melanoma cells.

Confocal microscopy with the addition of Aryscan processing (resolution: 140 nm) suggested that siRNA-mediated LRP down-regulation in early (A375) and late (A375SM) stage malignant melanoma cells causes distinct changes in nuclear morphology that are specific to cells undergoing apoptotic induction. Therefore, to confirm that this crucial observation of apoptotic induction has indeed occurred in both of the malignant cell lines, Annexin V-FITC/PI assays were performed.

Upon transfection of A375 cells with Human RPSA siRNA (Fig. 26), 91.4% of the cells underwent total apoptotic induction when compared to the untreated cells. Treatment of A375SM cells with Human RPSA siRNA resulted in 97.4% of cells experiencing total apoptotic induction in comparison to the untreated cells (Fig. 27). In addition, treatment of both malignant cell lines with the negative control esiRNA-RLUC did not result in apoptotic induction (Fig. 27 and 28). PCA was used as the effective positive control and led to high percentages of total apoptotic induction in both cell lines; 98.3% in the A375 cells and 97.8% in the A375SM cells (Fig. 26 and 27).

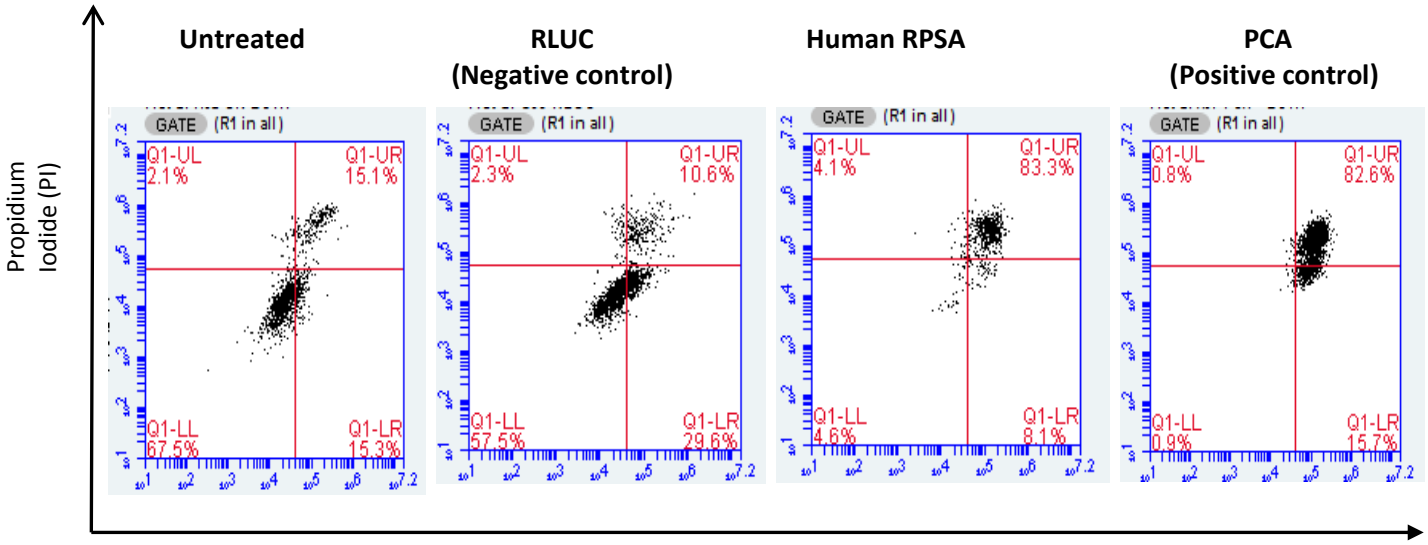


Figure 26: Induction of apoptosis in early stage (A375) malignant melanoma cells after Human RPSA siRNA transfection. The non-transfected/untreated plot shows a majority of cells in the lower left quadrant (Q1-LL) as it is representative of normal living cells. Treatment of the cells with the negative control esiRNA-RLUC also resulted in the majority of the cells appearing in the lower left quadrant, while the transfection of the 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. 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.

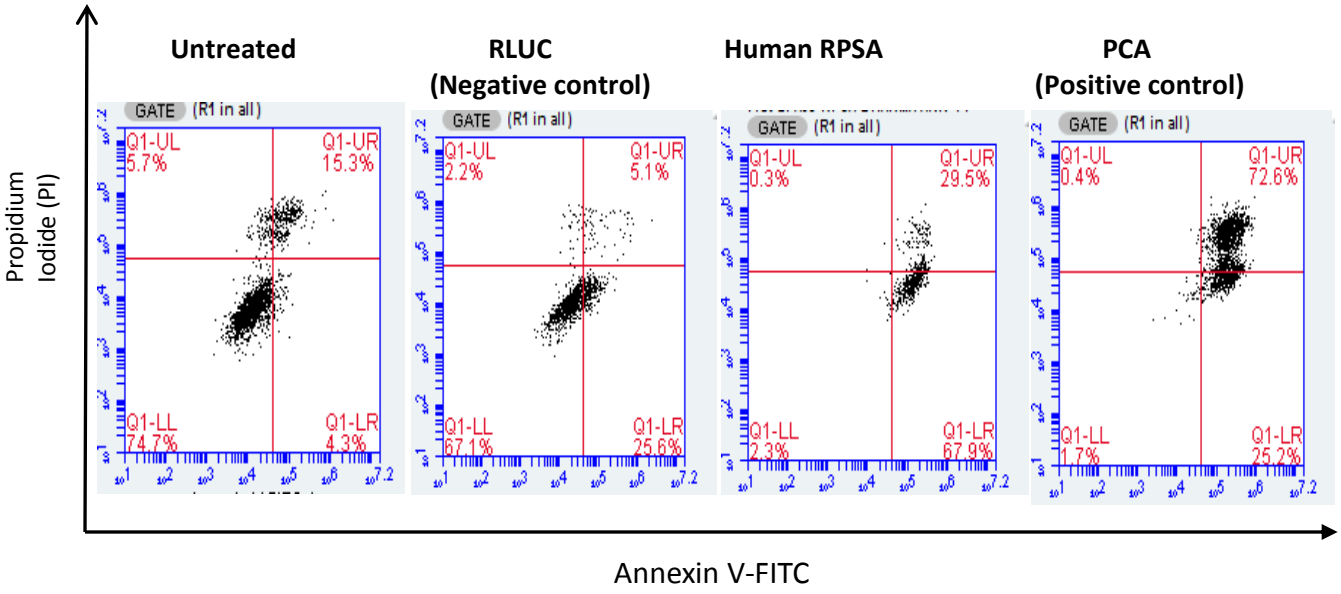


Figure 27: Apoptotic Induction in late stage (A375SM) malignant melanoma cells after siRNA transfection. Majority of the cells in the untreated and esiRNA-RLUC treated plot appeared in the lower left quadrant, indicative of normal living cells. 67.9% of cells transfected with Human RPSA siRNA appeared to be undergoing early apoptosis as shown in the lower right quadrant, 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. Cells treated with PCA, appeared mostly in the upper right quadrant, indicative of cells undergoing late apoptosis. 20 000 cells were counted per sample.

4.9 The level of apoptotic induction after siRNA-mediated LRP down-regulation is significantly increased in early and late stage malignant melanoma cells.

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. 26 and 27) in triplicate. This was used as an indication of total apoptotic levels and it was observed that 7% and 76% of A375 cells underwent early and late apoptosis, respectively. In addition, 65% and 31% of A375SM cells underwent early and late apoptosis, respectively. A375 and A375SM cells underwent 61% and 78% more total apoptosis in comparison to the non-transfected cells, after Human RPSA siRNA transfection, respectively.

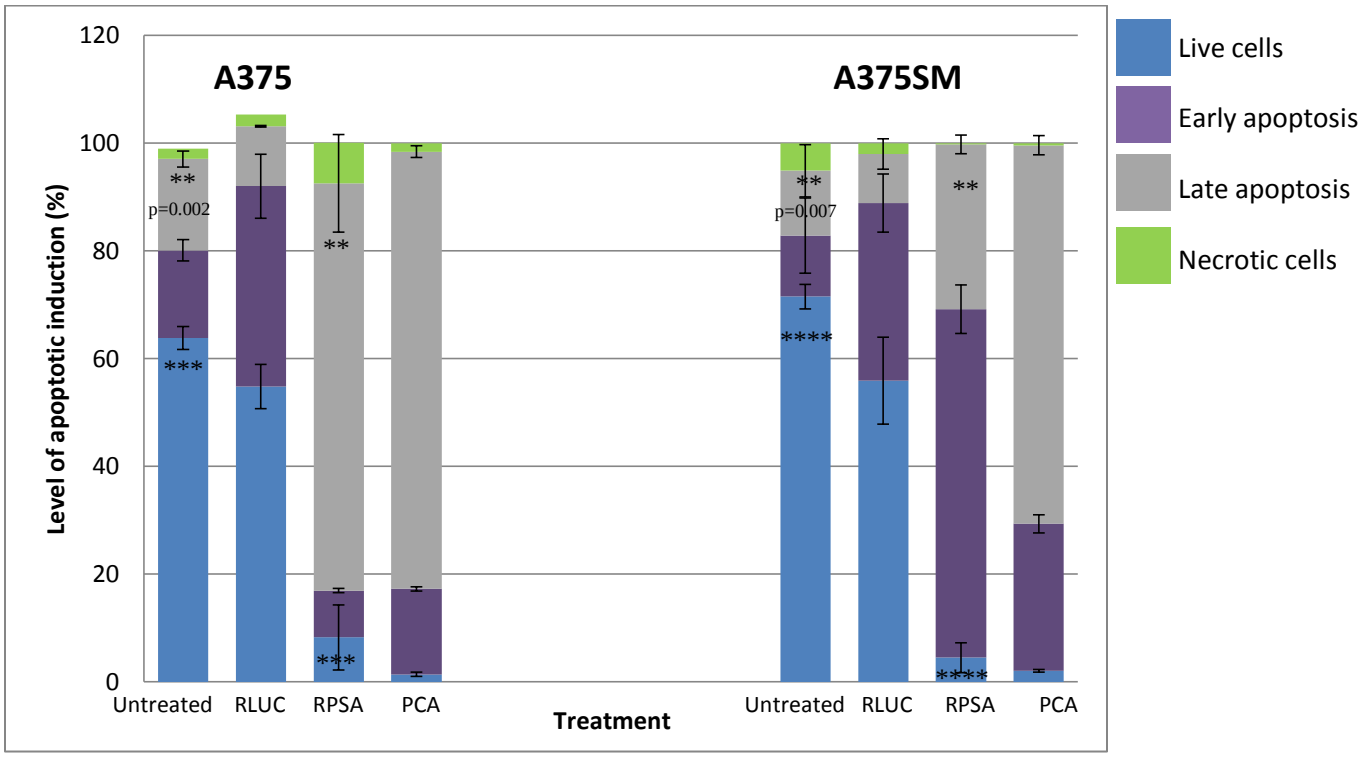


Figure 28: Total levels of apoptotic induction in early (A375) and late (A375SM) stage malignant melanoma cells after siRNA-mediated LRP down-regulation. Post transfection of cells with Human RPSA siRNA, 7% and 76% of A375 cells underwent early and late apoptosis, respectively. 65% and 31% of A375SM 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

A High correlation was observed between total LRP levels post Human RPSA treatment and total levels of apoptosis for both cell lines (Table 7).

Table 7: Person's correlation between total LRP levels post Human RPSA transfection and total levels of apoptosis.

Cell Lines	Correlation between total LRP levels post Human RPSA transfection and total levels of apoptosis (r-value)
A375	0.7
A375SM	0.8

4.10 siRNA-mediated down-regulation of LRP expression leads to a significant increase in caspase-3 activity 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.29). In addition, treatment of both cell lines with the negative control Mission esiRNA-RLUC, showed no significant difference in caspase-3 activity when compared to the non-transfected cells which were set to 100% (Fig. 29).

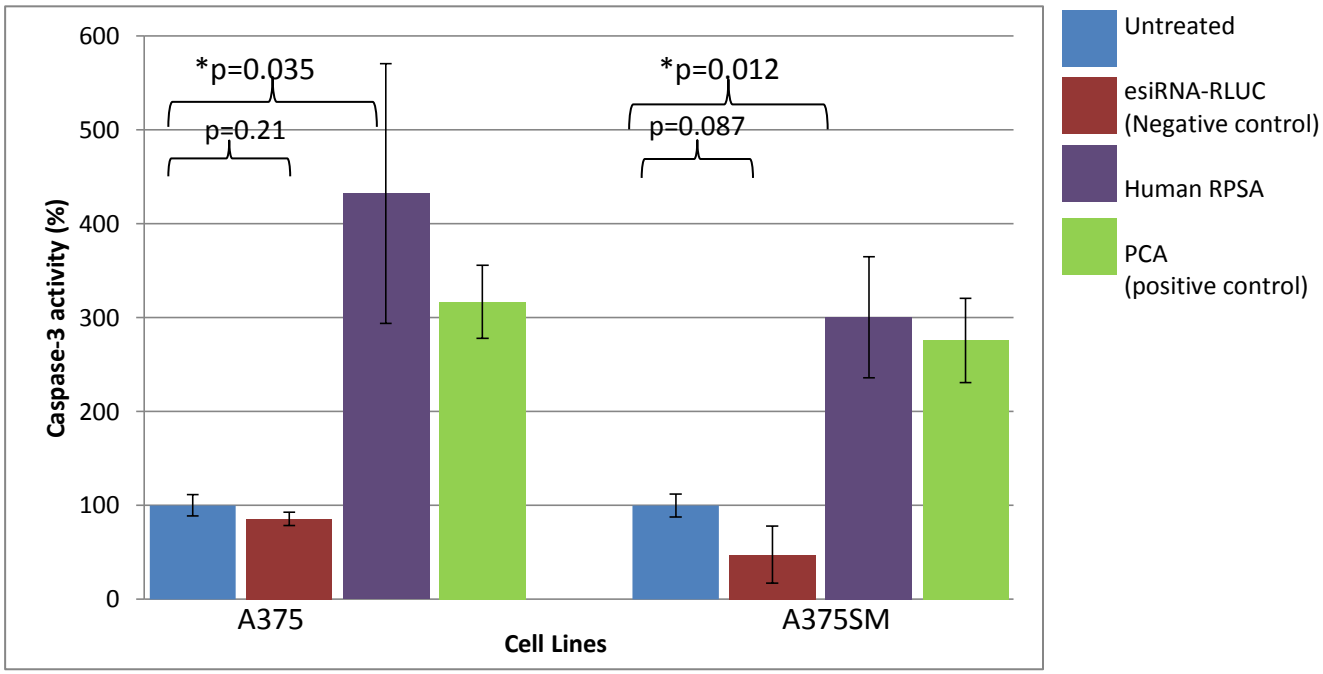


Figure 29: Analysis of the effect of siRNA-mediated LRP down-regulation on caspase-3 activity in early (A375) and late (A375SM) stage malignant melanoma cells. Caspase-3 activity assay indicated that post transfection of cells with Human RPSA siRNA, A375 and A375SM cells showed a significant 4-fold and 3-fold increase in caspase-3 activity, respectively when compared to the non-transfected cells (which was set to 100%). A non-significant difference was observed when both malignant melanoma cell lines were treated with the negative control esiRNA-RLUC and compared to non-transfected cells. Protocatechuic acid (PCA) was used as the positive control. The graph is 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

A high correlation was obtained between total levels of LRP post Human RPSA transfection and the increases in caspase-3 activity after RPSA treatment, thus indicating the increase in apoptosis (Table 8).

Table 8: Pearson's correlation between total LRP levels post Human RPSA transfection and increases in caspase-3 activity after Human RPSA treatment.

Cell Lines	Correlation between total LRP levels post Human RPSA transfection and increases in caspase-3 activity after Human RPSA treatment (r-value)
A375	0.63
A375SM	0.9

4.11 A375 cells exhibit a significant increase in caspase- 8 activity after siRNA-mediated down-regulation of LRP expression.

Caspase-8 activity assays were performed in order to further confirm that siRNA-mediated LRP down-regulation induces apoptosis in early (A375) and late (A375SM) stage malignant melanoma cells, and in addition to gain insight into whether or not the caspase-8 mediated extrinsic pathway is used by each of the cell lines under study to induce apoptosis post transfection with DHARMACON Human RPSA. Post transfection of both cell lines with Human RPSA, A375 cells showed a significant 66% increase in caspase-8 activity when compared to the non-transfected cells, which were set to 100% (Fig. 30). No significant difference was observed in caspase-8 activity in A375SM cells treated with Human RPSA when compared to the non-transfected cells. Moreover, the treatment of both cell lines with the negative control esiRNA-RLUC resulted in no significant difference in caspase-8 activity, when compared to the non-transfected cells (Fig. 30).

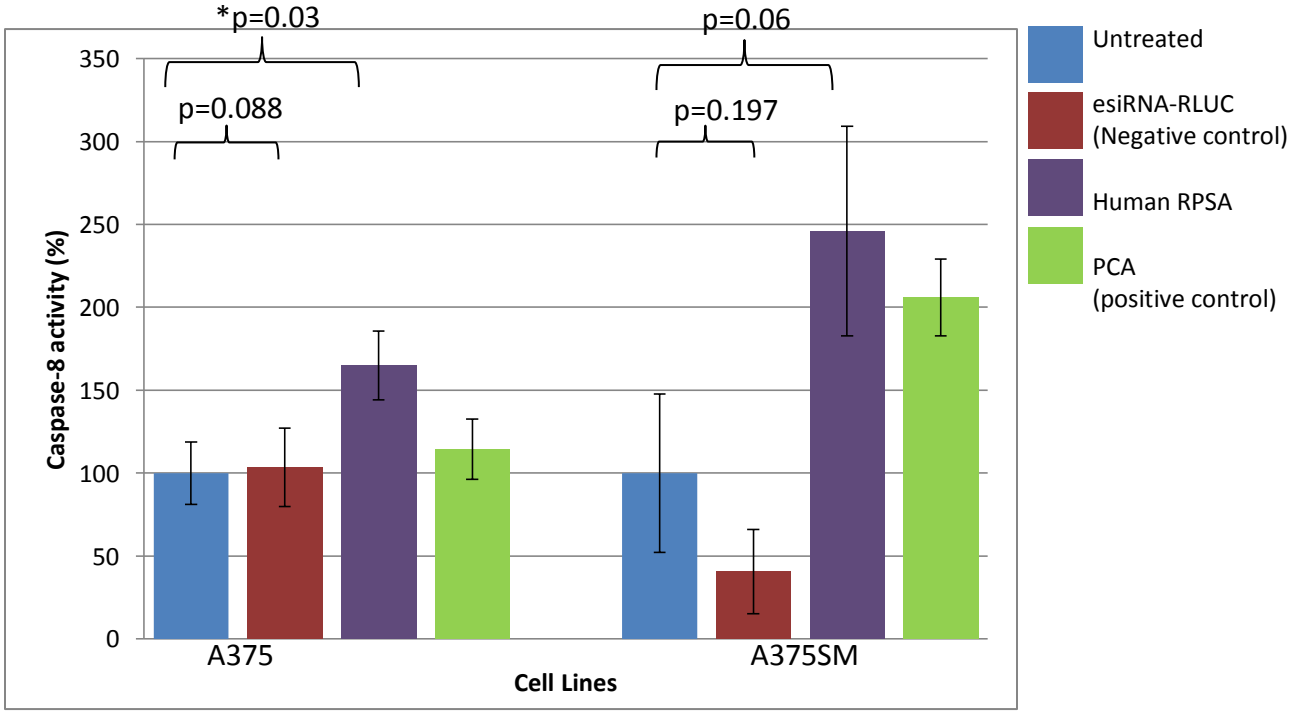


Figure 30: The effect of siRNA-mediated down-regulation of LRP expression on caspase-8 activity in early (A375) and late stage (A375SM) malignant melanoma cells. DHARMACON Huaman RPSA transfected A375 cells showed a significant 66% increase in caspase-8 activity, when compared to non-transfected cells (set to 100%). Treatment of A375SM cells with

DHARMACON Human RPSA showed no difference in caspase-8 activity by comparison to non-transfected cells. Treatment of both cell lines with the negative control esiRNA-RLUC resulted in no significant difference in caspase-8 activity when compared to non-transfected cells. PCA was used as a positive control. The graph is 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

4.12 A375SM cells show a significant increase in caspase-9 activity after siRNA-mediated LRP down-regulation.

Caspase-9 activity assays were performed in order to establish whether early (A375) and late (A375SM) stage malignant melanoma cells use the caspase-9 –mediated intrinsic pathway to induce apoptosis after siRNA-mediated LRP down-regulation. A375SM cells show a significant 3-fold increase in caspase-9 activity in comparison to the non-transfected cells, which were set to 100% (Fig. 31). A non-significant difference was observed in caspaase-9 activity in A375 cells treated with Human RPSA by comparison to non-transfected cells. In addition, treatment of both cells lines with the negative control esiRNA-RLUC resulted in a non-significant difference in caspase-9 activity in comparison to the non-transfected cells (Fig. 31).

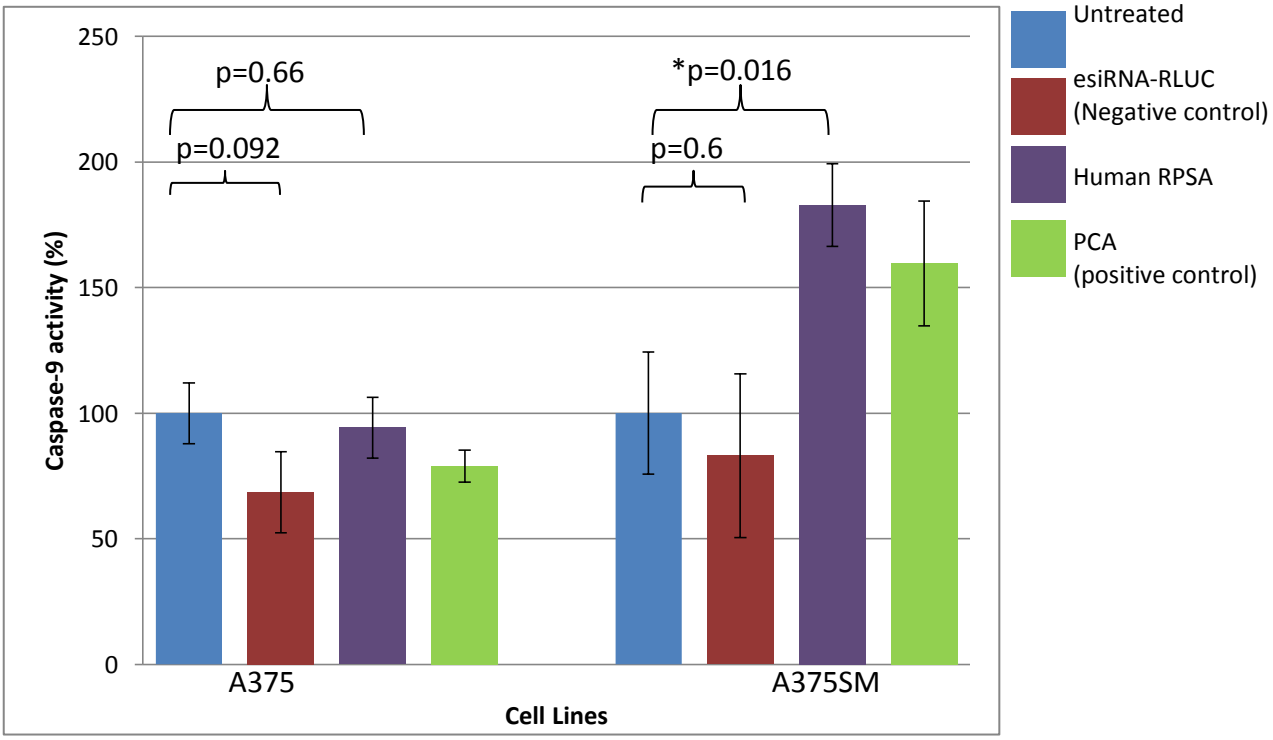


Figure 31: The effect of siRNA-mediated down-regulation of LRP expression on caspase-9 activity in early (A375) and late (A375SM) stage malignant melanoma cells. DHARMACON Human RPSA transfected A375SM cells showed a significant 3-fold increase in caspase-9

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-9 activity after treatment of cells with the negative control esiRNA-RLUC, and by comparison to non-transfected cells. PCA was used as a positive control. The graph is 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

Chapter 5: Discussion

Cancer is seen as a global burden affecting both economically developed and developing countries. It is a disease that exhibits high incidence and mortality rates and due to the alarming statistics previously outlined in section 1.1, it remains crucial to continuously investigate possible therapeutic tools for this disease. LRP/LR is a key contributor in the promotion of the tumorigenic phenotype, enhancing tumor progression through processes such as metastasis, tumor cell proliferation, apoptotic evasion by cancer cells and the maintenance of tumor cell viability. Therefore, the use of RNA interference technology to down-regulate LRP expression has been deemed an effective means of hampering the maintenance of tumor cell viability and in particular siRNAs directed to LRP/LR of lung (A549), cervical (HeLa) liver (Hep3B), pancreatic (AsPC-1) and neuroblastoma (IMR-32) cancer cells has resulted in decreased viability of these tumourigenic cells. Each cancer type has its own characteristic behavior and therefore it is critical to examine the use of LRP-specific siRNAs on other cancer types- which has been investigated in this study.

5.1 Analysis of the difference in cell surface LRP/LR levels between early and late stage malignant melanoma cells

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, however these percentages are representative of the percentage of cells within the population that contain a certain amount of LRP/LR on the cell surface. The actual LRP/LR expression levels present on the surface of both malignant melanoma cell lines was then further investigated and quantified using median fluorescent intensity (Section 4.2, Table 1) and it was revealed that A375SM cells display significantly higher cell surface LRP/LR in comparison to A375 cells. As indicated by *Munien et al. (2017)* [136] 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. It has been shown that Human umbilical vein endothelial (HUVE) cells [95], liver cancer cells [94], pancreatic and neuroblastoma cells [137] as well as colorectal cancer cells [138] display high levels of LRP/LR on the cell surface in comparison to the poorly invasive breast cancer (MCF-7) cells. These findings indicate that several cancer cell lines rely on LRP/LR to perform certain cellular processes. Therefore 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 transmembrane receptor may be responsible for.

In addition, the bacterial protein chloramphenicol acetyltransferase (CAT) was used as the negative control as it is not present in eukaryotic cells. CAT was used as an IgG isotype and therefore served as a highly efficient control due to the non-significant shift in the percentage of cells expressing LRP/LR on the cell surface thus confirming the specificity and recognition of IgG1-iS18 for LRP/LR.

5.2 Assessment of the difference in total LRP levels between early and late stage malignant melanoma cells

LRP/LR is predominantly found as a transmembrane receptor however, as previously described it can also be found in other cellular locations. Therefore, in addition to the differences observed in cell surface LRP/LR levels between A375 and A375SM cells, total LRP levels were also determined. Quantification of total LRP levels between the two malignant melanoma cell lines included the presence of this receptor in the cytosol, nucleus, perinuclear region and also on the cell surface. Western blotting and densitometric analysis revealed that A375SM cells contain higher total LRP in comparison to the early (A375) stage malignant melanoma cells. The role of LRP/LR in the nucleus is to maintain nuclear structures and to complete translational processes in the cytosol [86-91], 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 high correlation observed in Table 2 is crucial as LRP/LR present on the cell surface is predominantly implicated in the maintenance of cellular viability and apoptotic evasion [107], therefore total LRP levels can be used to assess cell viability and the induction of apoptosis.

5.3 siRNA mediated down-regulation of LRP

LRP was down-regulated via RNA interference using the siRNA called Dharmacon ON-TARGET Human RPSA, in order to assess the effect of this receptor on cellular viability. The Human RPSA siRNA targets the mRNA of the 37kDa laminin receptor precursor (LRP) form, targeting both sense and antisense strands of the human LRP gene via the ON-TARGET*plus*

modification pattern to reduce off-target effects (sequence provided in appendix). Western blotting was used to detect down-regulated LRP levels with the use of the anti-LRP/LR specific antibody IgG1-iS18, which only effectively detects the 37kDa LRP. Treatment of A375 and A375SM cells with Dharmacon Human RPSA showed significant reductions in LRP expression, when compared to the non-transfected cells- thereby proving the efficiency of Dharmacon Human RPSA on LRP expression.

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. With regards to the preparation of lysates, the same density of cells from both cell lines was treated with the same volume and concentration of Human RPSA siRNA. Therefore, it can be suggested that the volume of Human RPSA used was effective in targeting more LRP in A375 cells due to lower total LRP levels present in this cell line prior to siRNA transfection. It can therefore be said that the volume of Human RPSA was less effective in targeting LRP in A375SM cells due to higher total LRP levels prior to siRNA transfection. High total LRP levels before siRNA transfection, therefore leads to less LRP down-regulation post siRNA treatment and vice versa.

The 37kDa LRP is the precursor of the 67kDa LR form, therefore reduced LRP expression would in turn result in the reduced maturation of the LR form through the post-translational modification known as fatty acylation [83]. However this maturation does not occur through direct transcription or translation and therefore as previously addressed, the Human RPSA siRNA specifically targets the 37kDa LRP as the 67kDa LRP is unable to be directly targeted.

It is important to note that the negative control Mission esiRNA-RLUC was used to eliminate the effects of Dharmacon Human RPSA by not targeting arbitrary genes within the cell. This negative control siRNA is also designed to exert minimal or no effect on cellular viability as well as have no significant effect on LRP expression in both A375 and A375SM cells (Fig 20 A and 20B).

As observed experimentally, 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 called Mission esiRNA-

RPSA was used to target the mRNA of the 37kDa 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 37kDa LRP mRNA, while esiRNA-RPSA targets nucleotides 521-929 of the mRNA sequence (as per Appendix A). Treatment of A375 and A375SM cells with esiRNA-RPSA resulted in significant reductions in LRP expression, respectively. The high correlation obtained further confirms that higher total LRP levels before siRNA treatment results in less LRP down-regulation post siRNA transfection.

The two siRNAs referred to above, yielded similar effects even though their target sequences differ. It can therefore be said that the results obtained upon treatment with Dharmacon Human RPSA are a direct outcome of LRP down-regulation. As previously mentioned, esiRNA-RLUC was again used as the negative control and no significant difference in LRP expression was observed when A375 and A375SM cells were treated with this negative control siRNA, therefore also proposing high specificity of esiRNA-RPSA for LRP.

5.4 Relationship between LRP expression and the maintenance of cellular viability

LRP/LR plays a crucial role in the maintenance of cellular viability, however in order to gain further insight into this role, siRNAs were used to down-regulate LRP expression and the effect thereof on the cellular viability of early and late stage malignant melanoma was evaluated using MTT assays. Treatment of A375 and A375SM cells with Dharmacon Human RPSA resulted in significant reductions in cellular viability, when compared to the non-transfected cells. The high correlations further confirm 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 the alternative esiRNA-RPSA resulted in significant reductions in cellular viability, respectively when compared to the non-transfected cells. 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.

In addition, upon treatment of A375 and A375SM cells with the negative control esiRNA-RLUC, no effect on cellular viability was observed thus confirming the high efficacy of this negative control as well as confirming the specificity of the respective target siRNAs used in the

study. The cells were also treated with 8 mM protocatechuic acid (PCA) - which is a known apoptotic inducer and therefore used as the positive control in this study. After treatment of A375 and A375SM cells with PCA, reductions in cellular viability were observed- parallel to that of the cells treated with Dharmacon Human RPSA and Mission esiRNA-RPSA, as predicted.

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 [78]. 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 [126]. 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 [106]. 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 [126]. *Khumalo et al, 2015* [108] and *Chetty et al, 2017* [110] 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 a reduction in the LRP/LR-Midkine interactions thus being responsible for the observed reductions in cellular viability. In addition, *Lu et al, 2016* [127] 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 upregulation of specific pro-apoptotic proteins and a downregulationn in anti-apoptotic proteins.

5.5 Effect of down-regulated LRP expression on the induction of apoptosis

5.5.1 Nuclear morphological alterations

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 addition of Airyscan processing was performed. The two malignant

melanoma cell lines were treated with the Human RPSA siRNA, and after transfection, nuclear morphological alterations were highly evident- 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. These changes were furthermore observed in the PCA treated cells (positive control), therefore suggesting that siRNA-mediated LRP/LR down-regulation induces apoptosis. It has been found that 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 [128]. Therefore down-regulation of LRP/LR results in reduced nuclear integrity and altered nuclear morphology. These observable Human RPSA siRNA- induced changes to the nuclear integrity and morphology of A375 and A375SM cells are indicative of apoptotic induction [34, 35] in these two malignant melanoma cell lines. Furthermore, disruptions in the structure of cellular DNA is characteristic in cells undergoing apoptotic induction [129].It has previously been shown that siRNA-mediated down-regulation causes morphological changes in breast and oesophageal [108], lung and cervical [109] as well as pancreatic cancer and neuroblastoma cells [110]. However, transfection of both malignant melanoma cell lines with the negative control esiRNA-RLUC showed no disruptive alterations in nuclear morphology, confirmed by similarities to the non-transfected cells.

5.5.2 Confirmation of Apoptotic induction via Annexin V-FITC/PI assays

Even though confocal microscopy indicated the occurrence of apoptosis in A375 and A375SM cells, further confirmation as well as quantification of apoptotic induction was required and this was achieved by performing the Annexin-V-FITC/PI assay. Induction of apoptosis results in reductions in cell membrane integrity as previously described 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 [129]. Important to note, the Annexin-V protein is bound to the FITC fluorochrome therefore allowing for the PS to be detected through 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. In particular, 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.

It was observed that 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. Subsequently, Human RPSA-treated and PCA- treated (8mM) A375 and A375SM cells portrayed positive staining for Annexin-V, 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. This process of cell membrane blebbing exposes PS to the outer cell membrane leaflet, favoring the binding of Annexin-V, as previously described. 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. 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.

It has been shown by Khumalo et al, 2015 [108], Moodley et al, 2013 [109] and Chetty et al, 2017 [110] that several cancerous cell lines undergo apoptosis via the early and late apoptotic stages through siRNA-mediated LRP/LR down-regulation. Small percentages of both cell lines underwent necrosis after treatment (upper left quadrant). One possible explanation for this observation could be due to the 72-hour incubation or transfection period being too long, thus also applying to the necrotic cells observed for the non-transfected condition. However, this

would have to be further investigated by shortening the incubation or transfection period and analysing the resultant observable effects.

5.5.3 Mechanistic molecular pathways of apoptosis

Nuclear morphological studies using confocal microscopy along with Annexin-V-FITC/PI assays proved that LRP down-regulation via siRNA-mediated action leads to apoptotic induction in A375 and A375SM cells. In addition, caspase-3 assays were performed to further confirm the apoptosis inducing effects of siRNA-mediated LRP down-regulation. The effector caspase-3 has increased activity in cells that are actively undergoing apoptosis [131]. 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. 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* [132] 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 (Fig.12B) [132]. Thereby it can be stated that siRNA-mediated LRP down-regulation in the current study may reduce the LRP/LR-FAK interaction, resulting in apoptotic induction. LRP/LR is furthermore known to play a role in the biogenesis of ribosomes by facilitating the maturation process of the 21S pre-rRNA into the 18S rRNA, while associating with and acting as a component of the 40S ribosomal small unit and assisting in protein translation and synthesis [133, 134, 135]. Therefore the down-regulation of LRP/LR could moreover 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. This could be 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-3 cleaves internal cellular substrates in order to initiate the induction of apoptosis and it is therefore an effector caspase. The observed increase in caspase-3 activity post Human RPSA-mediated LRP down-regulation can only be an indication of the occurrence of apoptosis since caspase-3 is involved in both the intrinsic and extrinsic apoptotic pathways (Fig. 7). Caspase-8 and caspase-9 activity assays were performed in order to evaluate which apoptotic pathways A375 and A375SM cells initiate in order to undergo apoptosis after Human RPSA-mediated down-regulation of LRP expression as different cancer cell lines may utilize different mechanistic caspase pathways to undergo apoptosis as shown by Khumalo et al, 2015 [108], Moodely et al, 2013 [109] and Chetty et al, 2017 [110]. 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. The late stage malignant melanoma also displayed an increase in caspase-8, however it was non-significant. 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. 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 [141]. However, to obtain more insight in this regard, further analysis of the apoptotic proteins involved in the regulation of caspase-8 (extrinsic pathway) and caspase-9 (intrinsic pathway) should be investigated.

Chapter 6: Conclusion and Future work

6.1 Conclusion

The present study indicated that siRNA-mediated down-regulation of LRP expression significantly reduced the cellular viability of both the early (A375) and late (A375SM) stage malignant melanoma cells through the induction of apoptosis. In particular, apoptosis occurred via the extrinsic pathway in A375 cells and via the intrinsic pathway in A375SM cells.

These significant findings demonstrate the important role played by LRP/LR in the maintenance of cellular viability and the evasion of apoptosis in both malignant melanoma cell lines. The specific siRNAs known to target LRP expression may be considered as a possible therapeutic tool for the treatment of both early and late stage malignant melanoma cells.

6.2 Future Work

The present study only focused on two malignant melanoma cell lines (early and late stage) and therefore several other cancer types remain to be investigated in terms of siRNA-mediated LRP down-regulation and cellular viability. Additional cell lines within a specific cancer type that are at different stages of their life cycle (i.e. early, middle and late) could be considered in order to determine at which stage siRNA-mediated LRP down-regulation is most effective at reducing cellular viability. In addition, dose and time- dependent studies could be investigated in order to determine optimal transfection periods as well as siRNA dosages. Further apoptotic studies could be performed in order to gain further insight into the regulation of the intrinsic and extrinsic pathways via analysis of apoptotic proteins such as Bcl-2 and *Bax*.

LRP/LR plays several physiological roles in normal cells, and therefore it may be worthwhile to investigate the effects of siRNA action on LRP expression in normal cells. This should be investigated in order to determine if the down-regulation of LRP exerts a minimal or no effect on the functioning of normal cells and in turn to further the study using animal models such and nude mice *in vivo.* Therefore, future studies could explore the use of siRNAs together with anti-LRP/LR specific antibodies to treat several tumor enhancing processes. Animal trials, if successful may lead to clinical trials, therefore allowing the effect of siRNA-mediated LRP down-regulation and anti-LRP/LR specific antibodies to be evaluated in the human body.

Chapter 7: Animal study to investigate the role of IgG1-Is18 as a potential therapeutic to reduce the metastatic potential of malignant melanoma

The efficacy of the anti-LRP/LR specific antibody IgG1-iS18 as a therapeutic tool for the treatment of malignant melanoma, using an *in vivo* MF-1 nude mouse model.

7.1 Rationale and research question

LRP/LR has been seen to be over-expressed in numerous cancerous cells, including breast, colon, cervical, prostate and in particular early (A375) and late (A375SM) stage malignant melanoma cells thus promoting tumor progression. This over-expression promotes enhanced LRP/LR-laminin-1 interaction resulting in elevated tumor aggressiveness and enhanced metastatic potential (as described in section 1.3.7). A direct correlation between LRP/LR levels and the metastatic and invasive potential of cells has been observed [136] and according to *Munien et al, 2017* [137], treatment of cells with the anti-LRP/LR specific antibody IgG1-iS18 has led to a significant reduction in the adhesion and invasion of A375 and A375SM malignant melanoma cells *in vitro*. Therefore the aim of the present study was to further investigate the significance of blocking LRP/LR with the IgG1-iS18 antibody and if this will be effective in the treatment of malignant melanoma, *in vivo,* using a MF-1 nude mice model.

7.2 Hypothesis

Blocking LRP/LR with the IgG1-iS18 antibody will significantly reduce the metastatic potential of malignant melanoma and therefore aid in the treatment of this cancer type, *in vivo.*

7.3 Aim

To further investigate the significance of blocking LRP/LR with the IgG1-iS18 antibody and to investigate whether the antibody is effective in the treatment of malignant melanoma *in vivo.*

7.4 Objectives

1. To assess the efficiency of the IgG1-iS18 antibody for the treatment of malignant melanoma using nude mouse models.

2. To analyse the ability of IgG1-iS18 with respect to tumor cell shrinkage and reductions in tumor cell metastasis *in vivo*

7.5 Materials and methods

The MF-1 nude mice model was employed to investigate the response of the IgG1-iS18 antibody on malignant transformation, invasion and metastasis. In this model, the human late stage malignant melanoma cells (A375SM) were subcutaneously injected into immunocompromised mice that do not reject human cells (Animal Ethics Clearance Number (AESC No.) 2015-04-C).

This model allows for the investigation of different therapeutic approaches on human cancers and it is effective as the actual human tumor tissue or cancer cell lines are used thus featuring the complexity of genetic and epigenetic abnormalities of the human tumor population as well as the ability to obtain experimental results within a few weeks from a human tumor biopsy [141].

The experimental approach involved an initial pilot study (which is presented here) using MF-1 nude mice in order to assess the feasibility of the experimental design for the full *in vivo* study.

7.5.1 Preparation of late stage malignant melanoma cells

The A375SM cells were detached using 1X Trypsin/EDTA and re-suspended in full growth medium (DMEM). Thereafter, the cells were pelleted by centrifugation at 1200 rpm for 10 minutes and then washed in 1X PBS prior to a second round of centrifugation at 1200 rpm. The resultant pellet was re-suspended in 10 ml media; cell counted and added into separate eppendorf tubes at a density of $5x10^6$ cells/ml. The cells were then centrifuged, and the pellet re-suspended in 110 µl media. Prior to subcutaneous injection of the cells, 110 µl cold Matrigel was added to the media and cell mixture.

7.5.2 Transplantation procedure

The 9 subject mice were divided into three groups; with 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 subcutaneous injection of the late stage malignant melanoma cells. The cell mixture (220 µl) was then drawn up into a syringe and carefully

injected into the flank/ hind leg region of each mouse using either a 25 or 27 gauge needle. The tumors were then allowed to establish for 3 weeks (Fig.32).

7.5.3 Analysis of tumor formation

The mice were monitored externally for tumour formation by visually observing the mice, weighing the mice as well as quantifying the size and volume of the tumour via 3D caliper measurements. This occurred over the 3 week induction period prior to the initiation of treatment.

7.5.4 Treatment regime

Once the mice had visible tumour formation, within the 3 week induction period, group 1 was treated with 0.885 mg/ml of the IgG1-iS18 antibody, group 2 was treated with the Phosphate Buffered Saline (PBS) vehicle control solution and the last group remained untreated. The IgG1-iS18 antibody was administered at 2.5mg/kg body weight twice a week, intraperitoneally over a period of 4 weeks (Fig. 33) [142]. For the vehicle control, Phosphate Buffered Saline (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 [143]. 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 (Fig.33). The antibody was injected into the lower left or right quadrant so as to avoid hitting vital internal organs such as the bladder and liver. 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.

After the four week treatment period, all nine mice were euthenized with 0.5 ml euthenaze and assessed for internal and external tumor formation. The tumors were harvested, then weighed and the tumor volumes determined using the following formula: V= (Length x width x width)/ 2.

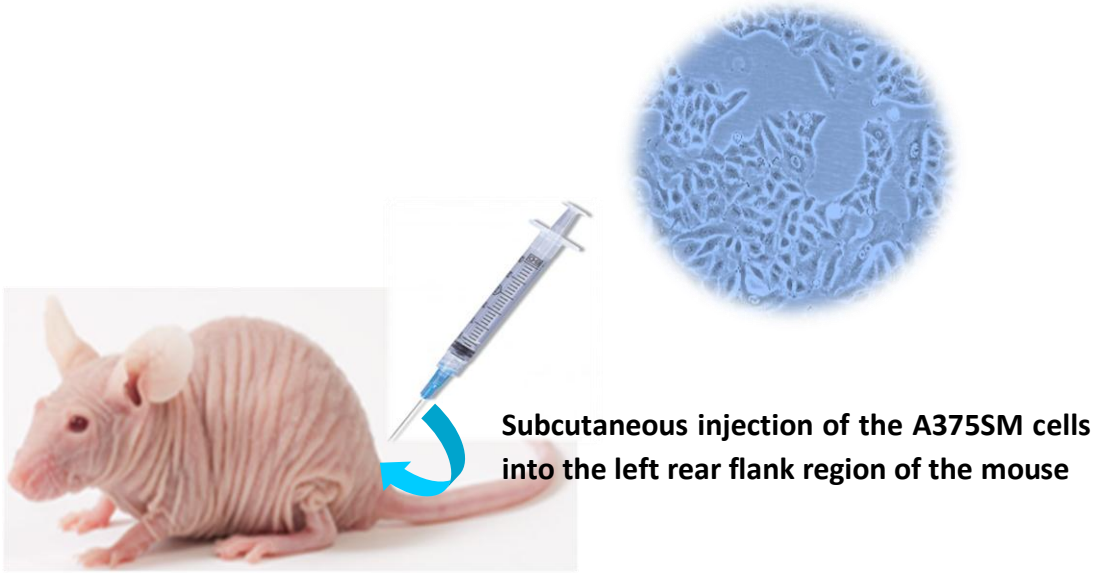


Figure 32: The injection procedure of the A375SM cells in MF-1 nude mice. The A375SM cells were pelleted at a density of $5x10^6$ cells/ml and prepared in 100 µl Matrigel before subcutaneous injection into the rear flank region of the 9 subject mice. The cells were injected and allowed to establish into tumors over a period of 3 weeks.

Figure 33: The treatment procedure of the MF-1 nude mice model using the IgG1-iS18 antibody. The IgG1-iS18 antibody was administered twice a week for 4 weeks intraperitoneally and tumor size was monitored.

7.6 Results and Discussion

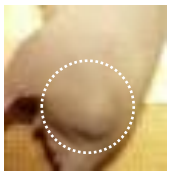
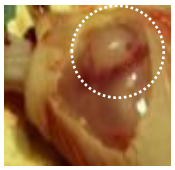
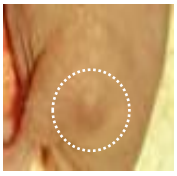
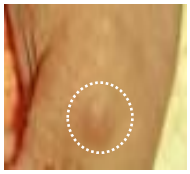
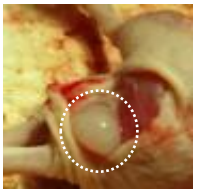
The results of this pilot study indicated that three of the nine mice developed external tumor formation: one of the mice being from the untreated group with a tumor volume of $352mm^3$ and a weight of 0.77g. The other two mice were from the IgG1-iS18 treatment group, both tumors having a tumor weight of 0.2g and a tumor volume of $40mm^3$ and $63mm^3$, respectively (Table 8).

When comparing the tumor formation from the two groups, untreated and treated, there was a tumor volume difference of 312mm^3 and 289mm^3, respectively and a weight difference of 0.575g (Table 8). Due to the reduction in tumor weight and volume post treatment, it can be concluded that the IgG1-iS18 antibody may act as a possible therapeutic tool for the prevention or treatment of malignant melanoma. This however is currently being investigated by performing the full *in vivo* study with a total of 24 MF-1 nude mice, 12 receiving IgG1-iS18 antibody treatment and 12 receiving the PBS vehicle control. Due to visible tumour formation in the mice and evident reductions in tumour volume upon antibody treatment, it can be stated that the parameters from the pilot study are favourable in order to perform the full study.

Malignant melanoma is the most deadly form of skin cancer and if it is not treated in its early stages it can become fatal as it is able to metastasise to distant sites in the body. Therefore, directly targeting the overexpressed LRP/LR receptor in malignant melanoma cells with the anti-LRP/LR specific antibody IgG1-iS18, may act as a highly effective treatment option as it is directed towards reducing metastasis via the LRP/LR- laminin interaction [136]. With regards to the treatment options available, IgG1-iS18 is more target specific to each cancer type in that it directly targets metastasis while having a limited effect on normal cells which is not the case in terms of chemotherapy.

Obtaining favourable results *in vivo* will allow for further research on the antibody as a possible novel therapeutic option for the treatment of malignant melanoma, and that may involve clinical studies and trials on human patients.

Table 9: Summarized experimental parameters and outcomes observed in the pilot study MF-1 nude mice model.

	Mouse 1	**Mouse 2**	**Mouse 3**
Treatment group	Untreated	IgG1-iS18 antibody	IgG1-iS18 antibody
Density of cells injected (cells/ml)	$5x10^6$	$5x10^6$	$5x10^6$
Induction period for Tumour formation (weeks)	3	3	3
Visual representation of tumor formation **<u>Surface view of tumor:</u>** **<u>Internal view of tumor:</u>**	 	 	 
Tumour volume (mm^3)	352	40	63
Tumor volume difference	----	312	289
Tumor weight (g)	0.77	0.2	0.2
Tumour weight difference (g)	----	0.575	0.575

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Appendix A

Reagents and Materials

- Media (DMEM)- GE Life sciences /Hyclone
- BSA, Ethanol -VWR Life sciences
- Matrigel Matrix- Corning
- Molecular weight marker -New England Biolabs Inc.
- penicillin/streptomycin, PBS, Opti-MEM -biowest/Gibco
- Tween, DMSO, lysis buffer-Merck Millipore
- Methanol, Paraformaldehyde -Associate chemical enterprise (ace)
- MTT powder -Duchefa Biochemie
- Trypsin/EDTA, Pen/Strep Amphoteracin B 100X -Lonza
- Trypan Blue, PCA-Aldrich chemistry
- HEPES – Thermo Scientific
- FCS- Capricorn Scientific
- L-glutamine, sodium pyruvate, non-essential amino acids, BCA reagents , APS, acrylamide, TEMED, fluoromount mounting fluid, SDS, Triton-X– Sigma Life science
- PVDF membrane – Pall corporation
- Chemiluminescent substrate, Cell counting slides - Biorad
- Microscopy slides and coverslips – Labocare
- 24-well, 6-well and cell culture flasks – Corning Inc.

Antibodies and siRNAs

- IgG1-iS18 – Affimed Therapeutics
- Anti-human IgG-HRP, anti-human PE, anti-rabbit APC– Abcam
- Anti-β actin peroxidase, anti-CAT, DAPI, esiRNA-RPSA, esiRNA-RLUC, Mission transfection reagent – Sigma
- Dharmacon (5nmol and 20nmol)- GE Life sciences (Dharmacon)
- DharmaFect transfection reagent –GE Life sciences (Dharmacon)

Kits

- Annexin V-FITC/ PI kit – BD Biosciences
- Caspase 3,-8 and -9 kits – Merck Millipore

Equipment list

- Flow cytometer – BD Accuri C6
- Confocal microscope – Zeiss LSM 710 3-channel (images were captured using the blue laser, 63X magnification using Zen software and the Airyscan processing tool).
- Laminar flow – Labotec
- ELISA reader – Tecan (using Magellan software)
- Centrifuge – Eppendorf 5417C
- Pipettes and micropipettes – Eppendorf research
- Gel casting and running apparatus – Biorad
- Biorad TC20 Cell Counter- Biorad
- pH meter – Eutech instruments

Software

- ImageJ
- IrfanView
- LSM Image Browser

Table A1: Dharmacon™ ON-TARGETplus SMARTpool Human RPSA, Mission esiRNA-RPSA and esiRNA-RLUC target sequences

Name of siRNA (catalogue/reference #)	Organism	Target sequence
ON-TARGETplus SMARTpool siRNA, RPSA	Homo Sapiens	
1. J-013303-05		CGACAUGAGUUGUACUUCU
2. J-013303-06		GAUUGCAUAUCAAAGCAUA
3. J-013303-07		GGUCAUGCCUGAUCUGUAC
4. J-013303-08		UAUCAUAAAUCUCAAGAGG
Mission esiRNA-RPSA	Homo Sapiens	CCTCTCACGGAGGCATCTTATGTTAACCTACCTACCATTGCGC TGTGTAACACAGATTCTCCTCTGCGCTATGTGGACATTGCCAT CCCATGCAACAACAAGGGAGCTCACTCAGTGGGTTTGATGTGG TGGATGCTGGCTCGGGAAGTTCTGCGCATGCGTGGCACCATTT CCCGTGAACACCCATGGGAGGTCATGCCTGATCTGTACTTCTA CAGAGATCCTGAAGAGATTGAAAAAGAAGAGCAGGCTGCTGCT

		GAGAAGGCAGTGACCAAGGAGGAATTTCAGGGTGAATGGACT GCTCCCGCTCCTGAGTTCACTGCTACTCAGCCTGAGGTTGCAG ACTGGTCTGAAGGTGTACAGGTGCCCTCTGTGCCTATTCAGCA ATTCCCTACTGAAGACTGGAGCG
Mission esiRNA-RLUC		GATAACTGGTCCGCAGTGGTGGGCCAGATGTAAACAAATGAAT GTTCTTGATTCATTTATTAATTATTATGATTCAGAAAAACATGC AGAAAATGCTGTTATTTTTTTACATGGTAACGCGGCCTCTTCT TATTTATGGCGACATGTTGTGCCACATATTGAGCCAGTAGCGC GGTGTATTATACCAGACCTTATTGGTATGGGCAAATCAGGCAA ATCTGGTAATGGTTCTTATAGGTTACTTGATCATTACAAATAT CTTACTGCATGGTTTGAACTTCTTAATTTACCAAAGAAGATCAT TTTTGTCGGCCATGATTGGGGTGCTTGTTTGGCATTTCATTAT AGCTATGAGCATCAAGATAAGATCAAAGCAATAGTTCACGCTG AAAGTGTAGTAGATGTGATTGAATCATGGGATGAATGG

Table A2: Transfection procedure using Dharmacon™ ON-TARGETplus SMARTpool Human RPSA and Mission esiRNA-RLUC

Lypholized Human RPSA siRNA (5 nmol/20 nmol) were reconstituted in 250 µl/1000 µl of 1X RNAse- free siRNA buffer before use, and esiRNA-RLUC (200 ng/ µl) was purchased reconstituted. The table below shows the amounts of siRNA and corresponding components used for transfections per well of a 6-well and 24-well plate.

	6-well plate (Total volume of 2 ml)	**24-well plate (Total volume of 500 µl)**
Opti-MEM media for addition of siRNA (µl)	190 µl	47.5 µl
Human RPSA (µl)	10 µl	2.5 µl
Opti-MEM media for addition of DharmaFect Transfection reagent (µl)	190 µl	47.5 µl
DharmaFect/ Transfection reagent (µl)	10 µl	2.5 µl
DMEM media (µl)	1595 µl	398.5 µl

Basic procedure followed:

Reconstituted Human RPSA was added to the corresponding volume of Opti-MEM media in an Eppendorf tube. In a second Eppendorf tube, transfection reagent was added to the corresponding volume of Opti-MEM media. Both tubes were incubated for 5 minutes at room temperature and then the contents of both tubes were mixed together and the Human RPSA siRNA mixture was incubated for a further 20 minutes at room temperature. The suggested volumes of DMEM media were added into the 6-well/24-well plates containing the seeded cells and thereafter the resultant siRNA solution was added to the cells. The esiRNA-RLUC and esiRNA-RPSA siRNA solutions were prepared according to the table below.

Table A3: Transfection procedure using Mission 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 per well of a 6-well and 24-well plate.

	6-well plate (total volume of 2 ml)	24-well plate (Total volume of 500 µl)
Opti-MEM media for addition of siRNA (µl)	250 µl	50 µl
esiRNA-RPSA or esiRNA-RLUC (µl)	5 µl	1.5 µl
Opti-MEM media for addition of Mission Transfection reagent (µl)	250 µl	50 µl
Mission Transfection reagent (µl)	5 µl	1.5 µl
DMEM media (µl)	1490 µl	397 µl

Basic procedure followed:

esiRNA-RPSA or esiRNA-RLUC was added to the corresponding volume of Opti-MEM media in an Eppendorf tube. In a second Eppendorf tube, transfection reagent was added to the corresponding volume of Opti-MEM media. Both tubes were incubated for 5 minutes at room temperature and then the contents of both tubes were mixed together. Thereafter, the suggested volumes of DMEM media

were added into the 6-well/24-well plates containing the seeded cells and the resultant siRNA solution was added to the cells.

Table A4: Composition of the 12% polyacrylamide gels prepared and used for SDS-PAGE (per gel)

	Separating gel	Stacking gel
Distilled water	2.15 ml	1.83 ml
40% Acrylamide	1.5 ml	313 µl
Separating Tris buffer	1.25 ml	---
Stacking Tris buffer	---	313 µl
10% SDS	50 µl	25 µl
APS	50 µl	25 µl
TEMED	2 µl	2.5 µl

Table A5: Caspase-3, -8 and -9 Assay Mixture components

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

Sample	5x Assay Buffer	Caspase-3, -8 or -9 sample	Inhibitor	DI H_2O	Caspase-3, -8 or -9 Substrate	Total Volume
Buffer Blank	20 µl	0 µl	0 µl	80 µl	0 µl	100 µl
Substrate Blank	20 µl	0 µl	0 µl	70 µl	10 µl	100 µl
Test Sample	20 µl	X µl	0 µl	(70-X) µl	10 µl	100 µl

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STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2015/04/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 and lung cancer in BALB/c nude mice

Number and Species

6 mice

Approval was given for to 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.

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: 13th March 2015

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: 12th March 2015

cc: Supervisor: N/A
Director: CAS

Works 2000/lain0015/AESCCert.wps

AESC 2012 M&E

Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND

ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Prof Stefan FT Weiss

b. Department: School of Molecular and Cell Biology

c. Experiment to be modified / extended

		AESC NO	
Original AESC number	2015	04	C
Other M&Es :			Yes

d. Project Title: Verification of the therapeutic potential of anti-LRP/LR antibody IgG1-iS18 for the treatment of metastatic colorectal and lung cancer in BALB/c Nude mice.

		No.	Species
e.	Number and species of animals originally approved:	6	*BALB/c Nude mice*
f.	Number of additional animals previously allocated on M&Es:	12	*BALB/c Nude mice*
g.	Total number of animals allocated to the experiment to date:	18	*BALB/c Nude mice*
h.	Number of animals used to date:	0	

i. Specific modification / extension requested:

1) Please change species from BALB/c nude mice to MF1/nude mice.

2) Please change title of the project to:

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.

3) Please change the age of the mice to 4-6 weeks.

j. Motivation for modification / extension:

1) No BALB/c nude mice are currently available in South Africa. MF1 nude mice are available from UCT and both strains are Athymic Nude Mice and can be used for this study.

2) It is recommended that the mice should be 4-6 weeks old when the xenografts are established (Protocol online, http://www.protocol-online.org/prot/Protocols/Xenograft-Tumor-Model-Protocol-3435.html).

3) Since the cancer type has changed to malignant melanoma (as per previous M&E) and the species of mice have changed the title should be changed accordingly.

Date: 2017-03-31 Signature: 

RECOMMENDATIONS: Approved. i. Change of species from BALB/c nude mice to MF1/nude mice. ii. Change of title of the project to: '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'. iii. Use of mice aged 4-6 weeks in the study .

Date: 11 April 2017 Signature: 

Chairman, AESC

SFT Weiss M&E 2015-04-C 2017

Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND

ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Prof Stefan FT Weiss

b. Department: School of Molecular and Cell Biology

c. Experiment to be modified / extended

		AESC NO	
Original AESC number	2015	04	C
Other M&Es :			No

d. Project Title: Verification of the therapeutic potential of anti-LRP/LR antibody IgG1-iS18 for the treatment of metastatic colorectal and lung cancer in BALB/c Nude mice.

		No.	Species
e.	Number and species of animals originally approved:	6	*BALB/c Nude mice*
f.	Number of additional animals previously allocated on M&Es:		
g.	Total number of animals allocated to the experiment to date:	0	
h.	Number of animals used to date:	0	

i. Specific modification / extension requested:

1) Please add the following MSc students as co-workers for this project:

Ms Thalia Rebelo; ID: 9312190041087; Student number: 600344
Ms Leila Vania; ID: 9401010092089; Student number: 668573

2) Please change the classification of the project to: research (including some BSc Honours degrees and higher degrees (MSc and PhD degrees).

3) IgG1-HD37 will no longer be used as a negative control antibody.

4) Colorectal cancer cells (SW480 or HT-29) will no longer be injected subcutaneously into the flanks of the mice but via orthotopic implantation using Matrigel. For orthotopic implantation, human colon cancer cells will be harvested from culture flasks after a brief trypsinization and transferred to serum-free medium. The nude mice will be anesthetized (ketamine and xylazine (half dose), and then maintained on isoflourane) and a small median abdominal incision will be made, and the cecum exteriorized. The colon cancer cells ($2.0 \times 10^6 - 5 \times 10^6$) in 100 µL serum- free medium containing 10 µg/µL Matrigel will be inoculated

into the cecal walls using 27-30-gauge needles (Sasaki et al. 2008).

Protocol according to Tseng et al. 2007
Mouse Preparation:
1. The depth of anesthesia is assessed using toe pinch. There should be no withdraw reflex with toe pinch.
2. Antibiotics may be given at this point.
3. The anesthetized mouse, which was previously shaved, is properly positioned.
4. The abdomen is prepped with a betadine solution.
5. The abdomen and surgical site are draped in a sterile fashion.

Laparotomy:
1. A small nick is made in the skin
2. The abdominal wall musculature is grasped and lifted up
3. The abdominal cavity is entered and a single blade of the scissors is used to push the intra-abdominal contents away
4. The incision is extended to 2-3 cm

Exposure of the Cecum:
1. The cecum with its blind ending pouch is identified and exteriorized
2. The cecum is isolated from the rest of the mouse using a pre-cut, sterile gauze
3. Warm saline is used to keep the cecum moist

Injection of Cells into the Cecal Wall:
1. A 27 G or finer needle is used to inject a 50 µL volume of cells into the cecal wall
2. The needle is removed
3. The injection site is inspected to ensure no leakage
4. The cecum is returned to the abdominal cavity

Analgesia (meloxicam @ 1mk/kg or TramaHexal @ 2mg/kg) will be administered subcutaneously as required. Ten to 14 days after injection of the cancer cells, mice will be anesthetized using isoflourane and will receive treatment intraperitoneally: Three mice will receive 100 µl of PBS, three will receive 2.5 mg/kg body weight of IgG1-iS18 antibody in a maximum of 100 µl PBS and three will receive no treatment. Mice will be treated twice a week for 4 weeks and will be terminated 7 days after the final treatment. No tumour measurements will be made during the treatment period but mice will be monitored throughout. A full body analysis will be performed after the mice were terminated to determine if the IgG1-iS18 treatment prevented tumour formation and metastasis. Tumours will be harvested for histological analysis.

3) Please change A549 lung cancer cells to A375SM melanoma cells. The nude mice will be anesthetized using isoflourane and 5×10^6 – 1×10^7 A375SM metastatic melanoma cells will be injected subcutaneously. The cells will be injected in a 200-µl suspension consisting of 100 µl of PBS and 100 µl of Matrigel (White et al. 2009). Ten to 14 days after injection of the cancer cells, mice will be anesthetized using isoflourane and receive treatment intraperitoneally: Three mice will receive 100 µl of PBS, three will receive 2.5 mg/kg body weight of IgG1-iS18 antibody in a maximum of 100 µl PBS and three will receive no treatment. Mice will be treated twice a week for 4 weeks. The size of visible tumours will be determined by calliper, once a week for the duration of the study. These measurements will only be made on visible tumours on the skin while the mice are anesthetized just prior to treatment. The mice will be terminated 7 days after the final treatment. A full body analysis will be performed to determine if the IgG1-iS18 treatment prevented tumour formation and metastasis. Tumours

will be harvested for histological analysis.

6) GFP fluorescence imaging or Bioluminescence imaging will no longer be performed to evaluate tumour formation.

7) Please change number of animals to 18 and gender to male and/or female.

8) Please extend study to 31 December 2017

j. Motivation for modification / extension:

1) This project forms part of Ms Rebelo and Ms Vania's MSc projects.

2) Since the project forms part of MSc degrees the classification of the project should be research.

3) This control is unnecessary since a PBS control is included.

4) Despite metastasis as an important cause of death in colorectal cancer patients, current animal models of this disease are scarcely metastatic. The traditional subcutaneous tumor model is less than ideal for studying colorectal cancer. Orthotopic mouse models of colorectal cancer, which feature cancer cells growing in their natural location, replicate human disease with high fidelity. Orthotopic cell microinjection procedure induces tumor foci in the most clinically relevant metastatic sites: colon-draining lymphatics, liver, lung, and peritoneum. The replication of the clinical pattern of dissemination makes it a good model for advanced colorectal cancer (Céspedes et al., 2007; Tseng et al., 2007)

5) All cell lines have to be authenticated before used. The available A549 lung cancer cell line has not been authenticated. It is furthermore difficult to establish lung metastasis in xenograft models with other lung cancer cell lines.

Skin cancer is the most common cancer in South Africa with about 20 000 reported cases every year and 700 deaths. South Africa has the second highest incidence of skin cancer in the world after Australia (CANSA). Malignant melanoma can metastasise or spread to other parts of the body, such as the lungs, brain, or other organs and tissue and if this occurs the 10-year survival rate is only 10 to 15 percent (American Cancer Society). It is therefore imperative to find a novel treatment to prevent metastasis of malignant melanoma.

6) In order to minimize stress on the animals, GFP fluorescence imaging or Bioluminescence imaging will no longer be performed to evaluate tumour formation. Treatment will commence 10-14 days after injection of the cancer cells and evaluation of the effectiveness of the treatment will be performed by calliper measurements if any tumours are visible on the skin (melanoma study) or after the mice have been terminated.

7) In order to perform statistical analysis on the results obtained, at least 3 mice are required per group. Therefore 3 untreated, 3 for PBS treatment and 3 mice for IgG1-iS18 antibody treatment for both the colorectal as well as melanoma study.

8) Due to a delay in shipment of cell lines required, the study could not commence on the date previously proposed.

SFT Weiss M&E 2015-04-C 2017

References

Céspedes MV, Espina C, García-Cabezas MA, Trias M, Boluda A, Gómez del Pulgar MT, Sancho FJ, Nistal M, Lacal JC, Mangues R. (2007) Orthotopic microinjection of human colon cancer cells in nude mice induces tumor foci in all clinically relevant metastatic sites. Am J Pathol. 170(3):1077-85.

Hiroyuki Sasaki, Koh Miura, Akira Horii, Naoyuki Kaneko et al. (2008) Orthotopic implantation mouse model and cDNA microarray analysis indicates several genes potentially involved in lymph node metastasis of colorectal cancer. Cancer Sci. 99;4: 711–719

Tseng W., Leong X., Engleman E. (2007). Orthotopic Mouse Model of Colorectal Cancer. JoVE. 10. http://www.jove.com/index/Details.stp?ID=484, doi: 10.3791/484

Nicholas White, Gillian E. Knight, Peter E. M. Butler and Geoffrey Burnstock. (2009) An in vivo model of melanoma: treatment with ATP. Purinergic Signalling. 5:327–333

CANSA. http://www.cansa.org.za/files/2012/05/SKIN_CANCER_Leaflet-2010.pdf

Date: 2017-02-15 Signature:

RECOMMENDATIONS: Approved.

1. Inclusion of postgraduate students: Ms Thalia Rebelo and Ms Leila Vania as co-workers
2. Modification of methodology as detailed above.
3. Twelve (12) additional male and/or female mice (total for study eighteen).
4. Extension of time to 31 December 2017

Conditions: i. A pilot study to be conducted on three (3) of the mice first to establish feasibility of the methodology. ii. The postgraduate students should attend a "first time user's session" facilitate by the CAS before working with the mice. iii. An annual report (including student publications and graduations linked to the study) to be submitted to the AESC.

Date: 23 February 2017

Signature:

Chairman, AESC

AESC 2012 M&E

Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND

ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Prof Stefan FT Weiss

b. Department: School of Molecular and Cell Biology

c. Experiment to be modified / extended

		AESC NO	
Original AESC number	2015	02/04	C
Other M&Es :			Yes

d. Project Title: Verification of the therapeutic potential of anti-LRP/LR antibody IgG1-i518 for the treatment of metastatic colorectal cancer and malignant melanoma in MF1 Nude mice.

		No.	Species
e.	Number and species of animals originally approved:	6	*BALB/c Nude mice*
f.	Number of additional animals previously allocated on M&Es:	12	*BALB/c Nude mice*
g.	Total number of animals allocated to the experiment to date:	19	*MF1 Nude mice*
h.	Number of animals used to date:	19	*MF1 Nude mice*

i. Specific modification / extension requested:

In a previous M&E, we stated that we will terminate the mice 7 days after the final treatment, which is Monday 10 July 2017 for the first group. We would like to extend this period after treatment with an additional 14 days before termination, thus 21 days in total.

j. Motivation for modification / extension:

The animals show no clinical symptoms of tumour formation or an appropriate tumour size where tumours are visible. Additional time will therefore allow further tumour growth. The animals will be terminated when they show distress, to avoid unnecessary suffering, or after the additional 14 days.

Date: 2017-07-06 Signature:

RECOMMENDATIONS: Approved. Extension of time before termination (7 days to 21 days).
Condition: Ensure vigilant monitoring for any signs of distress or pain as endpoints as indicated in the welfare monitoring scoresheet.

Date: 6 July 2017

Signature:

Chairman, AESC

IDEXX BioResearch
FINAL REPORT OF LABORATORY EXAMINATION
4011 Discovery Drive, Columbia, MO 65201
1-800-669-0825 1-573-499-5700
idexxbioresearch@idexx.com www.idexxbioresearch.com

IDEXX BioResearch Case # 9213-2017

Received: 3/17/2017
Completed: 3/21/2017

Submitted By

Pamela Nakajima
Fox Chase Cancer Center
Institute for Cancer Research
333 Cottman Ave
Cell Culture Facility
Philadelphia, PA 19111

Phone: 215-728-2486
Fax: 215-728-2412
Email: Pamela.Nakajima@fccc.edu

Specimen Description

Species: Human
Description: cells
Number of Specimens/Animals: 3

Purchase Order #: 6720037034

ID	Client ID	Cell Line	Species	ATCC #
1	1	A375SM	Human	CRL-1619
2	2	DU145	Human	HTB-81
3	3	PC-3	Human	CRL-1435

Services/Tests Performed: CellCheck 9 (9 Marker STR Profile and Inter-species Contamination Test)

Genetic evaluation for: Human 9-Marker STR Profile, Interspecies Contamination Test

Summary: Cell Check results are provided in the data results section for each sample. For human samples, an identity matching score above 80% indicates the sample is consistent with the cell line of origin. For human samples with less than an 80% matching score, please see individual comments for these samples in the detail section.

Please see the report for details.

CELL CHECK

Species-specific PCR Evaluation

Species	1	2	3
mouse	-	-	-
rat	-	-	-
human	+	+	+
Chinese hamster	-	-	-
African green monkey	-	-	-

Marker Analysis

Marker Name	1		2		3	
	Sample Results	A-375 (ATCC# CRL-1619)	Sample Results	DU-145 (ATCC# HTB-81)	Sample Results	PC-3 (ATCC# CRL-1435)
AMEL	X	X	X, Y	X, Y	X	X
CSF1PO	11, 12	11, 12	10, 11	10, 11	11	11
D13S317	11, 14	11, 14	12, 13, 14	12, 13, 14	11	11
D16S539	9	9	11, 13	11, 13	11	11
D5S818	12	12	10, 13	10, 13	13	13
D7S820	9	9	7, 10, 11	7, 10, 11	8, 11	8, 11
TH01	8	8	7	7	6, 7	6, 7
TPOX	8, 10	8, 10	11	11	8, 9	8, 9
vWA	16, 17	16, 17	17, 18	17, 18, 19	17	17
Identity Match	100%		> 80%		100%	

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

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON-JONES
HREC (MEDICAL)
2014 -08- 04
JOHANNESBURG

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

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