Targeting LRP/LR for the treatment of metastatic lung and colorectal cancer through impediment of telomerase activity

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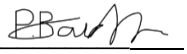
Signature 

Pavan Baichan

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List of abbreviations

A549	Lung Adenocarcinoma
AD	Alzheimer's disease
APC	Allophycocyanin
APP	Amyloid Precursor Protein
Aβ	Amyloid Beta
BCA	Bicinchoninic acid
BCL2	B-Cell Lymphoma 2
BSA	Bovine Serum Albumin
CHAPS	Dimethyl[3-(propyl].azaniumyl}propane-1-Sulphonate

Cu(II)S	Copper (II) Sulphate
DAPI	4',6-diamidino-2-phenylindole
DKC	Dyskerin
DLD-1	Late-Stage Colorectal Carcinoma
DMEM	Dulbecco's Modified Eagle's Media
DMSO	Dimethyl Sulphoxide
DNase	Deoxyribonuclease
dsRNA	Double-Stranded Ribonucleic Acid
ELISA	Enzyme-linked Immunosorbent Assay
FAK	Focal Adhesion Kinase
FBS	Foetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HEK293	Human Embryonic Kidney
HRP	Horseradish Peroxidase
hTERC	Human Telomerase RNA Component
hTERT	Human Telomerase Reverse Transcriptase Component
IgG1-iS18	Immunoglobulin G 1
LRP/LR	Laminin Receptor Precursor/High Affinity Laminin Receptor
MDA-MB-231	Mammary Gland Adenocarcinoma
MAPK/ERK	Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMD	Non-sense Mediated Decay
NRF	National Research Foundation
p53	Tumour Protein p53
PBS	Phosphate Buffered Saline
PCA	Protocatechuic Acid
PI3k/Akt	Phosphatidylinositol-4,5-bisphosphate 3-kinase/Protein Kinase B
POT1	Protection f Telomeres 1
PrP	Prion Protein
PrP^{c}	Cellular Prion Protein
PrP^{Sc}	Scrapie Prion Protein
PVDF	Polyvinylidene Fluoride
qPCR	Quantitative Polymerase Chain Reaction
Rap1	Ras-proximate-1
RdRp	RNA-dependent RNA polymerase
RIPA	Radioimmunoprecipitation Assay
RISC	RNA-Induced Silencing Complex
RMRP	RNA component of mitochondrial RNA processing
RNAi	Interfering Ribonucleic Acid
RPSA	Ribosomal protein SA
RT	Reverse Transcription

SAMRC	South African Medical Council
scFv	Single Chain Variable Fragment
SiRNA	Small Interfering Ribonucleic Acid
TERRA	Telomeric Repeat-Containing RNA
TIN2	TERF1-interacting nuclear factor 2
T-Loop	Telomere-Loop
TPP1	Tripeptidyl Peptidase 1
TRF1	Telomeric Repeat Factor 1
TRF2	Telomeric Repeat Factor 2

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1 Abstract

The 37 kDa/67 kDa laminin receptor (LRP/LR) plays a vital role in the malignancy of various cancer types contributing to invasion, adhesion, apoptosis evasion, proliferation and tumour angiogenesis. In addition, LRP/LR interacts with the catalytic reverse transcriptase subunit, TERT, of the ribonucleoprotein telomerase. Both LRP/LR and telomerase are implicated in cancer progression and knockdown of LRP/LR causes a decrease in telomerase activity in breast cancer cells. In the current study, LRP/LR was downregulated in lung adenocarcinoma (A549) and late-stage colorectal carcinoma (DLD-1) cells in an attempt to impede telomerase activity and ultimately impede cancer progression. Western blotting analysis showed a significant decrease in LRP/LR levels in HEK293 (Human Embryonic Kidney Cells) and A549 cells after siRNA mediated LRP/LR knockdown. To confirm LRP/LR knockdown confocal microscopy was performed; a reduction in LRP/LR protein levels was observed which also resulted in a subsequent decrease in *hTERT* mRNA levels with a corresponding decrease in hTERT levels in HEK293, A549, and DLD-1 cell lines. Furthermore, siRNA mediated knockdown of LRP/LR significantly decreased telomerase activity in HEK293, A549, and DLD-1 cells. The effect of LRP/LR downregulation on cellular viability was investigated via the MTT assay and a significant decrease in cell viability in A549 and DLD-1 cells was observed. Since downregulation of LRP/LR impedes telomerase activity and decreases cell viability, siRNAs directed against LRP mRNA acts as potential alternative therapeutic tools for treatment of lung adenocarcinoma and late-stage colorectal carcinoma.

2 Introduction

2.1 Cancer

Cancer is among the leading causes of death worldwide. In 2012, there were approximately 14 million new cancer cases and 8.2 million deaths related to cancer (Cancer Fact Sheet Nº297, 2017). A 70% increase in new cancer cases is expected in the next two decades. Also, in 2015, cancer was responsible for 8.8 million (1 in 6) deaths worldwide (Cancer Fact Sheet Nº297, 2018). Approximately 70% of deaths related to cancer occur in middle- and low-income countries. In men and women, lung adenocarcinoma and colorectal carcinoma are among the top five most frequent locations for tumour development (Cancer Fact Sheet Nº297, 2017). Due to the frequency of these cancer types in both men and women, it is therefore necessary that these cancer types be thoroughly studied. Cancer onset is due to numerous environmental factors in conjunction with genetic factors. Environmental stimulants account for majority of

cancer cases (90-95%) with hereditary causes accounting for 5-10% of cancer cases (Figure 1a). Some environmental factors implicated in tumourigenic onset include: tobacco, ultraviolet radiation, diet and lifestyle, and infectious organisms, as can be seen in Figure 1c (Anand et al., 2008). Moreover, the cancer types which are most likely to be affected by hereditary factors are represented by Figure 1c (Anand et al., 2008).

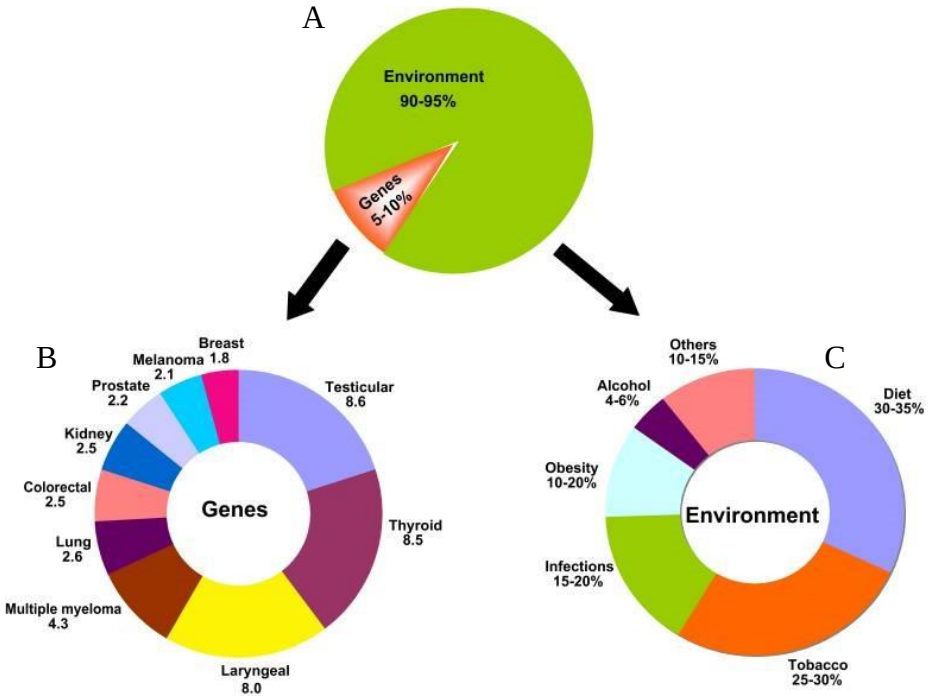


Figure 1: Contribution of environmental and genetic factors to cancer onset. This figure depicts the distribution of the environmental factors which lead to tumour carcinogenesis as well as the distribution of cancers in which genes play a role. (A) The distribution between genetic and environmental causes for cancer; 90%-95% of cancers are brought about by the environment and 5%-10% are due to genetic alterations. (B) The types of cancers due to genetic alterations and their prevalence due to genetic alterations. (C) The contributions of various environmental factors to tumourigenesis (Anand et al., 2008).

Tumourigenic onset is driven by these environmental factors which collectively function to promote DNA damage and mutations that ultimately alter normal cellular functioning (Irigaray et al., 2007). Single mutations are not sufficient to initiate the tumourigenic process, but rather rely on widespread damage and mutations to multiple genes in a multistep process (Irigaray et al., 2007). This loss of normal cell functioning allows for specific hallmarks of cancer to develop including uncontrolled growth and proliferation, amongst others. The accumulation of these alterations and specific hallmarks convey the ability to drive cancer progression, and in

turn also provide late stage tumours with the ability to metastasise (Hanahan and Weinberg, 2011). Moreover, with regards to genetic alterations, these new mutations can lead to the subsequent proteins being dysregulated (Croce, 2008). These dysregulations can either promote protein overexpression or underexpression, in relation to the protein functions (Croce, 2008). An example is the pro-apoptotic protein p53. In cancer, this protein is strictly underexpressed in order for the tumours to bypass apoptosis and continue proliferating. Another example is BCL2. This protein is involved in cell survival and proliferation, so in tumours this protein is severely overexpressed in order for the tumours to proliferate uncontrollably (Croce, 2008). Primary tumours contain heterogeneous cell populations; this means the cell population can differ in morphological and phenotypical profiles from one another (Chiang and Massague, 2008). Heterogeneous cell populations contain varied degrees of metastatic potential, due to the variation in phenotypic profiles. Therefore, the cell population constituents of malignant tumours contribute to metastasis (Fidler, 1978). Metastasis is the ability of malignant tumours to migrate from one location in the body to another, thus allowing for secondary and tertiary tumours to become established at different sites within the body (Chiang and Massague, 2008). In this regard, for a secondary tumour to be successfully established, the primary tumour must follow a series of phases (Hunter et al., 2008). These involve the separation of cells from the primary tumour, followed by invasion of the basement membrane through secretion of Collagenase IV (Al-Mehdi et al., 2000). Thereafter, these cells enter the circulatory or lymphatic system, where they will travel to distant tissues, and attach to form secondary or tertiary tumours (Hunter et al., 2008). Additionally, these cancer cells then proceed to adjust to the new microenvironment, proliferate, and induce angiogenesis ensuring the viability of the cells, as can be seen in Figure 2 (Al-Mehdi et al., 2000, Hunter et al., 2008). All of these processes combined allows for metastasis to occur and the formation of additional tumours. One important protein promoting tumour viability and metastasis is LRP/LR.

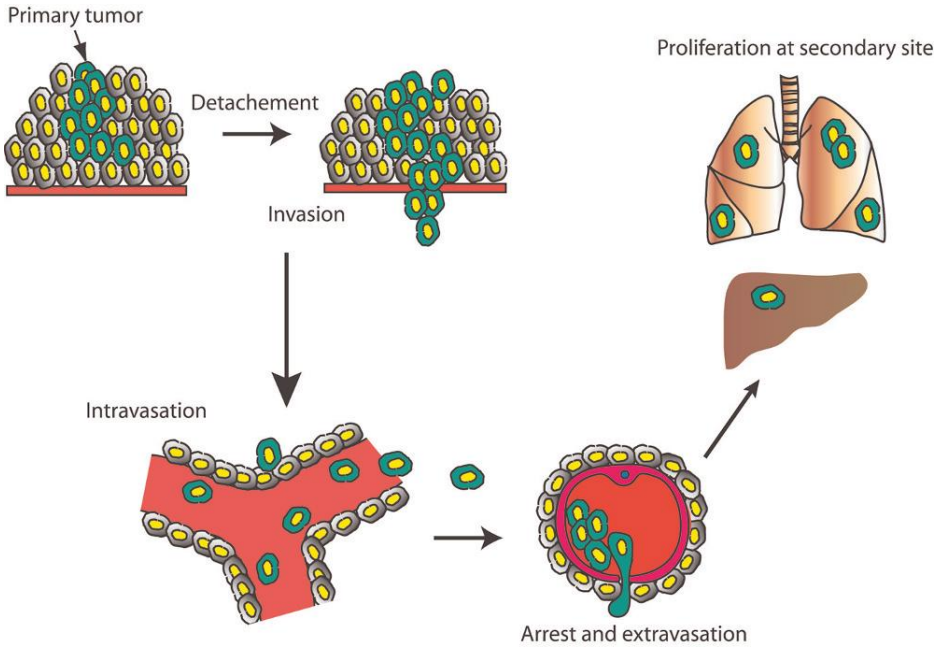


Figure 2: Schematic depicting possible mechanisms of metastasis. The mechanism of metastasis functions whereby cells break-off from the tumour, degrade the basement membrane, and enter the circulatory system. These cells then migrate and establish at a secondary site in the body to establish a secondary tumour (Hunter et al., 2008).

2.2 37 kDa/67 kDa High Affinity Laminin Receptor (LRP/LR)

The 37 kDa/67 kDa laminin receptor precursor/ high affinity laminin receptor (LRP/LR) is a multifunctional, non-integrin laminin cell surface receptor (Omar et al., 2012, Omar et al., 2011). This cell surface receptor is involved in various physiological processes such as promoting cell attachment, cell motility and viability, and cellular proliferation (Moodley and Weiss, 2013, Weiss, 2017). It is speculated that the 37 kDa LRP precursor forms the 67 kDa high affinity laminin receptor via acylation (Moodley and Weiss, 2013, Butò et al., 1998). In addition to the cell surface, LRP/LR is also found within the cytoplasm, the perinuclear compartment, and the nucleus (Jovanovic et al., 2015, Moodley and Weiss, 2013, Weiss, 2017). Due to LRP/LR being a cell surface receptor protein, it contains a transmembrane domain located at the N-terminal domain and an extracellular C-terminal domain. It also contains binding sites for proteins other than Laminin-1; these include Heparan Sulphate Proteoglycans, the prion protein (PrP), single-chain variable fragment (scFv), and the IgG1 antibody among other proteins and macromolecules, as depicted in Figure 3 (Mbazima et al., 2010).

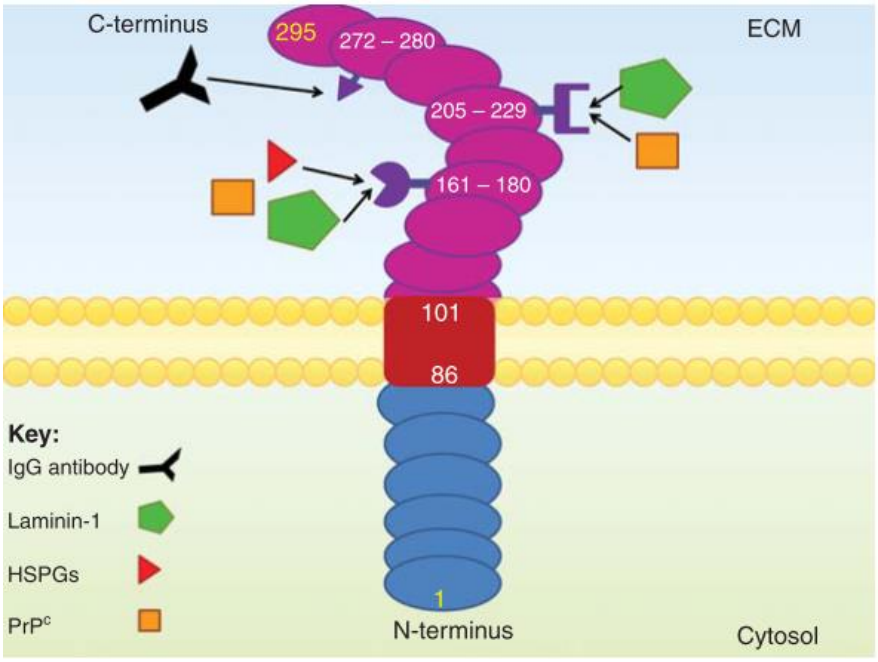


Figure 3: Representation of the structure of the 37 kDA laminin receptor precursor. This figure shows the functional structure of LRP/LR when embedded into the cell surface. Binding sites for various proteins such as PrP, Heparan Sulphate Proteoglycans, Laminin-1, and the IgG1 antibody are depicted. Various other biomolecules are able to bind LRP/LR (not shown) (Jovanovic et al., 2015).

2.2.1 Role of LRP/LR in Cancer

2.2.1.1 Adhesion and Invasion

LRP/LR has been implicated to play a role in cancer, most notably in the tumourigenic process metastasis. In addition, LRP/LR has also been observed to play a role in adhesion, invasion, cell viability, and cellular proliferation (Chetty et al., 2014, Jovanovic et al., 2015, Khusal et al., 2013, Rao et al., 1989). LRP/LR is overexpressed in many cancer types including lung, cervical, colon, ovarian, breast and many more (Sanjuán et al., 1996, Fontanini et al., 1997). LRP/LR interacts with Laminin-1 in the basement membrane; overexpression of LRP/LR increases this interaction and subsequently induces the tumourigenic processes (Khumalo et al., 2013, Omar et al., 2012, Munien et al., 2017, Rebelo et al., 2016, Vania et al., 2016). The LRP/LR-Laminin-1 interaction induces secretion of collagenase IV which results in the basement membrane being degraded, which can be seen in Figure 4 (Ardini et al., 2002, Jovanovic et al., 2015, Vania et al., 2016, Munien et al., 2017, Rebelo et al., 2016). Degradation of the basement membrane allows the tumourigenic cells to gain access to the circulatory system. Subsequently, these cells travel through the circulatory system to distant tissues and

invade causing secondary and tertiary tumours. (Turpeenniemi-Hujanen et al., 1986, Jovanovic et al., 2015).

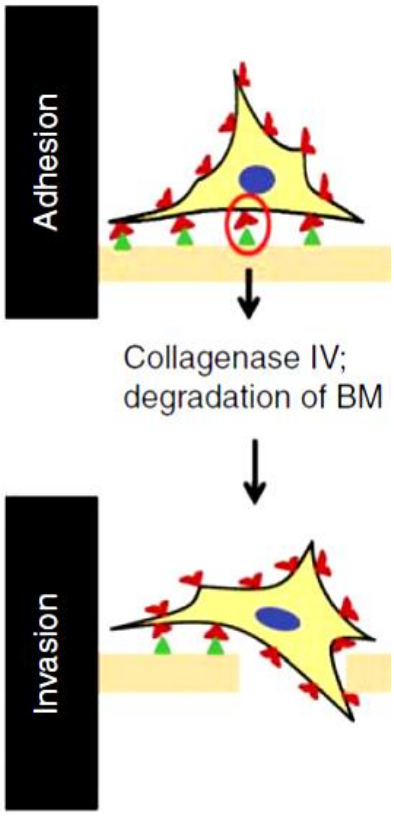


Figure 4: Adhesion and invasion of cancer cells to basement membrane. The figure above indicates the mechanism in which LRP/LR promotes adhesion and invasion by binding to Laminin-1 in the basement membrane and secreting Collagenase IV; this allows access to the circulatory system for metastasis to occur (adapted from Jovanovic et al., 2015).

2.2.1.2 Role of LRP/LR in Angiogenesis

Angiogenesis is the process where new blood vessels are formed from pre-existing capillaries (Dixelius et al., 2004). This process contributes to cellular adhesion and invasion (Figure 4) whereby access to blood vessels becomes more available and therefore easier to migrate from one location to another. Angiogenesis is important in normal processes such as embryonic development, wound healing, vascular remodelling, and tissue growth (Tanaka et al., 2000). Angiogenesis is strictly regulated in healthy individuals, but in cancer this process becomes dysregulated in order to promote tumour growth and metastasis due to the increased delivery of oxygen and nutrients (Khusal et al., 2013, Jovanovic et al., 2015) because oxygen and nutrients become depleted once tumours become very large (Khusal et al., 2013, Jovanovic et al., 2015). Figure 5 depicts the process of angiogenesis. Angiogenesis is promoted in tumour cells whereby LRP/LR interacts with Laminin-1, as previously described. Once again, the

LRP/LR-Laminin-1 interaction induces Collagenase IV secretion to degrade the basal lamina (Ardini et al., 2002, Khusal et al., 2013). This, in turn, causes a release of pro-angiogenic factors which causes endothelial cell paracrine stimulation in order to proliferate and differentiate into new blood vessels (Khusal et al., 2013). Moreover, it has been shown that targeting LRP/LR with anti-LRP/LR specific antibodies, namely IgG1-iS18, decreases tumour angiogenesis (Khusal et al., 2013).

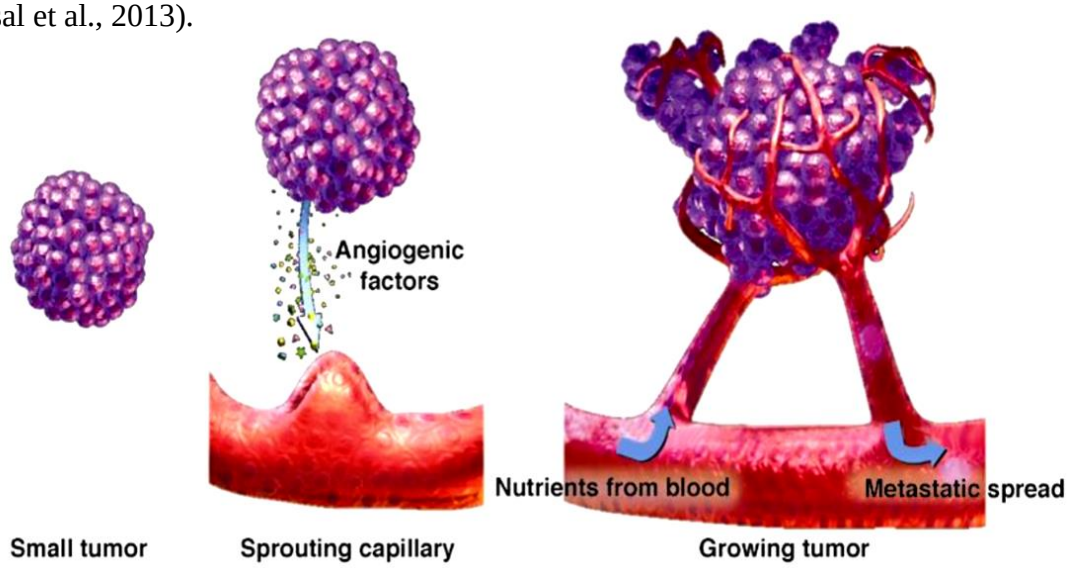


Figure 5: Possible mechanisms of tumour angiogenesis. One possible mechanism of angiogenesis is depicted. Angiogenic factors are secreted to cause budding of an existing capillary in order to form new capillaries to aid in tumour progression and metastasis (Adapted from http://www.rodallrich.com/advphysiology/Angiogenesis.html accessed on 2/11/17).

2.2.1.3 Role of LRP/LR in Cell Viability and Proliferation

One hallmark of tumours is the ability to prevent programmed cell death, also known as apoptosis. Apoptosis is necessary in order to maintain normal homeostasis of tissues (Wong, 2011, Jovanovic et al., 2015, Kerr et al., 1972). When LRP/LR interacts with Laminin-1, the LRP/LR-Laminin-1 complex interacts with focal adhesion kinases (FAKs) to promote cell survival pathways (Sun et al., 2014). Such survival pathways are the PI3K/Akt and MAPK/ERK pathways. Subsequently, these pathways increase the expression of BCL2 which is an anti-apoptotic protein and therefore increase cellular proliferation and survival (Sun et al., 2014, Jovanovic et al., 2015, Hehlgans et al., 2007, McLean et al., 2005). This furthermore inhibits caspase 8, caspase 3, and caspase 9 activation which are pro-apoptotic proteins. This can be seen in Figure 6. In addition to the survival pathways stated, LRP/LR can promote cellular viability via a different mechanism whereby it aids in retaining chromosomal stability.

This is achieved when the LRP/LR-Laminin-1 complex interacts with Midkine proteins to anchor the chromosomes onto the nuclear envelope (Khumalo et al., 2015, Salama et al., 2001). Blockage of LRP/LR via anti-LRP/LR specific antibodies and knockdown via LRP/LR specific siRNAs, decrease cell survival by increasing caspase activation (Chetty et al., 2017, Khumalo et al., 2015, Moodley and Weiss, 2013, Lu et al., 2016, Vania et al., 2018, Rebelo et al., 2018)). This is due to the fact that the LRP/LR-Laminin-1 complex is disrupted which, therefore, decreases the potential of LRP/LR to maintain cell viability and induce proliferation (Chetty et al., 2014, Omar et al., 2012)

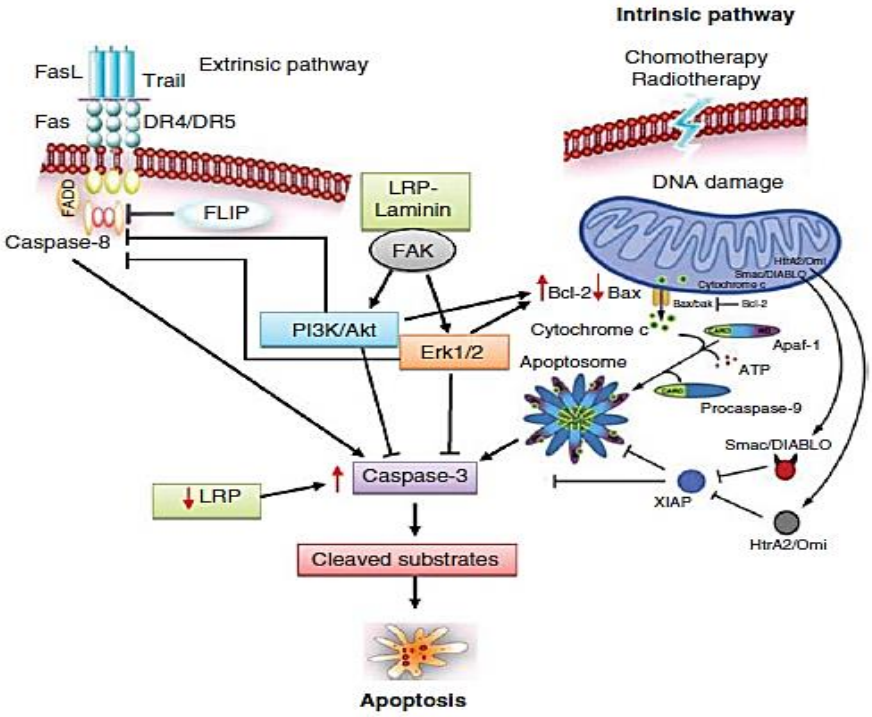


Figure 6: Possible roles of LRP/LR in cellular survival pathways. LRP/LR is involved in cell survival pathways when interacting with Laminin-1 to form the LRP/LR-Laminin-1 complex. This complex increases PI3K/Akt and MAPK/ERK cell survival pathways and decreases Caspase function to bypass apoptosis (Jovanovic et al., 2015, adapted from Favaloro et al., 2012).

2.2.1.4 LRP/LR Knockdown Mechanism

LRP/LR has been shown to be downregulated by small interfering RNA (siRNA). These siRNAs function in the RNA interfering (RNAi) pathway (Cheema et al., 2007). The mechanism functions to introduce a dsRNA molecule into a cell via a vector; this siRNA is complementary to the mRNA of the target protein to be downregulated. The siRNAs are

subsequently cleaved by Dicers, which are dsRNA-specific endonucleases. The resulting siRNA then forms a complex with protein components to form the RNA-induced Silencing Complex (RISC) (Cheema et al., 2007). This RISC complex then binds to the target mRNA causing degradation of the mRNA via exonuclease activity (Cheema et al., 2007). This, in turn, causes a decrease in expression of the LRP/LR protein.

2.2.2 Role of LRP/LR in Neurodegenerative Disorders, Diseases, and Ageing

It has been found that LRP/LR is the receptor for cellular prion protein (PrP^{C}) as well as infectious prion protein (PrP^{Sc}) (Gauczynski et al., 2001, Gauczynski et al., 2006). PrP^{C} is linked to Alzheimer's disease (AD) which is a neurodegenerative disease where amyloid precursor protein (APP) is cleaved into Aβ (Gonsalves et al., 2012, Jovanovic et al., 2013). PrP^{C} serves to bind Aβ to induce a signal transduction cascade which results in neuronal death. However, PrP^{C} lacks a transmembrane domain and so relies on LRP/LR to induce the signal transduction as well as aiding in PrP^{C} internalisation (Gauczynski et al., 2001, Jovanovic et al., 2015). Moreover, it has been shown that blockage of LRP/LR reduces the amount of Aβ shedding in AD mouse models (Ferreira et al., 2018). Also, LRP/LR serves as a receptor for Aβ, more specifically $Aβ_{42}$, in AD (Jovanovic et al., 2015, Da Costa Dias et al., 2013, Jovanovic et al., 2013, Jovanovic et al., 2014). LRP/LR has also been identified to play a role in Aβ shedding by directly interacting with γ-secretase and possibly indirectly with β-secretase (Jovanovic et al., 2015, Jovanovic et al., 2014). LRP/LR in AD seems to accelerate γ-secretase and β-secretase functioning to cleave APP into Aβ. This drives AD by increasing the amount of Aβ aggregation (Jovanovic et al., 2015, Jovanovic et al., 2014, Da Costa Dias et al., 2013, Jovanovic et al., 2013). It has been shown that blockage of LRP/LR with the anti-LRP/LR specific antibody IgG1-iS18 and knockdown of LRP/LR with shRNAs reduces Aβ shedding in AD models (Jovanovic et al., 2015, Jovanovic et al., 2014, Da Costa Dias et al., 2013, Jovanovic et al., 2013). In an AD mouse model, blockage of LRP/LR with anti-LRP/LR specific antibody IgG1-iS18 rescues mice from Aβ shedding and protects against neurodegeneration (Ferreira et al., 2018).

Moreover, LRP/LR has been shown to play a role in ageing. It interacts with a ribonucleoprotein known as telomerase and overexpression of the 37-kDa LRP::FLAG (full length LRP with a FLAG tag to differentiate between overexpressed and endogenous levels of LRP) has been shown to increase telomerase activity and decrease ageing markers in fibroblast cells (Otgaar et al., 2017). In contrast, Naidoo *et al*. (2015) showed that knockdown of LRP/LR

reduced telomerase activity in a cancer model, (Naidoo et al., 2015) indicating that LRP/LR and telomerase are able to play dual roles in an ageing as well as a cancer context.

2.3 Telomerase and Telomeres

2.3.1 Telomerase

An additional protein intricately involved in the tumourigenic process, is telomerase. Telomerase is a holoenzyme, only active once combined with a coenzyme. Telomerase is also a ribonucleoprotein, combination of RNA and RNA-binding proteins, and functions to maintain telomeres in cells. It achieves this by introducing TTAGGG repeats onto the 3´ overhang on the ends of chromosomes (Naidoo et al., 2015, Greider and Blackburn, 1989). The telomerase protein was discovered in *Tetrahymena* (Greider and Blackburn, 1985) and is expressed in highly proliferative cells such as embryonic cells, germ cells, and most tumour cells (Kim et al., 1994, Zhang et al., 2016). There are two main components of telomerase, the reverse transcriptase catalytic subunit, hTERT, and the RNA integral component, hTERC (Lai et al., 2007). Moreover, there are other proteins that form part of the telomerase protein complex, which contributes to the stability of the protein. These proteins include: dyskerin (DKC), NOP10, GAR1, and NHP2 (Wang and Meier, 2004, Zhang et al., 2016). Moreover, hTERC functions as a template for *de novo* synthesis of telomeres by synthesising additional telomeric repeats as hTERT uses hTERC as a template (Figure 7) (Harley et al., 1994).

Stimulation of telomerase activity has been implicated in carcinogenesis progression, ageing/senescence prevention, and apoptosis prevention (Bodnar et al., 1998, Greider and Blackburn, 1989). This is due to the fact that elongated/stabilised telomeres prevent the induction of cellular senescence as well as apoptosis which is a known aspect of tumour progression (Greider and Blackburn, 1989, Bodnar et al., 1998). Telomeric repeat-containing RNA (TERRA) is a non-coding RNA which is expressed using telomeric and subtelomeric sequences (Luke and Lingner, 2009). Moreover, TERRA regulation occurs at the telomeres via nonsense-mediated RNA decay (NMD) factors. Furthermore, TERRA is involved in inhibiting telomerase activity as described by Redon *et al* (2010). This is achieved as TERRA binds to hTERC which inhibits telomerase from binding to telomeres and extending the telomeric repeats (Redon et al., 2010). In various cancers, TERRA has been observed to be downregulated. Therefore, this possibly provides a link to elongated telomeres via telomerase-mediated chromosome telomere lengthening (Ng et al., 2009, Cheetham et al., 2013).

It has been observed that telomerase has extra-telomeric properties in addition to its telomeric functioning (Saretzki, 2014).

Table 1 indicates the functions telomerase performs outside of telomere extension. Most notably, telomerase plays a role in signalling pathways both in the nucleus and the cytoplasm. Moreover, in the mitochondria, telomerase protects against mtDNA damage and decreases apoptosis (Saretzki, 2014).

Table 1: Subcellular Localisation of Telomerase (Saretzki, 2014)

nuclear	cytoplasmic	mitochondrial
• Maintenance of telomeres and genomic stability • Regulation of chromatin structure, gene expression and DNA damage response • Interaction with various signalling pathways • TERT interacts with the RNA component of RMRP and synthesises dsRNA that can be processed into siRNA • Nucleolus: TERT interacts with nucleolar proteins	• TERT binds to stress particles under non-stressed conditions • TERT interacts with signalling pathways • Storage in lymphocytes outside the nucleus without stimulation	• Decrease of mitochondrial ROS • Decrease of apoptosis • Binding to mt DNA • Protection against mtDNA damage • Improved respiration and increased complex I activity • Reverse transcriptase with mitochondrial tRNAs

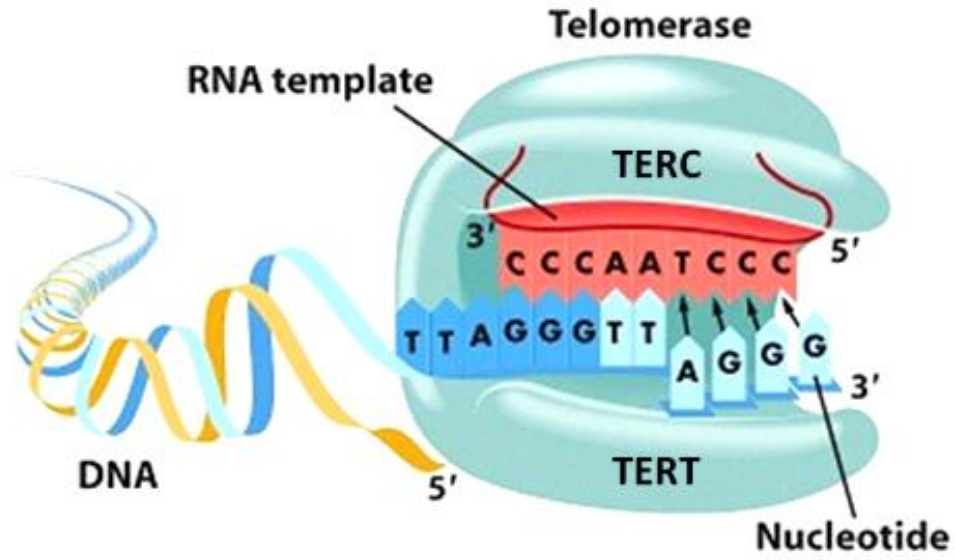


Figure 7: The Mechanism of Telomerase Functioning. The mechanism of telomerase functioning is shown above where hTERC (red) associates with the 3´ overhang, and then the telomere is extended using hTERT (turquoise). Additionally, hTERC then shifts to associate with the overhang again and recruits hTERT to further increase the telomere length (https://sussexdrugdiscovery.wordpress.com/2015/12/02/telomerase-might-have-an-important-role-in-the-mechanism-of-action-of-various-psychiatric-medications/ Accessed on 28/02/2016).

There is further evidence that telomerase plays a role in signalling pathways. It has been identified than an additional RNA molecule interacts with hTERT, known as the RNA component of mitochondrial RNA processing endoribonuclease (RMRP). RMRP is similar to hTERC as it as a small nucleolar RNA and it is also found within the mitochondria (Maida et al., 2009, Tollervey and Kiss, 1997). Maida *et al*. (2009) discovered that hTERT-hTERC and hTERT-RMRP complexes are similar in abundance even though hTERC expression is five-fold higher than RMRP. Additionally, telomerase activity was not detected for the hTERT-RMRP complex. Moreover, the hTERT-RMRP complex contains a RNA-dependent RNA polymerase (RdRp) function (Martinez and Blasco, 2011). The hTERT-RMRP functions as an RdRp by processing RMRP into small dsRNA which is further processed resulting in generation of siRNA molecules (Martinez and Blasco, 2011, Maida et al., 2009). In addition, the siRNA molecules then, in turn, regulate endogenous RMRP expression levels indicating that hTERT-RMRP regulates RMRP expression via a negative feedback loop (Cong and Shay,

2008, Martinez and Blasco, 2011). Furthermore, it is speculated that this complex is able to generate additional siRNA molecules in order to regulate various pathways, although none have been identified as yet. Therefore, this further supports the idea of hTERT in regulating signalling pathways (Maida et al., 2009, Martinez and Blasco, 2011).

2.3.2 Telomeres

Telomeres are specialised structures which consist of DNA, RNA, and multi-protein complexes which are maintained by telomerase (Artandi, 2002, Blackburn, 2001). They are located at the end of eukaryotic linear chromosomes (Blackburn and Szostak, 1984). Telomeric DNA comprising of double-stranded TTAGGG hexamers of up to 25kb in humans and the single-stranded 3′ overhang form telomere loops (Baird et al., 2006, Blackburn, 1991, Capper et al., 2007). Telomere-loop structures, or t-loop structures (Figure 8) are formed when an end of a telomere curls over to invade the proximal region of the telomere (De Lange, 2005, Griffith et al., 1999). Telomeres serve to protect chromosomes from being recognised as double stranded breaks in the DNA (di Fagagna et al., 2003). Additionally, this serves to prevent the "end-replication" problem from taking effect due to the semi-conservative mechanism of DNA replication (Harley et al., 1990). The "end-replication" problem occurs after every mitotic event, telomeres shorten slightly due to the semi-conservative mechanism of DNA replication (Allsopp et al., 1995, Harley et al., 1994). The semi-conservative mechanism states that DNA polymerase cannot synthesise DNA strands at the ends of the chromosome which results in shortened telomeres (Allsopp et al., 1995). The t-loop conformation of telomeres protects further degradation of telomeres which leads to recombination, end-to-end fusion, and ultimately genomic instability which is a hallmark of cancer (Letsolo et al., 2010, Capper et al., 2007).

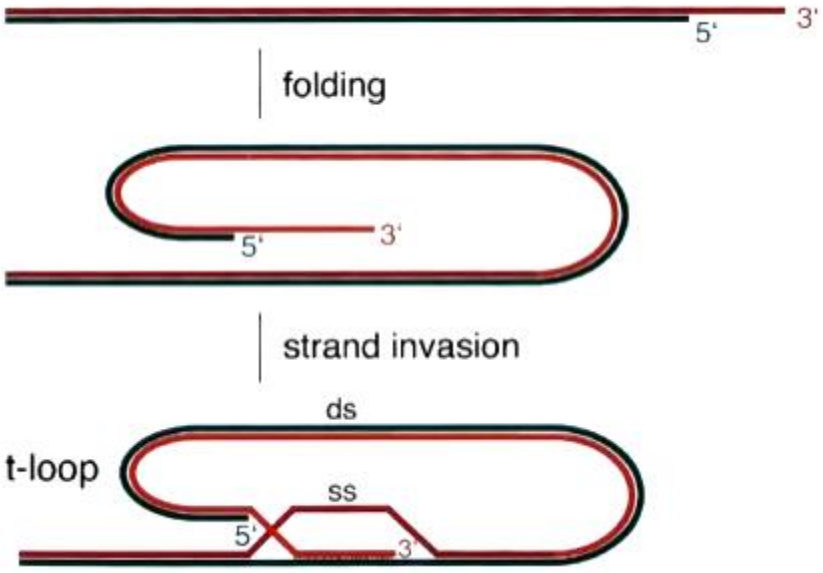


Figure 8: Formation of T-Loop Structures in Telomeres. Depiction of the formation of t-loop structures, whereby the telomeres fold over and the 3′ overhang invades and interacts with the telomere in order to form a stable t-loop structure protecting the telomere from further degradation (De Lange, 2005).

2.3.3 Shelterin Complex

TRF1, TRF2, and POT1 are proteins which bind to telomeric repeats; together with TIN2, TPP1, and Rap1 they form a protein complex known as the shelterin complex (De Lange, 2005). This complex only localises to the telomeres of chromosomes and are apparent during the entire life cycle of the cell (De Lange, 2005). Furthermore, there have been three main functions of shelterin discovered; it determines telomeric structure, aids in t-loop generation, and controls telomere elongation via telomerase activity (De Lange, 2005). Additionally, Rap1 has been found to not only interact with telomeres. Martinez *et al* has shown Rap1 to interact with subtelomeric regions of chromosomes in addition to the telomeres (Martinez et al., 2010). Moreover, Rap1 still recognises the TTAGGG consensus sequence when binding to extra-telomeric regions (Martinez et al., 2010).

TRF1 is known to be a negative regulator of telomere length (van Steensel et al., 1998, Van Steensel and De Lange, 1997). It has been shown previously that with TRF1 overexpression, the length telomeres gradually shortened until a new length was reached. Conversely, partial inhibition of TRF1 caused telomeres to lengthen until a new equilibrium length was achieved (Smogorzewska and de Lange, 2004). Also, the rate of telomere shortening in telomerase negative cells was not affected by TRF1 levels. However, TRF1 does not affect telomerase

activity *in vivo* or *in vitro* so it is not understood how TRF1 performs its regulation of telomere length (Soohoo et al., 2011).

2.3.4 Telomerase and Cancer

As previously mentioned, telomerase has been found to interact with LRP/LR (Naidoo et al., 2015). As both of these proteins are active in tumourigenic processes, this creates a new area of research in possible therapeutics in cancer. Both LRP/LR and telomerase are overexpressed in tumours (Naidoo et al., 2015, Harley and Villeponteau, 1995, Harley et al., 1994). Telomerase functions in cancer to maintain critically short telomeres to allow tumours to bypass apoptosis and senescence (Shammas et al., 2005). Additionally, telomerase has also been observed to be present in mitochondria in order to protect mtDNA from degradation (Saretzki, 2014). These processes allow for tumours to proliferate uncontrollably, regardless of the shortened telomeres (Shammas et al., 2005). Disrupting the LRP/LR-telomerase interaction could serve as a potential therapeutic against cancer. It was shown by Naidoo *et al.* that knockdown of LRP/LR via siRNA technology that telomerase activity significantly decreased in HEK293 and MDA-MD-231 cell lines (Naidoo et al., 2015). Interestingly, it has been found that the hTERT gene promoter in cancer is hypermethylated which corresponds to lower expression patterns (Devereux et al., 1999). Zinn *et al.* then investigated why hTERT is overexpressed in many cancer types due to the hypermethylation patters. Zinn *et al*. discovered that some alleles of the hTERT promoter are spared from hypermethylation which allows for continued expression of hTERT in many cancers (Zinn et al., 2007). As previously mentioned, telomerase, and hTERT, plays a role in cell signalling pathways. The tumour suppressor protein p53 regulates cell survival and cell growth. Also, it has been shown that p53 downregulates telomerase expression (Xu et al., 2000, Rahman et al., 2005). It has also been shown that an increase in hTERT expression in cancer cells allows for inhibition of p53 as well as p53-dependent apoptosis (Rahman et al., 2005). Interestingly, hTERT did not have to be functionally active in order to inhibit p53 functioning (Rahman et al., 2005). The Wnt/β-catenin pathway is another signalling pathway hTERT is involved in. This pathway has been known to cause carcinogenesis (Logan and Nusse, 2004, Saretzki, 2014, Martinez and Blasco, 2011) The Wnt/β-catenin directly transcribes hTERT leading to carcinogenesis (Zhang et al., 2012). This is one of the reasons for hTERT overexpression in many cancer types. Since downregulation of LRP/LR impedes telomerase activity and decreases cell viability, the hypothesis is whether the siRNAs directed against LRP mRNA act as potential alternative therapeutic tools for treatment of lung adenocarcinoma and late-stage colorectal carcinoma.

3 Hypothesis, Aims, and Objectives

Cancer is one of the top causes of death worldwide, there has been a great need to find alternate therapeutic strategies which are able to combat tumours while preserving normal tissue. Since it has previously been shown that downregulation of LRP/LR impedes telomerase activity in HEK293 and MDA-MB-231 cells, and decreases cellular viability, the hypothesis of this study was to determine whether siRNAs directed against LRP mRNA may act as a potential alternative therapeutics tools for treatment of lung adenocarcinoma and late-stage colorectal carcinoma

3.1 Aims

To investigate the role of the 37 kDa/67 kDa high affinity laminin receptor (LRP/LR) on telomerase activity in A549 (late stage lung adenocarcinoma) and DLD-1 (late stage colorectal carcinoma) cells lines.

3.2 Objectives

- To determine downregulation of LRP/LR and telomerase via transfection with siRNA in lung adenocarcinoma and late stage colorectal carcinoma cells.
- To investigate the co-localisation of the LRP/LR and hTERT intracellularly, before and after siRNA transfections in lung adenocarcinoma and late stage colorectal carcinoma cells.
- To determine whether knockdown of LRP/LR via siRNA technology affects telomerase activity in lung adenocarcinoma and late stage colorectal carcinoma cells.
- To investigate whether knockdown of LRP/LR via transfection with siRNA affects hTERT expression levels in lung adenocarcinoma and late stage colorectal carcinoma cells.
- To investigate cellular viability of cells before and after transfection with siRNA in lung carcinoma and late stage colorectal carcinoma cells.

4 Methods and materials

4.1 Cell culture and Transfections

Lung adenocarcinoma (A549), late-stage colorectal carcinoma (DLD-1), and human embryonic kidney (HEK293) cells were cultured in a humidified incubator at 37 °C and 5% CO_2. The A549 and HEK293 cells were cultured using Dulbecco's Modified Eagle Medium

(DMEM) and the DLD-1 cells were cultured in Ham's F12, all media was supplemented with 20% Foetal Bovine Serum (FBS) and 3% penicillin/streptomycin/amphotericin B. Cell passaging was performed when the cells reached 75%-80% confluency in T25 (25 cm^2) cell culture flasks. Prior to passaging, cells were washed using 2 ml of phosphate buffered saline (PBS). Thereafter, cells were incubated in 1 ml Trypsin/EDTA at 37 °C for 7-10 minutes to detach cells from the flask. Once the incubation was complete, 4 ml of the relevant media was added in order to inactivate the Trypsin/EDTA. A 1:4 cell passaging was performed and the relevant media was added to make a final volume of 5 ml. Cell transfections were carried out using small interfering RNAs (siRNAs) in order to knockdown LRP/LR. The following siRNAs were used; Dharmacon On-Target™ RPSA, Mission® Universal Negative, and Mission® esiRNA RPSA. The On-Target™ RPSA was used to knockdown LRP/LR, the Mission® esiRNA RPSA as an alternative siRNA, and Mission® Universal Negative as the negative control to show no off-target effects are occurring. Supplementary Table 1 below indicates the required volumes of siRNA, transfection reagent, and serum-free media in order to perform transfections. Transfections were performed when cell confluency reached 40%-50%. In order to perform transfections, each of the components (siRNA and transfection reagent) was prepared in separate microfuge tubes (Tube 1 and Tube 2). This was followed by incubation at room temperature for 5 minutes. Thereafter, Tube 1 and Tube 2 were mixed together and incubated for 20 minutes at room temperature. Once this was completed, the entire liquid content was then transferred into the well/dish and incubated for 72 hours. Once the treatments were completed (after 72-hour incubation), the cells were harvested by collecting the media into 15 ml centrifugation tubes. The cells were then detached by using 1 ml of Trypsin/EDTA for 5 minutes at 37 °C. After incubation, the cells were scraped and collected into the 15 ml tubes containing the harvested media. The cell suspensions were then centrifuged for 10 minutes at 5600 X *g*. Once complete, the excess media was removed and the cell pellets stored at -80 °C until required for downstream applications.

4.2 Western Blotting and Protein Extraction and Quantification

4.2.1 Protein extraction and BCA Assay

Western blotting is a widely used technique in molecular biology. It is utilised in order to identify specific proteins extracted from cells. It separates proteins by size using SDS-PAGE (Mahmood &Yang, 2012). Prior to western blotting, protein lysates were prepared. Harvested cell pellets were incubated with 100 µl of 1X RIPA (Radioimmunoprecipitation Assay) buffer

for 10 minutes at 4 °C. In order to quantify the protein extracted, a BCA (Bicinchoninic Acid) assay was performed. In order to construct a standard curve, BSA (Bovine Serum Albumin) standards of 0 mg/ml, 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml, and 1 mg/ml were pipetted in triplicate at 25 µl each into a 96 well plate. The crude protein lysate was then added into separate wells, 5 µl of raw lysate in triplicate to 20 µl ddH_2O (5 times dilution) in the wells. A solution of 97% BCA and 3% Cu(II)S was then prepared and 200 µl of this solution was added to each well. The plate was then incubated for 30 minutes at 37 °C. Thereafter the absorbance was measured at 562 nm using an ELISA Plate Reader. The protein concentration was then calculated, taking the dilution factor into account, and a protein stock of a fixed concentration was prepared.

4.2.2 Western Blotting

This technique was utilised in order to determine whether LRP/LR knockdown was successful. This technique involves SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) in order to resolve and separate whole protein lysates according to molecular weight. SDS-PAGE gels, at a concentration of 10%, were prepared according to supplementary Table 2. The proteins were separated at 120V until the dye front reached the bottom of the gel. Thereafter, the proteins were transferred onto PVDF membranes via the semi-dry electroblotting mechanism; 150V was applied to the apparatus for 45 minutes. Following the electroblotting step, the PVDF membranes were transferred into a blocking solution (3% BSA in PBS/0.1% Tween) for 1 hour. This step was performed in order to reduce non-specific binding of the antibodies. Once the blocking step was completed, anti-LRP/LR specific antibody IgG1-iS18 was used for LRP/LR detection (1:5000 in blocking solution) and mouse anti-human HRP-conjugated Actin-Peroxidase for β-actin detection (1:7500 in blocking solution). This was then incubated overnight at 4 °C in the dark with gentle agitation. On the second day after primary antibody incubation, the antibody solution was removed and the PVDF membranes were washed 5 times in PBS/0.1% Tween for 5 minutes each. The secondary antibody was then added; Anti-Human-HRP (1:7500 in blocking solution) for LRP/LR detection. No secondary antibody was required for β-actin as the primary antibody had the HRP conjugated. This was incubated for 1 hour at room temperature in the dark. Upon completion of the incubation, the antibody solution was removed and the PVDF membranes were washed 5 times in PBS/0.1% Tween for 5 minutes each. Thereafter, the PVDF membranes were transferred into Clarity™ Western ECL substrate and incubated for 5 minutes at room temperature in the dark. The Bio-Rad Gel Doc with High Sensitivity and signal accumulation

time mode settings was used for detection. The images generated were saved and used for downstream densitometry analysis.

4.3 Confocal Microscopy

Confocal microscopy is used for visualisation of specifically fluorescently-tagged proteins within the cell. This allows for identification of spatial occupation of the fluorescently-tagged proteins to determine in which compartments of the cell these proteins occupy. In preparation for confocal microscopy, cells were seeded onto microscope cover slips and transfected as stated previously. Once transfections were completed, the cover slips were washed 2 times using 2 ml of PBS/0.1% Tween. The cells were then fixed onto the cover slips using 4% Paraformaldehyde for 20 minutes at room temperature. The excess Paraformaldehyde was discarded and the cover slips were washed once again, 2 times using 2 ml of PBS/0.1% Tween. In order to observe the intracellular protein localisation, the cells on the cover slips were permeabilised by incubation with 0.1% Triton-X for 20 minutes at room temperature. Thereafter, the excess Triton-X solution was discarded and the cover slips washed, 2 times using 2 ml of PBS/0.05% Tween. Following the washes, the cover slips were incubated for 15 minutes at room temperature in 2 ml blocking solution (0.5% BSA in PBS/0.1% Tween) in order to reduce non-specific binding. Subsequently, the appropriate antibodies were added to the blocking solution; anti-LRP/LR specific antibody IgG1-iS18 was used for LRP/LR detection (1:100 in blocking solution) and anti-rabbit hTERT was used for hTERT detection (1:100 in blocking solution). No primary antibody was added to the secondary only control cover slips. The cells were then incubated overnight at 4 °C in the dark while shaking. The cover slips were removed from the antibody solution and rinsed 8 times in PBS/0.1% Tween. Thereafter, the cover slips were incubated with secondary antibody. Anti-Human-FITC (1:200 in blocking solution) was used for LRP/LR detection and Anti-Rabbit-APC (1:200 in blocking solution) was used for hTERT detection. For the secondary only control, only single secondary antibodies were added at the same dilutions. The secondary antibody solutions were incubated in the dark for 40 minutes at room temperature where after 2 µl of 1mg/ml DAPI nuclear staining was added and further incubated for 20 minutes under the same conditions. Upon completion of antibody and nuclear staining incubation, the cover slips were rinsed 8 times with PBS/Tween. The cover slips were then mounted with Fluoromount™ Aqueous Mounting Medium onto microscope slides. These slides were stored at 4 °C in the dark until required. The slides were viewed with the Zeiss LSM 780 confocal microscope. Excitation and emission

wavelengths are as follows; FITC at 490nm and 525 nm, respectively, and APC at 348 nm and 395 nm, respectively.

4.4 qPCR Analysis for hTERT mRNA levels

4.4.1 RNA Extraction

This technique is used to determine mRNA levels for specific proteins using basic PCR principles and light to detect fluorescence emitted by fluorophores. This allows for accurate relative quantification of mRNA levels for a desired protein. Cells were seeded into 6-well dishes, transfected, and harvested as specified in section 4.1. The Zymo Research *Quick-RNA*™ Miniprep Kit was utilised in order to extract RNA from cells. In preparation of RNA extraction, 96 ml of 100% ethanol was added to the RNA Wash Buffer and lyophilised DNase I. All steps were performed at room temperature unless otherwise stated and centrifugations were performed at 16000 X g. In order to lyse cells, 100 µl of RNA Lysis Buffer was added to cell pellets and vortexed briefly. Thereafter, 1 volume (1:1 ratio) of 100% ethanol was added and mixed well. The mixture was then transferred to a Zymo-Spin™ IIICG Column with a collection tube and was centrifuged for 30 seconds and the flow-through discarded. An additional optional step was performed to remove excess gDNA. A mixture of DNase I (5 µl) and DNA Digestion Buffer (75 µl per sample) was prepared and added to each column. The mixture was incubated for 15 minutes then centrifuged for 30 seconds. Subsequently, 400 µl of RNA Prep Buffer was added to each column, centrifuged for 30 seconds and, the flow-through was discarded. Afterwards, 700 µl of RNA Wash Buffer was added into each column, centrifuged for 30 seconds and the flow-through was discarded. Next, 400 µl of RNA Wash Buffer was added into each column and centrifuged for 2 minutes; the flow-through was discarded. Thereafter, the columns were transferred into fresh RNase-free microfuge tubes. To elute the extracted RNA, 50 µl of nuclease-free water was added into the columns and centrifuged for 1 minute. The RNA was stored at -80 °C until required.

4.4.2 cDNA Synthesis and Quantification

The extracted RNA was converted into cDNA before further downstream applications. The ProtoScript® II First Strand cDNA Synthesis Kit was utilised for this. A mixture of oligo $d(T)_{23}$ VN (50 µM) and random primer mix was prepared (2 µl each) for each sample. Thereafter, 6 µl of RNA sample was transferred into fresh RNase-free PCR tubes and 4 µl of the primer mixture was added. The RNA samples were denatured by incubating the mixture for 5 minutes at 65 °C. Next, the mixture was microfuged briefly and placed on ice. Thereafter, a mixture of

the following components was prepared per sample: 10 µl of 2X ProtoScript II Reaction Mix and 2 µl of 10X ProtoScript II Enzyme Mix. Subsequently, 12 µl of this mixture was added to each of the samples. Supplementary Table 3 below indicates the cycling conditions performed in order to convert the RNA into cDNA; due to the use of the random primer mix it is important to note an additional 25 °C cycling step was performed._Moreover, a no-reaction negative control was also prepared. This contained all of the previous components except for the 10X ProtoScript II Enzyme Mix. Subsequently, the cDNA was quantified using the Nanodrop ND-1000 spectrophotometer. Next, the cDNA was diluted to 500 ng/µl and stored at -20 °C for downstream applications.

4.4.3 qPCR Analysis of hTERT mRNA Levels

In order to determine hTERT mRNA levels, qPCR was performed. Moreover, GAPDH was utilised as a reference gene to ensure any changes in hTERT expression levels was due to the treatment. Supplementary Table 4 below indicates the master mix solution that was prepared per sample. A standard curve was generated using cDNA lysate from cells known to express hTERT (e.g. HEK293 cells). A 10-times serial dilution was prepared: 950 ng/µl, 95.0 ng/µl, 9.50 ng/µl, 0.950 ng/µl, 0.0950 ng/µl. Nuclease-free water was used as a negative control. HEK293 lysates were utilised as a positive control as it is known to express hTERT. Thereafter, 9 µl of the master mix solution was added into each well of a sterile 96-well plate. Afterwards, 1 µl of standards, negative control, positive control, and experimental samples were added in triplicate. The mixture was thoroughly mixed. Subsequently, an adhesive protection sleeve was placed on top of the plate. Supplementary Table 5 below indicates the qPCR cycling conditions for analysis of GAPDH and hTERT RNA expression levels. The instrument utilised to conduct this technique was the Roche LightCycler® 480. Afterwards, the data was collected and analysed.

4.5 Detection of Telomerase Activity through qPCR

4.5.1 Protein Extraction and Quantification

The TRAPeze® RT Telomerase Detection Kit utilises qPCR. The telomerase protein will extend synthetic telomeres and releases fluorometric signals which are detected. This, in-turn, provides relative activity of the telomerase protein extracted from cells. Cells were seeded onto 6-well plates and transfected as stated in section 4.1. Once completed, the cells were harvested as previously stated. In order to extract active, native functioning telomerase protein CHAPS Lysis buffer was used. A positive telomerase activity control cell pellet was also provided in

the Merck TRAPeze® RT Telomerase Detection Kit and functioning telomerase protein was extracted alongside the experimental cell pellets. The cell pellets were resuspended in 200 µl CHAPS Lysis buffer and incubated for 30 minutes on ice. Thereafter, the lysate was centrifuged at 16000 X g for 20 minutes at 4 °C. The supernatant was removed and transferred into fresh microfuge tubes; 190 µl for further application and 10 µl for Nanodrop quantification. The lysates were then snap-frozen using dry ice. Subsequently, whenever the protein lysates were thawed down, dry ice was used to snap-freeze the lysates before storing at -80 °C. In order to quantify the protein concentration, the Nanodrop ND-1000 Spectrophotometer instrument was utilised to measure absorbance at 280 nm. Subsequently, two protein stocks at a concentration of 500 ng/µl were prepared. One was used to determine telomerase activity and the other served as a heat-treated negative control.

4.5.2 qPCR for Telomerase Activity

The TRAPeze® RT Telomerase Detection Kit was utilised for this procedure. Supplementary Table 5 below indicates the reagents required to create a master mix. A standard curve of TSR8 was provided in the kit and the following concentrations constructed the standard curve; 0.02 ng/µl, 0.2 ng/µl, 2 ng/µl, and 20 ng/µl. CHAPS Lysis buffer and nuclease-free water were utilised as negative controls. Two separate master mixes were prepared, a reaction master mix for the native protein samples and a control master mix for the heat-treated protein samples. Supplementary Table 6 indicates the reaction master mix recipe and the control master mix recipe. The heat-treated samples were incubated at 95 °C for 20 minutes in order to denature the telomerase protein. Once the master mixes were prepared, 12.5 µl of the appropriate master mix was pipetted into each well of a 96-well plate. Thereafter, 2 µl of standards, negative controls, positive control, heat-treated sample, and experimental samples were added in triplicate. The mixture was thoroughly mixed. Subsequently, an adhesive protection sleeve was placed on top of the plate. Supplementary Table 7 below indicates the qPCR cycling conditions. The instrument utilised to conduct this technique was the Roche LightCycler® 480. Thereafter, the data was collected and analysed.

4.6 Cellular Viability Detection via the MTT Assay

The MTT Assay is a widely used technique to determine cellular viability via a colourimetric assay. The MTT tetrazolium dye is reduced by live cells to generate formazan crystals, which only live cells have the capability of doing. This allows for relative determination of viable cells. All cell lines were seeded onto a 24-well plate and transfected as mentioned previously

in section 4.1. Protocatechuic Acid (PCA) was used as a positive control and was incubated with the cells for the same duration as the transfections; 500 µl 8 mM PCA was added to 1 ml media. After transfection, 300 µl of 1 mg/ml MTT solution was added into each well and incubated for 2 hours at 37 °C. Thereafter, the media with the MTT solution in the wells was removed. To dissolve the resulting formazan crystals, 400 µl of filter-sterilised Dimethyl Sulphoxide (DMSO) was added to each well and incubated for 10 minutes at 37 °C. Subsequently, 100 µl was transferred to a 96-well plate, in triplicate and the absorbance was measured at 570 nm using an ELISA plate reader.

4.7 Statistical Evaluation and Analysis

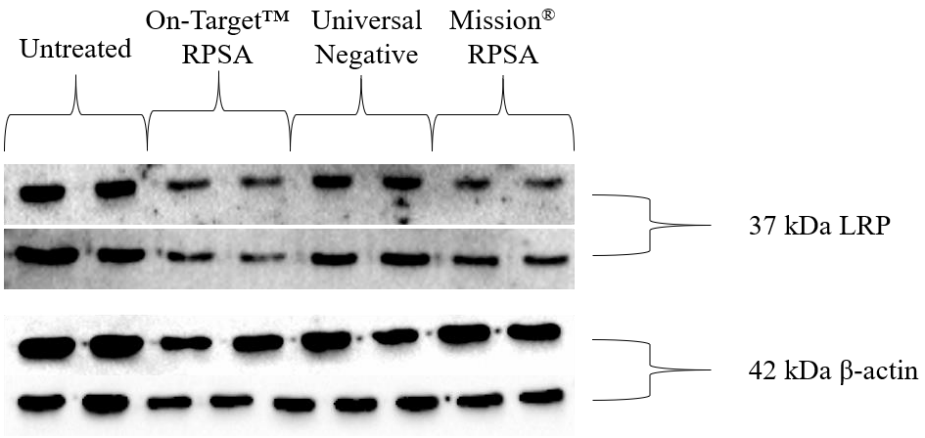
All of the data obtained from the experimental procedures were interpreted and statistical analysis performed in order to assess the significance. Appropriate statistical analytical tests (Student's T-Test) was performed at a confidence interval of 95% (*$p < 0.05$, **$p < 0.01$, ***$p<0.001$).

5 Results

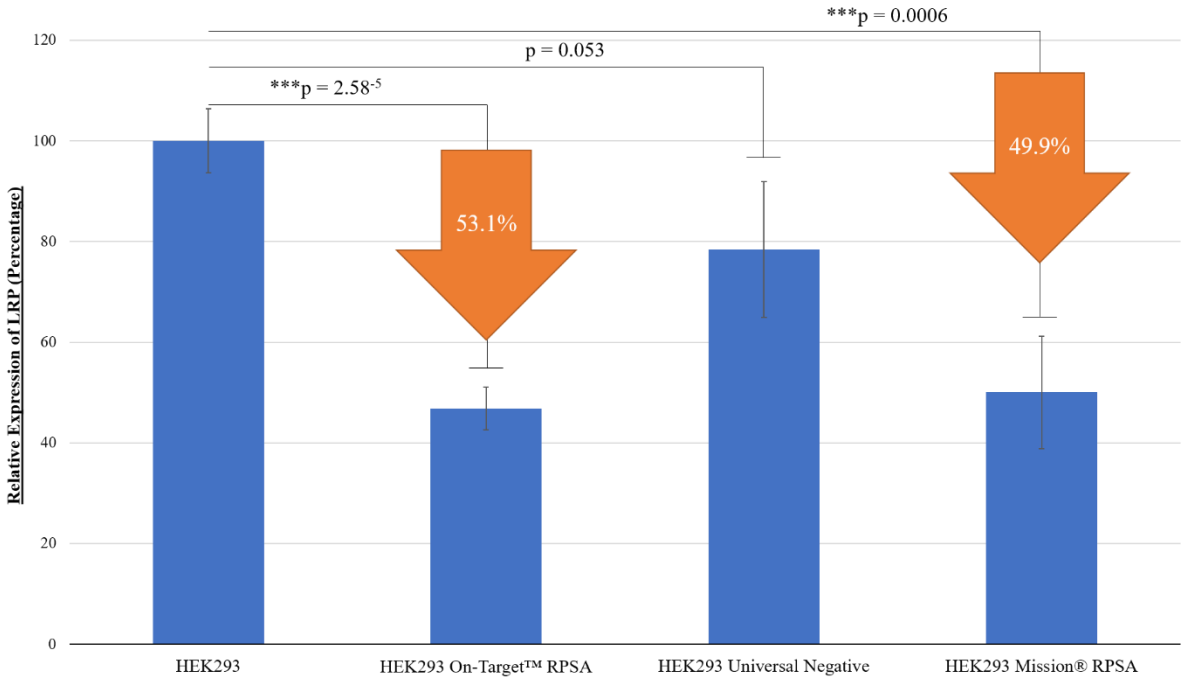
5.1 Knockdown of LRP/LR Through Use of RPSA siRNAs

The siRNA RPSA targets the mRNA sequence of LRP to knockdown LRP/LR. The processed LR receptor is also knocked down as there is little LRP to be modified and processed into LR. The On-Target™ siRNA contains a cocktail of various siRNAs in order to target various sequences of the LRP mRNA sequence (Supplementary Table 8). Western blotting analysis was utilised in order to determine the effect of the RPSA siRNA on LRP/LR levels. The loading control chosen was β-actin as this is a regular house-keeping protein; this was to ensure equal loading and that any changes in LRP/LR was due to siRNA treatment. Western blotting below indicates analysis of LRP levels for all cell lines (Figure 9).

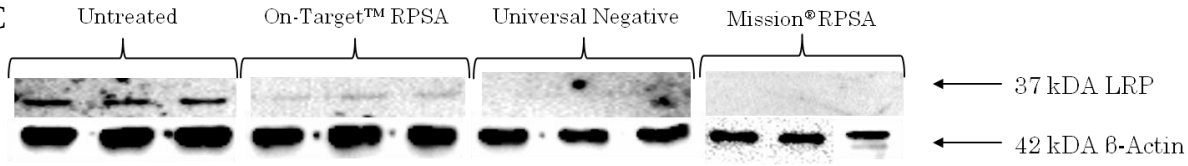
A


Untreated
On-Target™ RPSA
Universal Negative
Mission® RPSA
37 kDa LRP
42 kDa β-actin

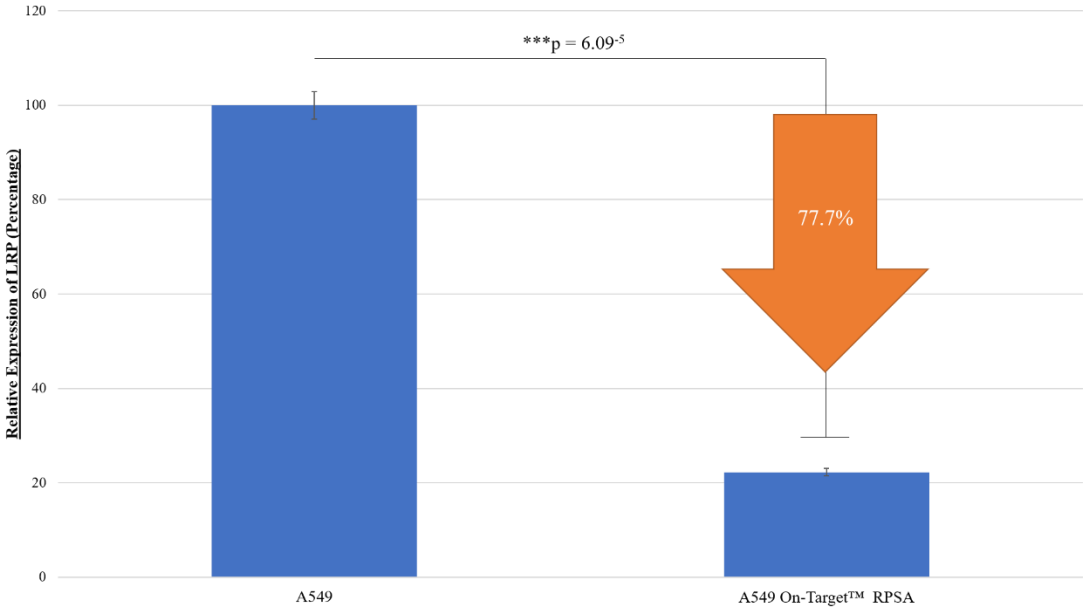
B


***p = 0.0006
p = 0.053
***p = 2.58-5
53.1%
49.9%
Relative Expression of LRP (Percentage)
120
100
80
60
40
20
0
HEK293
HEK293 On-Target™ RPSA
HEK293 Universal Negative
HEK293 Mission® RPSA

C


Untreated
On-Target™ RPSA
Universal Negative
Mission®RPSA
37 kDA LRP
42 kDA ß-Actin

D


***p = 6.09-5
77.7%
Relative Expression of LRP (Percentage)
120
100
80
60
40
20
0
A549
A549 On-Target™ RPSA

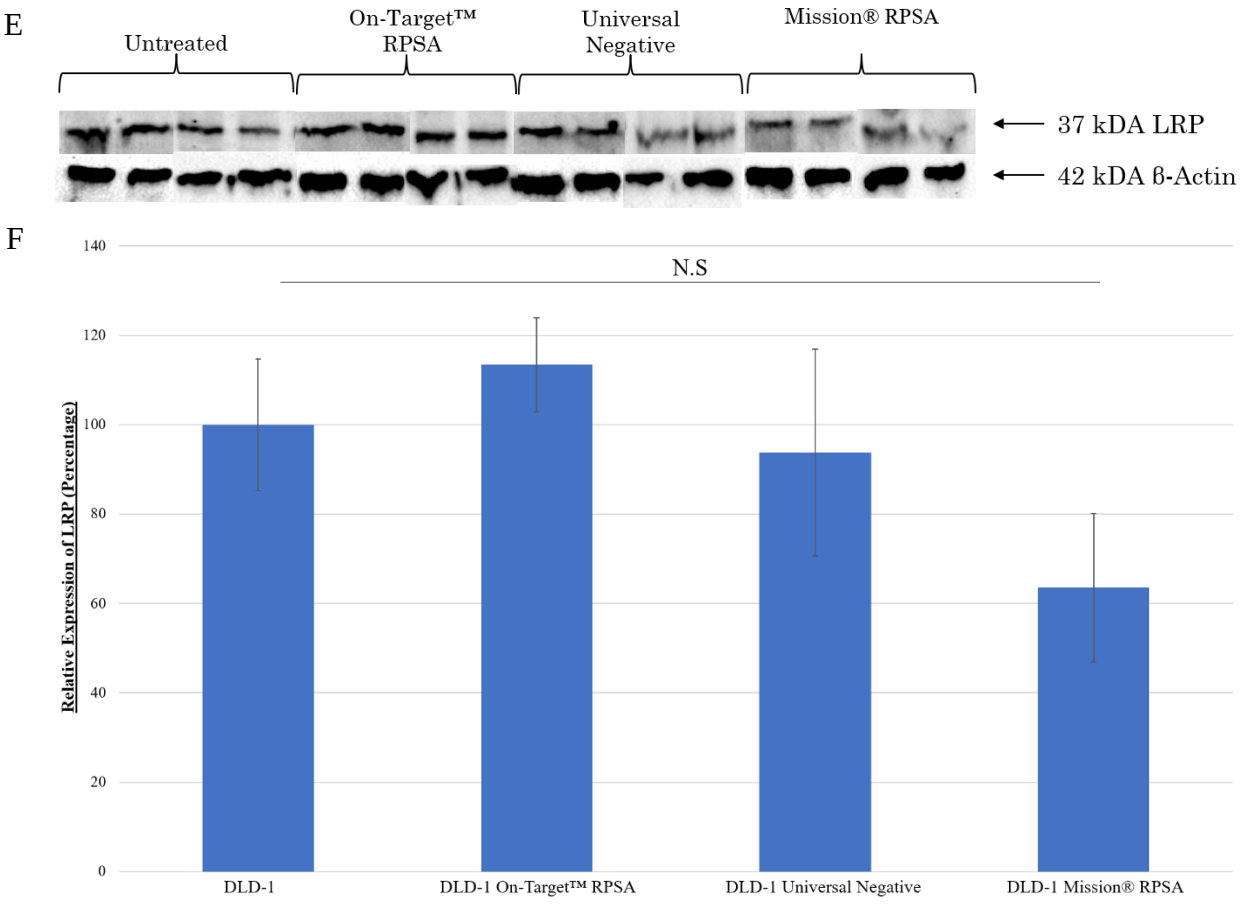


Figure 9: Downregulation of LRP/LR in HEK293, A549, and DLD-1 cell lines analysed by western blotting. (A) Western blotting banding pattern for A549 as detected by the Bio-Rad ChemiDoc™ MP Imaging System. (B) There is a significant difference in LRP levels between untreated HEK293 cells when compared to On-Target™ RPSA and Mission® RPSA siRNA transfected HEK293 cells indicating successful knockdown of LRP/LR. Also, there is no significant difference between the untreated HEK293 cells and the Universal Negative treated cells which indicates the Universal Negative has no effect on LRP levels. (C) Western blotting banding pattern for A549 as detected by the Bio-Rad ChemiDoc™ MP Imaging System. (D) There is a significant decrease in LRP levels between untreated and On-Target™ RPSA siRNA for the A549 cell line showing that knockdown of LRP/LR was successful. (E) Western blotting banding pattern for A549 as detected by the Bio-Rad ChemiDoc™ MP Imaging System. (F) There is no significant difference in LRP levels between untreated and siRNA transfected cell lines in the DLD-1 cells showing unsuccessful downregulation of LRP/LR (A549 n = 3, HEK293 and DLD-1 n = 4).

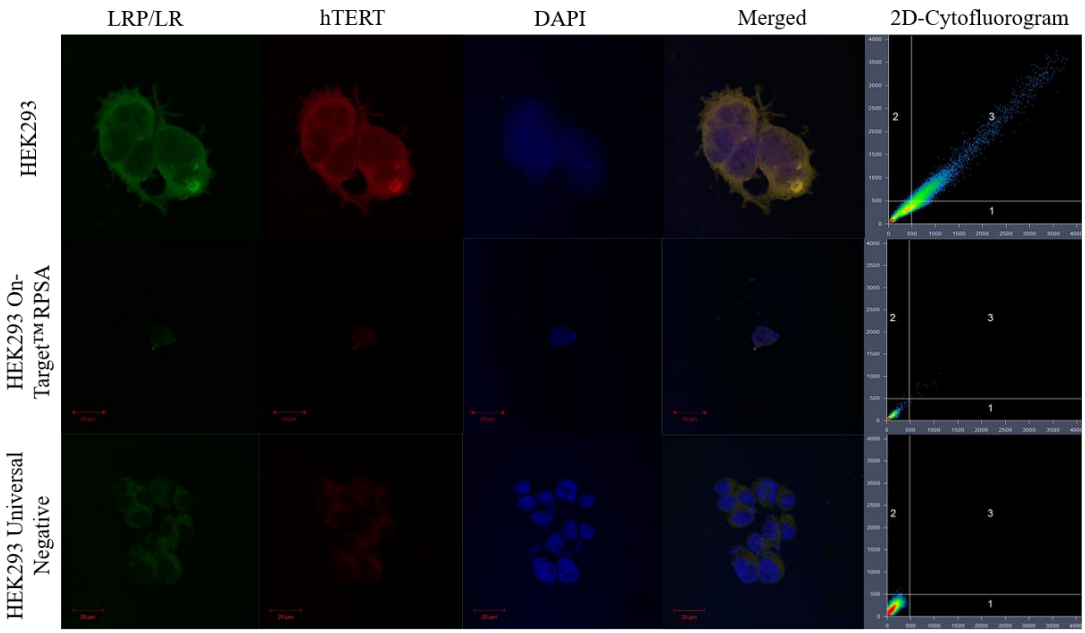
The HEK293 cell line shows a significant decrease in LRP/LR levels for both On-Target™ RPSA and Mission® RPSA. This indicates that treatment with the siRNAs successfully

knocked-down LRP/LR. The On-Target™ RPSA and Mission® RPSA decreases LRP/LR levels by 53.1% and 49.9%, respectively. Moreover, there is slight decrease in LRP/LR levels for the Mission® Universal Negative which can be attributed to the cellular stresses of the transfection treatment; however, the decrease is non-significant. The A549 cell line shows a 77.7% decrease in LRP/LR levels after On-Target™ RPSA treatment. There were no observable LRP/LR signals for the Mission® Universal Negative and the Mission® RPSA for the A549 cell line. The On-Target™ RPSA and Mission® RPSA treatments were unsuccessful on LRP expression levels when analysed by western blotting with regard to DLD-1 cells.

5.2 LRP/LR Knockdown and Co-localisation between LRP/LR and hTERT Analysed by Confocal Microscopy

Confocal microscopy was used in order to visualise the compartments of the cell in which LRP/LR and hTERT localise. It was also used in order to visualise the LRP/LR knockdown after treatments with On-Target™ RPSA and Mission® RPSA. The green fluorescence (FITC fluorophore) is indicative of LRP/LR and the red fluorescence (APC fluorophore) is indicative of hTERT. The nuclear staining utilised was DAPI. Figure 10 indicates the images obtained from the Zeiss LSM 780.

A



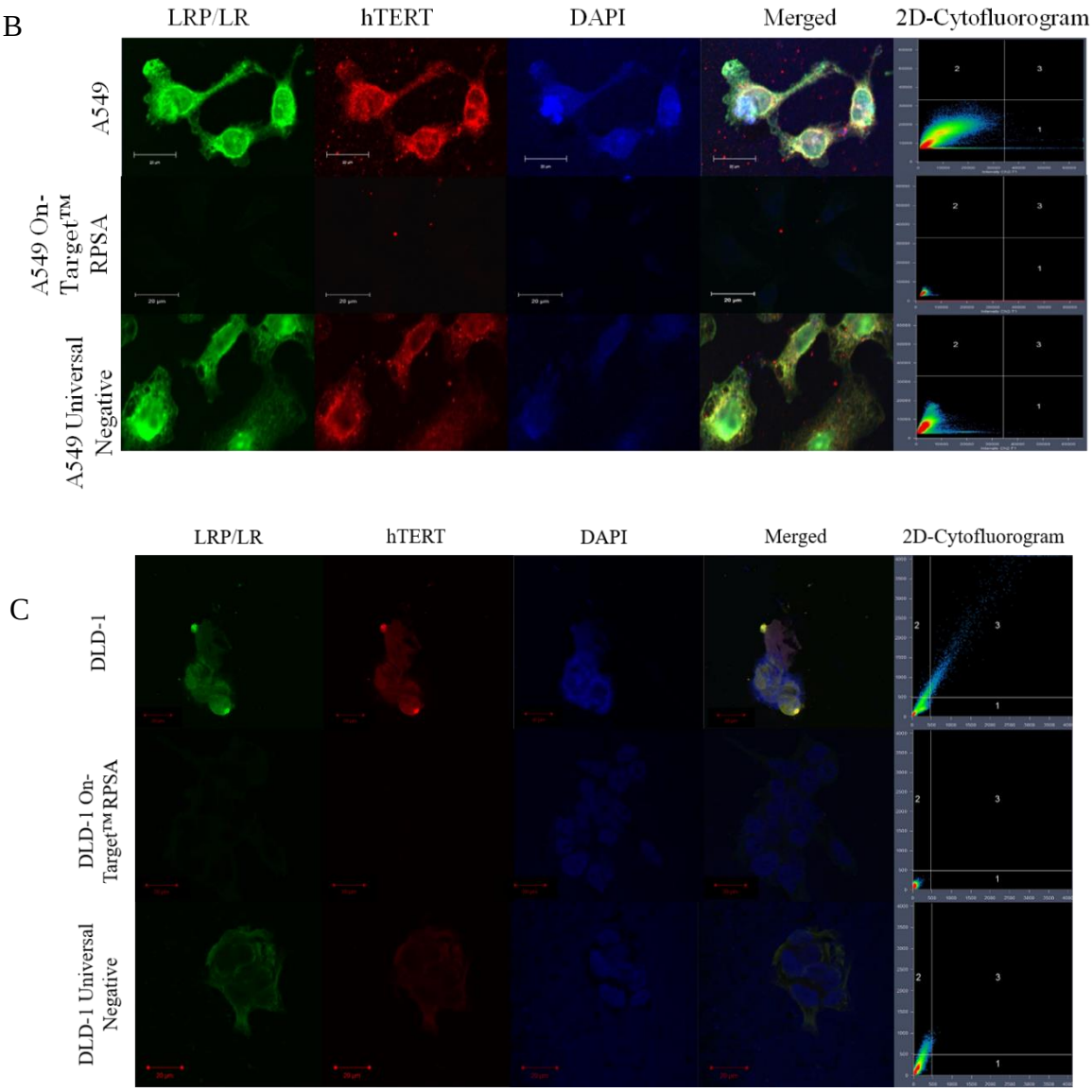


Figure 10: Downregulation of LRP/LR in HEK293, A549, and DLD-1 cell lines analysed by confocal microscopy. (A) Untreated HEK293 cells indicate the endogenous of LRP/LR and detectable levels of hTERT. The 2D-cytofluorogram indicates a strong correlation that both LRP/LR and hTERT localise within the same compartments of the cell for A549 cells. This further supports the fact that LRP/LR and hTERT interact within the cell as previously described by Naidoo *et al* (2015). Upon treatment with On-Target™ RPSA, LRP/LR levels decreased to almost non-detectable levels and subsequently hTERT levels decreased drastically. (B) The untreated A549 cells indicate LRP/LR levels and detectable hTERT levels, which is supported by the 2D-Cytofluorogram. The 2D-cytofluorogram indicates a co-localisation for both LRP/LR and hTERT localise within the same compartments of the cell for A549 cells. (C) The untreated DLD-1 cells indicate LRP/LR levels and detectable hTERT levels, which is supported by the 2D-Cytofluorogram. The 2D-cytofluorogram indicates a co-

localisation for both LRP/LR and hTERT localise within the same compartments of the cell for DLD-1 cells. In Figure 10, the On-Target™ RPSA successfully knocked down LRP/LR in A549 cells, as can be seen in a reduction in green fluorescence. This resulted in a subsequent decrease in hTERT protein levels, indicated by a reduction in red fluorescence. As previously mentioned, LRP/LR is involved in chromosomal and nuclear stability. Notably, the blue DAPI staining had a decrease in fluorescence. This might be due to the fact that the nuclear stability had been compromised. Moreover, the 2D-cytofluorogram indicated that LRP/LR and hTERT co-localise in the same compartments of A549 cells. The DLD-1 cells showed similar results as the A549 cells. There was notable decrease in green (LRP/LR) and red (hTERT) fluorescence after On-Target™ RPSA treatment. The nuclear stability was also compromised in the DLD-1 cells depicted by a reduction in blue DAPI staining. Similarly, the 2D-cytofluorogram also indicates that LRP/LR and hTERT co-localise to the same compartments in DLD-1 cells.

5.3 LRP/LR Knockdown Significantly Decreases Telomerase Activity

A telomerase activity assay was performed in order to determine whether the knockdown of LRP/LR, and subsequent decrease in hTERT levels, affected the functioning of the telomerase protein. This was done using the Merck TRAPeze® RT Telomerase Detection Kit and qPCR, as previously stated. Figure 11 indicates the relative telomerase activity for all cell lines. The exceptionally high decreases in telomerase activity across the cell lines can be attributed to the sensitivity of the hTERT protein. This protein is highly sensitive and the functioning is very securely regulated in non-tumourigenic cells (Bodnar et al., 1998). Moreover, it has been shown previously that knockdown of LRP/LR in HEK293 and MDA-MB-231 cell lines significantly decreased telomerase activity (Naidoo et al., 2015). This is possibly due to the interaction between LRP/LR and hTERT (Naidoo et al., 2015). Therefore, any changes in LRP/LR protein levels will have an effect on hTERT protein levels as well as telomerase activity.

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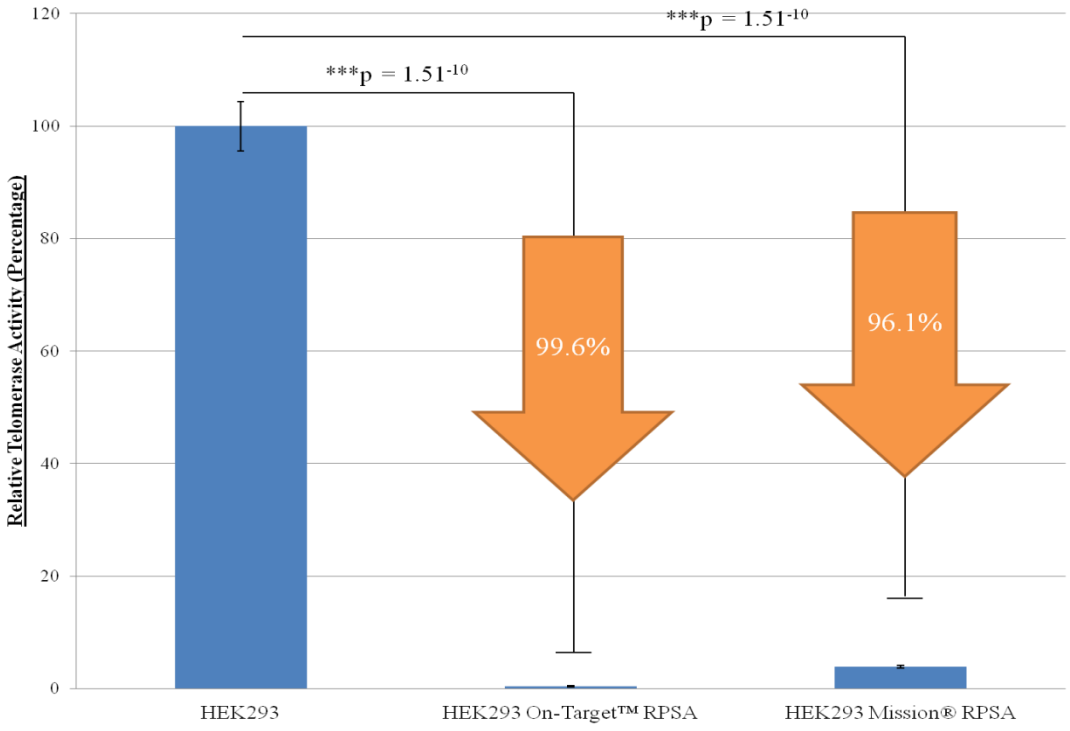
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***p = 1.51-10
Relative Telomerase Activity (Percentage)
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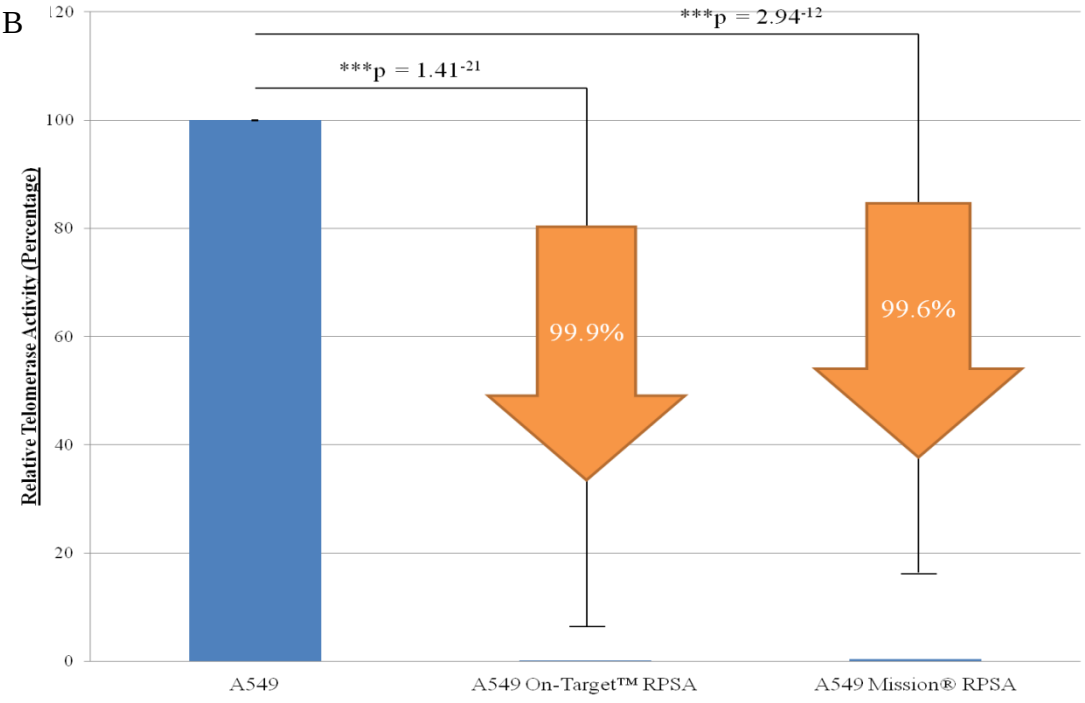
100
80
60
40
20
0
99.6%
96.1%
HEK293
HEK293 On-Target™ RPSA
HEK293 Mission® RPSA

B


***p = 2.94-12
***p = 1.41-21
Relative Telomerase Activity (Percentage)
120
100
80
60
40
20
0
99.9%
99.6%
A549
A549 On-Target™ RPSA
A549 Mission® RPSA

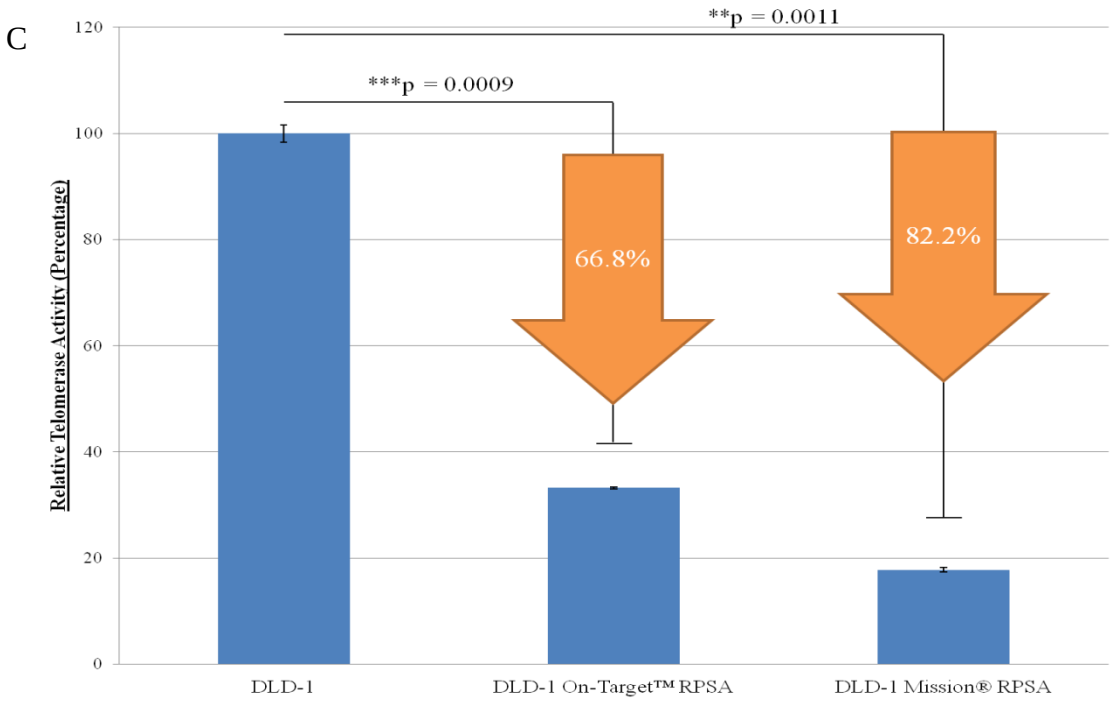


Figure 11: Knockdown of LRP/LR results in significantly decreased telomerase activity in HEK293, A549, and DLD-1 cell lines. (A) HEK293 cells after On-Target™ RPSA and Mission® RPSA treatment indicates a 99.6% and a 96.1% decrease in telomerase activity, respectively. (B) A549 cells after On-Target™ RPSA and Mission® RPSA treatments indicate a 99.9% and 99.6% decrease in telomerase activity, respectively. (C) DLD-1 cells after On-Target™ RPSA and Mission® RPSA treatments indicate a 66.8% and 82.2% decrease in telomerase activity, respectively (n=6).

Upon treatment of cells with On-Target™ RPSA and Mission® RPSA, telomerase activity in HEK293 cells decreased significantly by 99.6% and 96.1%, respectively. It has been previously shown by Naidoo *et al.* that treatment with RPSA siRNA decreases telomerase activity in HEK293 and MDA-MB-231 cells (Naidoo et al., 2015). This could be due to the fact that LRP/LR and hTERT interact which was shown by Naidoo *et al.* via the Pull Down assay (Naidoo et al., 2015). However, it is important to note that it is not yet certain whether this is a direct or an indirect interaction between LRP/LR and hTERT. Furthermore, with the same treatment in A549, cells a significant 99.9% and 99.6% reduction in telomerase was observed for On-Target™ RPSA and Mission® RPSA, respectively. Similarly, for the DLD-1 cells a significant reduction of 66.8% and 82.2% reduction were seen for On-Target™ RPSA and Mission® RPSA, respectively.

5.4 Knockdown of LRP/LR Decreases hTERT mRNA Levels

After a successful decrease in telomerase activity was observed, this lead to further investigation as to whether or not this decrease is attributed to a change in mRNA of hTERT. The technique utilised was qPCR in order to determine hTERT mRNA levels.Figure 12 indicates the results obtained. It can be seen that knockdown of LRP/LR induces a subsequent decrease in hTERT mRNA levels. Therefore, the siRNA treatment decreases not only the telomerase protein functioning, but the expression of hTERT mRNA levels as well.

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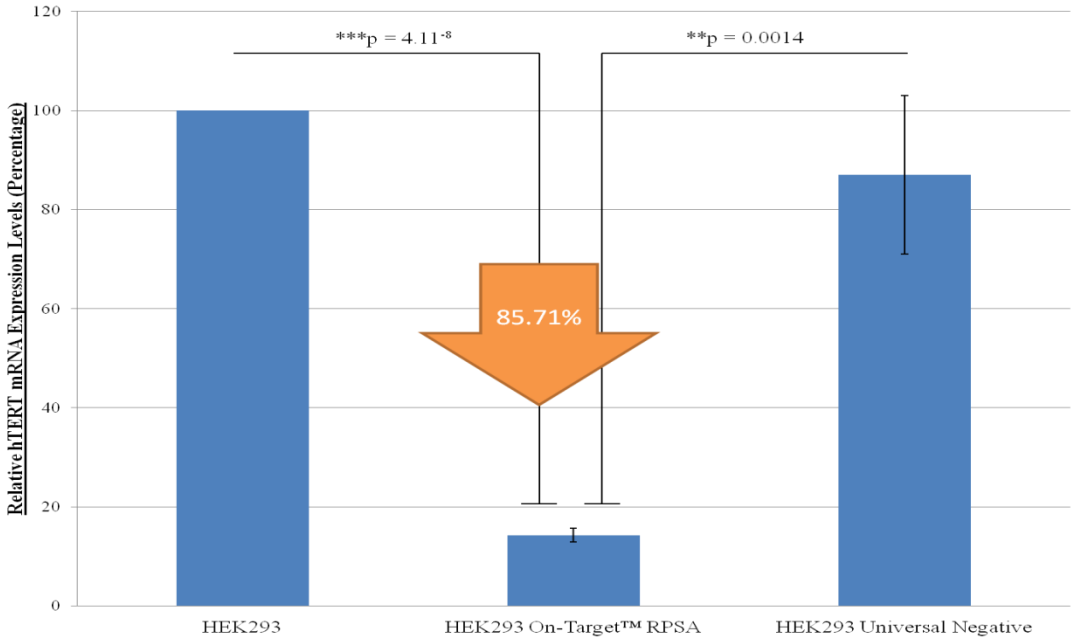

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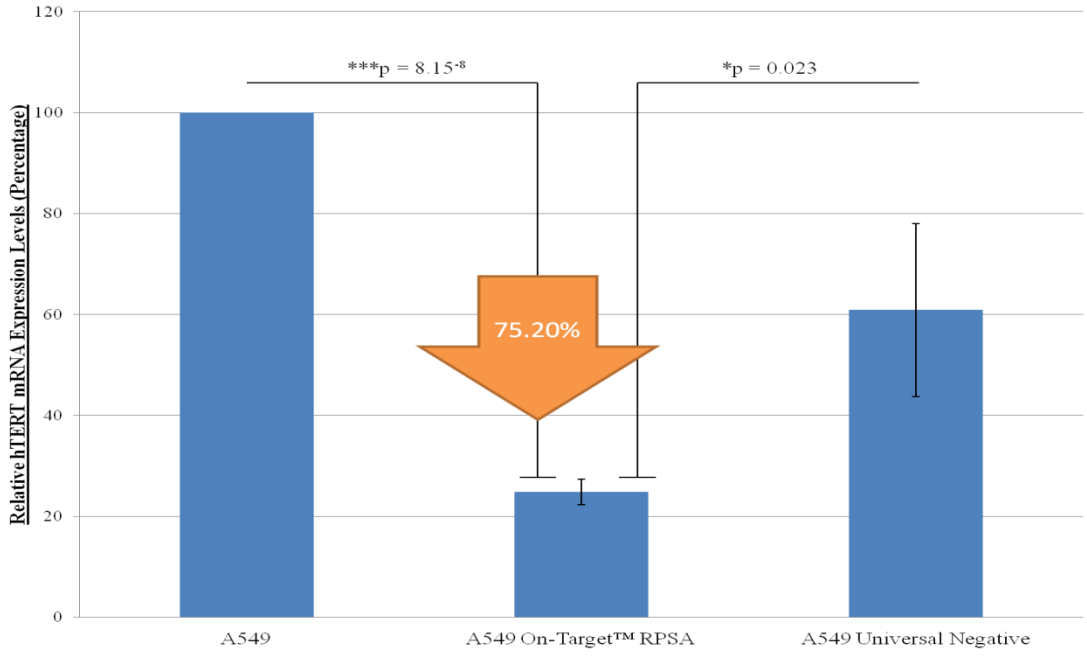

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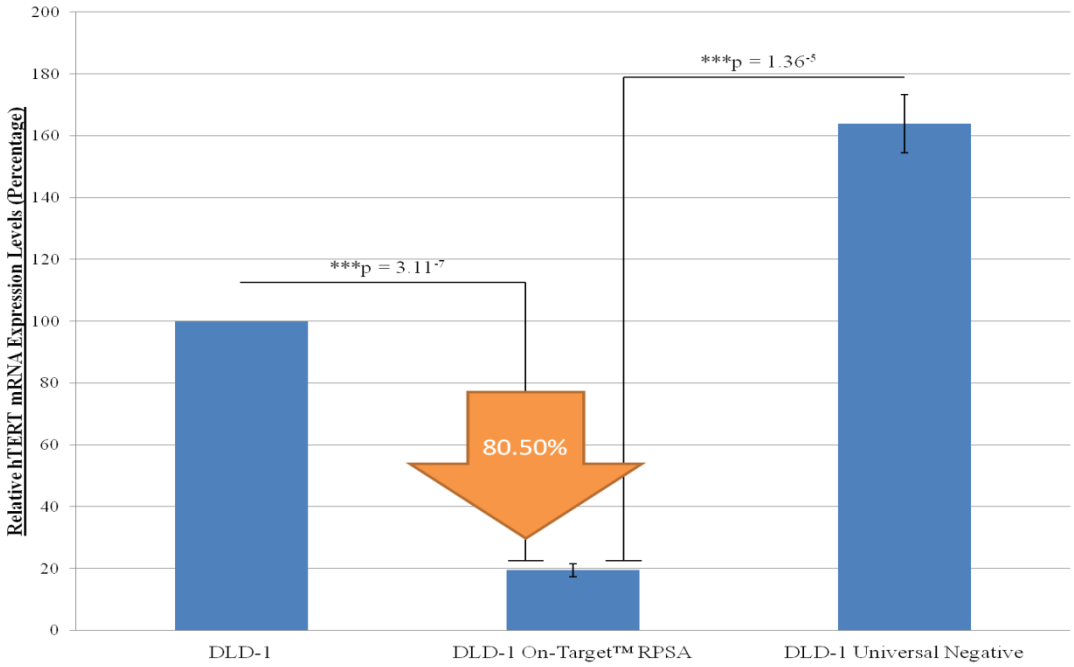

Figure 12: Knockdown of LRP/LR induces a subsequent decrease in hTERT mRNA expression levels. (A) There is a significant decrease of 85.71% in hTERT mRNA levels between in the untreated HEK293 and On-Target™ RPSA treated HEK293 cells. Also, there is a significant decrease when comparing the On-Target™ RPSA and Mission® Universal Negative. (B) With the A549 cells, there is a significant 75.20% decrease in hTERT mRNA levels when comparing the untreated and On-Target™ RPSA treated cells. Moreover, A significant decrease is also shown between On-Target™ RPSA and Mission® Universal Negative. (C) The DLD-1 cell line exhibited a significant 80.50% decrease in hTERT mRNA levels when comparing the untreated and On-Target™ RPSA. Also, there was a significant decrease between On-Target™ RPSA and Mission® Universal Negative (n=6).

Figure 12 indicates the hTERT mRNA levels after On-Target™ RPSA mediated knockdown of LRP in HEK293, A549 and DLD-1 cells. An 85.71%, 75.20%, and 80.50% reduction in hTERT mRNA levels was observed for HEK293, A549, and DLD-1 cells respectively. LRP/LR is known to localise in the nucleus where it has been hypothesised to play a role in nuclear structure and translation (Sato et al., 1996, Vana et al., 2009). Since LRP/LR plays a role in nuclear structure, it could potentially play a role in transcription in addition to translation. This could be a possible explanation as to the decrease in hTERT mRNA levels after On-Target™ RPSA treatment.

5.5 Cellular Viability Decreases Upon LRP/LR Knockdown

Further investigation of the On-Target™ RPSA treatment lead to determining whether this treatment affects the cellular viability of tumourigenic cell lines. Figure 13 indicates cellular viability for HEK293, A549, and DLD-1 cells via the MTT Assay.

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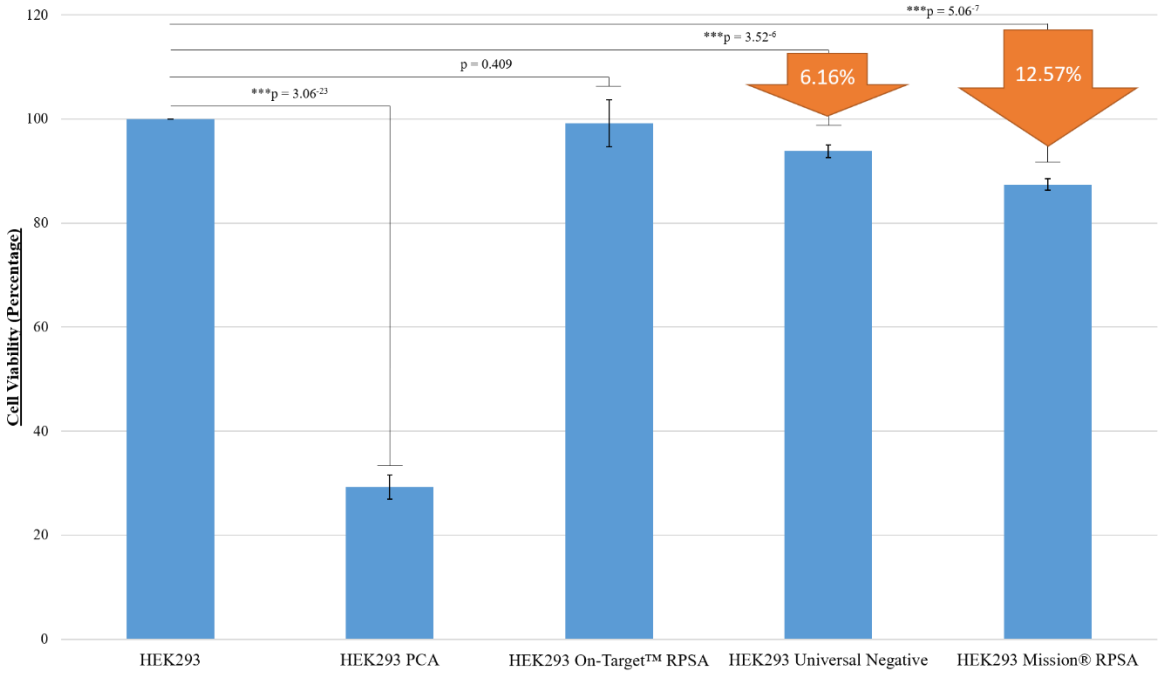

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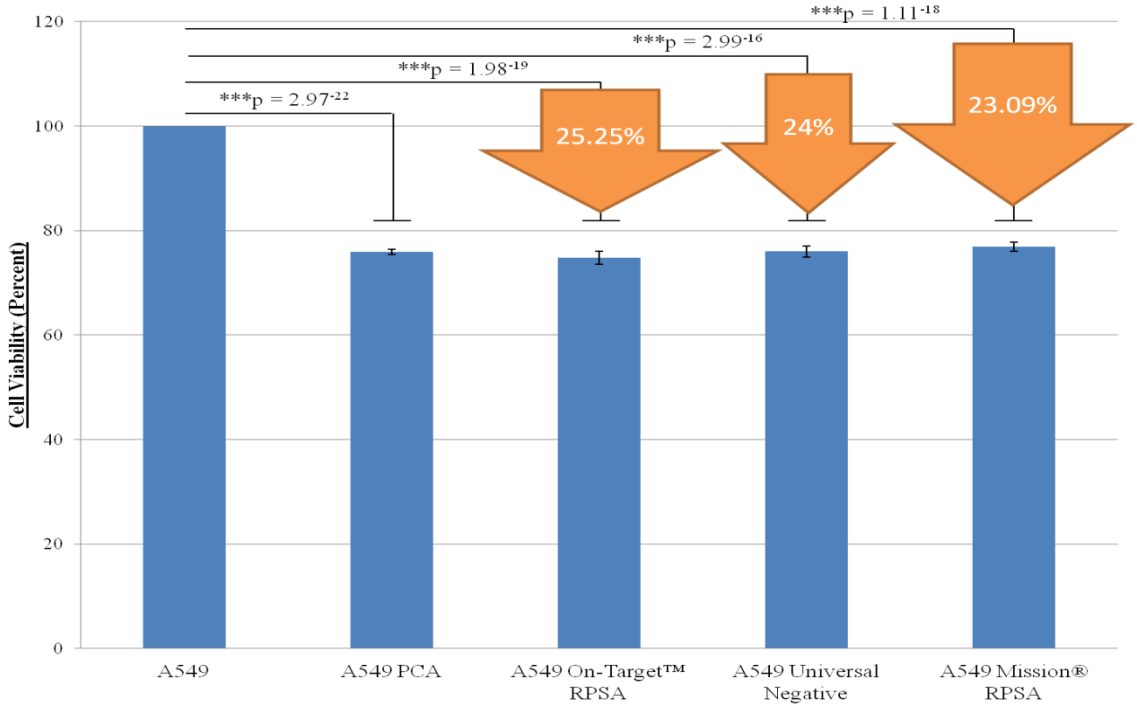

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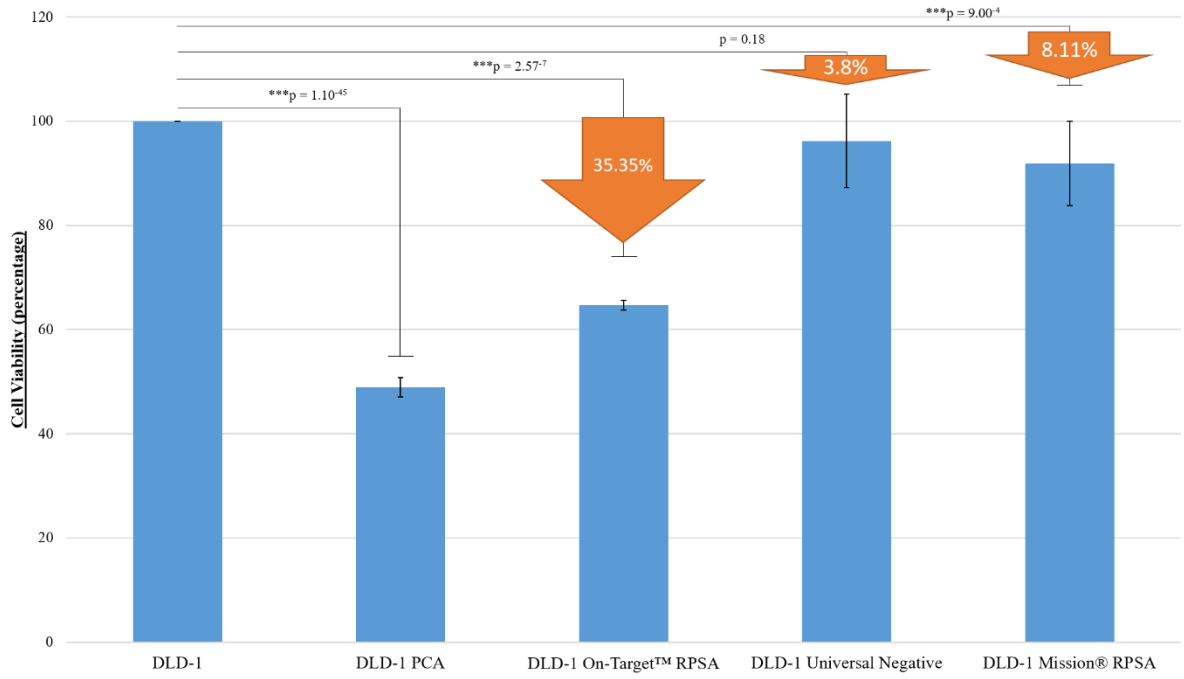

Figure 13: LRP/LR knockdown via RPSA treatments significantly decreases cellular viability in HEK293, A549, and DLD-1 cells. (A) There is no significant difference in cellular viability between untreated HEK293 cells and On-Target™ and Mission® RPSA treated HEK293 cells. (B) A significant decrease in cellular viability between untreated A549 cells and On-Target™ RPSA and Mission® RPSA treated cells can be observed with a 25.25% and 23.09% decrease, respectively. (C) A significant decrease between untreated DLD-1 cells and On-Target™ RPSA and Mission® RPSA treated cells can be observed with a 35.35% and 8.11% decrease, respectively. The PCA was used as a positive control in order as it is known to induce apoptosis in cells (n=4).

The insignificant difference in the HEK293 cells after On-Target™ RPSA treatment indicates the treatments has little effect on non-tumourigenic cells in comparison to tumourigenic cells. Although there is a significant difference between the untreated and Universal Negative Mission® RPSA treatments, the difference is minute and could possibly be attributed to the cytotoxic effects the transfection reagent is known to have (Kiefer et al., 2004). In A549 cells, treatment with On-Target™ RPSA and Mission® RPSA resulted in a decrease in cellular viability by 25.25% and 23.09%, respectively. In the DLD-1 cells, treatment with On-Target™ RPSA and Mission® RPSA resulted in a decrease of 35.35% and 16.04% in cellular viability, respectively. In A549 and DLD-1 cell lines that the On-Target™ RPSA is more effective in reducing cellular viability in tumourigenic cells than the Mission® RPSA. The PCA was used as a positive control as it is known to induce apoptosis in cells.

6 Discussion

6.1 Western Blotting and Confocal Microscopy Analyses after On-Target™ RPSA Treatment

It was shown via pull-down assay that LRP/LR and telomerase interacts (Naidoo et al., 2015); whether this interaction is direct or indirect has yet to be confirmed (Naidoo et al., 2015). The interaction between LRP/LR and telomerase was confirmed using the pull-down assay (Naidoo et al., 2015). A technique which can be utilised in order to determine direct/indirect interaction is Fluorescence/Förster Resonance Energy Transfer (FRET). This technique allows for more sensitive spatial resolution which, in turn, allows for determination of direct/indirect interactions (Sekar and Periasamy, 2003). Moreover, upon knockdown of LRP/LR via siRNA technology, Naidoo *et al.* (2015) showed that telomerase activity was significantly decreased in MDA-MB-231 breast cancer cells. In this study, this was also observed for HEK293, A549, and DLD-1 cells (Figure 11). Therefore, LRP/LR knockdown via siRNAs can become the basis for a possible novel therapeutic in the treatment of cancer as both LRP/LR and telomerase have been implicated in cancer and carcinogenesis (Jovanovic et al., 2015, Harley and Villeponteau, 1995) Moreover, the siRNA knockdown of LRP/LR is a strong candidate for novel cancer therapeutics as it affects non-tumourigenic cells to a lesser extent and could possibly have less adverse side effects in comparison to chemotherapy. The results clearly indicate a successful knockdown of LRP/LR in HEK293 and A549 cells after On-Target™ RPSA siRNA treatment (Figure 9). This is in line with what has been reported previously on MDA-MB-231 breast cancer cells (Naidoo et al., 2015), melanoma cells (Rebelo et al., 2018), pancreatic cancer cells and neuroblastoma cells (Chetty et al., 2017) as well as colorectal carcinoma cells (Vania et al., 2018). Confocal microscopy likewise showed a decrease in LRP/LR after On-Target™ RPSA siRNA treatment in HEK293, A549, and DLD-1 cell lines (Figure 10). Since it has previously been shown that downregulation of LRP caused a decrease in telomerase activity, confocal microscopy was furthermore utilized to determine the effect of siRNA mediated downregulation on hTERT levels. A decrease in hTERT was also observed for HEK293, A549, and DLD-1 cells after On-Target™ RPSA siRNA treatment (Figure 10). Since LRP/LR and telomerase are known to interact, this decrease is likely to be a result of the knockdown of LRP/LR. The 2D-Cytofluorograms for the untreated HEK293, A549, and DLD-1 cell lines all show strong co-localisation between LRP/LR and hTERT (Figure 10). The 2D-Cytofluorogram is constructed using the fluorescent intensities of each protein and overlapping

them, as seen in the Merged image (Figure 10), to create a diagonal to indicate co-localisation of the proteins. This indicates that these two proteins occupy the same compartments within the cells. The 2D-Cytofluorogram for the Universal Negative treatment also indicates co-localisation between LRP/LR and hTERT, although the signal is not as strong as that of the untreated. There is certainly a large decrease in the intensity of the 2D-Cytofluorogram after On-Target™ RPSA siRNA treatment (Figure 10). The decrease in the 2D-Cytofluorogram intensity further supports the evidence of the decrease of LRP/LR and subsequent decrease of hTERT fluorescent signals. The signals of both LRP/LR and hTERT had decreased substantially and this is further supported by the reduction in the 2D-Cytofluorogram co-localisation. However, whether the decrease in hTERT is purely due to the interaction with LRP/LR being disrupted, perhaps at the transcriptional or translational level, is not yet known. LRP/LR has been known to play a role in translation by interacting with ribosomal complexes (Auth and Brawerman, 1992, Pflanz et al., 2009, Gauczynski et al., 2001). One possibility is that after the knockdown of LRP/LR, the interaction between LRP/LR and the ribosomal complex is disrupted, therefore preventing the translation of the hTERT mRNA resulting in a decrease in hTERT protein levels. Another interesting observation after siRNA treatment was the decrease of fluorescence of the blue nuclear staining (DAPI). LRP/LR has been known to interact with chromosomes to stabilise the nuclear structure (Khumalo et al., 2015, Salama et al., 2001). Therefore, by knocking down LRP/LR this stabilisation is interrupted and the nuclear structure is comprised. This is possibly the reason for the decrease in nuclear staining after On-Target™ RPSA siRNA treatment (Figure 10).

There have been previous studies to suggest that the effect of siRNA treatments are not completely due to the siRNA treatment itself (Kiefer et al., 2004). It is vital to note that the transfection reagents are cytotoxic to the cells (Kiefer et al., 2004). Therefore, this could have possible off-target effects not attributed to the siRNA treatments. This is important to note as this could potentially affect non-specific genes which could generate skewed results (Jackson et al., 2003). The study performed by Jackson *et al.* (2003) indicated that even though targeted silencing of MAPK14 was still detectable after decreasing the siRNA *MAPK14-1* by 1000-fold, off-target gene regulation was also detected. After looking at temporal gene expression changes in MAPK14, it was determined that off-target gene regulations were not events due to MAPK14 function loss (Jackson et al., 2003).

The characteristic of cells and their proliferation profile is important for the efficiency of siRNA transfections (Rubis et al., 2013). The HEK293 and A549 both grow in a dispersed

manner whereas the DLD-1 cells have a high propensity to grow in colonies. These can be observed in Figure 10 where the DLD-1 cells aggregate and single cells cannot be identified. Due to this characteristic, the DLD-1 cells have lower transfection efficacy and it has been observed previously that siRNA functioning and efficacy is dependent on cell type (Rubis et al., 2013). Possibly this is a reason as to why the transfection efficacy and consistency for DLD-1 is varied. The Dharmacon On-Target™ RPSA is a cocktail of various siRNAs which each target different areas of the LRP mRNA sequence (Dzmitruk et al., 2015). This allows for more efficient and thorough degradation of the LRP/LR mRNA, and subsequently more efficient and thorough knockdown of LRP/LR. The RPSA siRNA nucleotide sequence functions to be complementary to the LRP/LR mRNA nucleotide sequence. Subsequently, this recruits the RISC complex in order to degrade the mRNA via exonuclease activity.

6.2 Decrease in Telomerase Activity after On-Target™ RPSA Treatment

It is widely known that increased telomerase activity is implicated in tumour progression and apoptosis evasion (Artandi, 2002, Counter et al., 1992, Cong and Shay, 2008, Saretzki, 2014). This is due to the fact that telomerase will maintain telomeres in tumourigenic cells at the critically short length. This is a vital process as this allows the tumourigenic cells to continue to proliferate indefinitely and bypass senescence and apoptosis (Shammas et al., 2005). Additionally, it has been shown that loss of hTERT impairs DNA damage response pathways, which include DNA/fragmented chromosome repair (Masutomi et al., 2005). Moreover, the lack of hTERT also induced radiosensitivity suggesting that hTERT is necessary for cancer cell maintenance (Masutomi et al., 2005). It was clearly observed that On-Target™ RPSA siRNA treatment significantly decreases telomerase activity in HEK293, A549, and DLD-1 cells (Figure 11), as previously observed in HEK293 and MDA-MB-231 cells (Naidoo et al., 2015). It is important to note that this treatment not only is effective in MDA-MB-231 breast cancer cells (Naidoo et al., 2015) but in other cancer types as well, namely lung adenocarcinoma and late-stage colorectal carcinoma. A reason for such large decreases in telomerase activity is that telomerase possibly translocates into the mitochondria, as it is known telomerase has extra-telomeric functions (Saretzki, 2014). A possible reason for the translocation of hTERT into the mitochondria is to render the cells less susceptible to mitochondrial DNA damage and subsequently cell death (Santos et al., 2006). Mitochondrial DNA (mtDNA) is circular so therefore contains no telomeres (Van Bruggen et al., 1966). Telomerase will attempt to repair the mtDNA rather than extending telomeres, which deviates

from the primary function of telomerase (Saretzki, 2014, Gordon and Santos, 2010). Possibly the decrease in telomerase activity is also attributed to the direct decrease of LRP/LR after treatment with On-Target™ RPSA siRNA since LRP/LR is known to interact with telomerase (Naidoo et al., 2015). It is likely that the LRP/LR-hTERT complex is vital for telomerase activity and functioning within the cells.

Decreasing telomerase activity via knockdown of LRP/LR is important in the context of cancer. More importantly, knockdown of LRP/LR could have a multi-pathway effect in the treatment of cancer; this includes a decrease in telomerase activity as well as an increase in caspase activation (Naidoo et al., 2015, Chetty et al., 2017). Cancer has been known to increase telomerase activity in order to maintain telomeres at the critically shortened state (Bodnar et al., 1998). This allows tumour cells to bypass apoptosis and promote uncontrolled proliferation (Greider and Blackburn, 1989). Therefore, by knocking-down LRP/LR, and subsequently decreasing telomerase activity, tumours are then unable to bypass apoptosis leading to cell death (Greider and Blackburn, 1989, Bodnar et al., 1998). In addition, LRP/LR knockdown furthermore induces apoptosis in tumourigenic cells via caspase activation (Chetty et al., 2017, Moodley & Weiss, 2013).

6.3 hTERT mRNA levels Decreased after On-Target™ RPSA Treatment

In order to determine whether or not LRP/LR regulates hTERT transcription, hTERT mRNA was quantified and analysed via qPCR. It was observed that there was a significant decrease in hTERT mRNA levels after On-Target™ RPSA siRNA treatment (Figure 12). This serves as evidence that LRP/LR plays a role in hTERT transcription. However, it is still not known whether the role played by LRP/LR is directly involved in transcription or whether it serves to recruit other transcription factors. This decrease is important as it shows that LRP/LR knockdown not only regulates hTERT at the protein level, but also regulates hTERT at the transcriptional level. This can be expected because even though LRP/LR is widely known as a cell surface receptor protein, it has been confirmed to localise to the nucleus where it is associated with nuclear structures, as well as the perinuclear compartment (Sato et al., 1996, Naidoo et al., 2015). This is due to LRP/LR being known to interact with chromosomes and stabilise them by anchoring the chromosomes to the nuclear membrane (Khumalo et al., 2015, Salama et al., 2001). Moreover, evidence to support the notion that LRP/LR is involved in transcriptional regulation is the fact that LRP/LR associates with histone proteins H2A, H2B, and H4 (Kinoshita et al., 1998, Zuber et al., 2007). Chromatin Immunoprecipitation (ChIP) is

a possible technique to elucidate if there is a direct correlation between LRP/LR and hTERT mRNA expression.

6.4 Cellular Viability Analyses after On-Target™ RPSA Treatment

It was imperative to determine if the On-Target™ RPSA siRNA treatment affects cellular viability of the tumourigenic cells. Ideally this treatment should be able to reduce the cellular viability of tumourigenic cells so they are not able to proliferate and thereby reduce tumour cell mass. As seen in Figure 13, the On-Target™ RPSA siRNA treatment decreases cellular viability significantly in A549 and DLD-1 cells, but not in non-tumourigenic HEK293 cells. In previous studies utilising the same siRNA, it was also observed that downregulation of LRP significantly decreased cellular viability in melanoma cells (A375 and A375SM) (Rebelo et al., 2018) as well as early-stage colorectal carcinoma (SW-480) (Vania et al., 2018). A cell proliferation assay could possibly be utilised in order to support the findings generated by the cellular viability MTT assay (Adan et al., 2016). Cell proliferation assays function to determine the number of proliferating cells in a sample which gives the indication the efficacy of treatments (Adan et al., 2016). It has been shown that knockdown of LRP/LR increases caspase activation, and subsequently, increases apoptosis in IMR-32, AsPC-1, SW-480, A375, and A375SM cell lines (Chetty et al., 2017, Vania et al., 2018, Rebelo et al., 2018). The main caspases involved are caspase 3, caspase 8, and caspase 9 (Vania et al., 2018, Rebelo et al., 2018). Upon treatment of On-Target™ RPSA siRNA, the activity of all the aforementioned caspases were significantly increased (Vania et al., 2018, Rebelo et al., 2018, Moodley & Weiss, 2013). Moreover, caspase activation had previously been elucidated for A549 (Moodley & Weiss, 2013) and DLD-1 (Vania et al., 2018) and the studies indicated that after On-Target™ RPSA siRNA treatment, caspase 3 activity significantly increased.

Unfortunately, the Mission® Universal Negative also significantly decreases cellular viability in HEK293, A549, and DLD-1 cells. A small but significant decrease could be due to the fact the transfection reagent is cytotoxic to the cells so this could be an off-target effect not attributed to the siRNA treatment (Kiefer et al., 2004, Jackson et al., 2003). Varied ratios of siRNA-to-transfection reagent will need to be tested to reduce the cytotoxic effect of the transfection reagent.

7 Conclusion

The siRNA mediated knockdown of LRP is a strong candidate as a potential new therapeutic for treatment of lung and colorectal cancer, and possibly other cancer types from data provided from previous publications. Although, further testing is required in order to reduce potential off-target effects of the transfection reagent or perhaps attempt other potential transfection reagents for compatibility with the On-Target™ RPSA siRNA treatment. Also, further testing is required in murine models in order to determine the effects this treatment has *in vivo*, most importantly, the effects on non-tumourigenic cells and the organism as a whole. Downregulation of LRP will be performed, *in vivo*, on lung adenocarcinoma or late-stage colorectal carcinoma mouse models. The *in vivo* treatment will involve various deliveries of the RPSA siRNA; this will include RPSA siRNA functionalised to nanoparticles or lentiviral vectors.

8 Acknowledgements

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10 Appendix

Supplementary Table 1: Required volumes for transfections per plating format

		Tube 1: siRNA (µl/well)		**Tube 2: DharmaFECT Transfection Reagent (µl/well)**	
Plating format (wells/plate)	**Surface area (cm^2)**	**Volume of 20 µM siRNA (µl)**	**Serum-free Media (µl)**	**Volume DharmaFECT reagent (µl)**	**Serum-free Media (µl)**
24	2	2.5	47.5	1	49
6	10	10	190	5	45
10 cm Culture Dish	78.5	40	760	15	785

Supplementary Table 2: 10% SDS-PAGE Formula

Reagent	Resolving Gel	Stacking Gel
ddH_2O	4.8 ml	3.65 ml
40% Acrylamide/Bisacrylamide	2.5 ml	625 µl
Resolving Buffer pH 8.8	2.5 ml	-
Stacking Buffer pH 6.8	-	625 µl
10% SDS	100 µl	50 µl
10% APS	100 µl	50 µl
TEMED	5 µl	5 µl

Supplementary Table 3: cDNA Synthesis Cycling Conditions

Temperature (°C)	Time (minutes)	Cycles
25	5	1
42	30	2
42	30	
80	5	1

Supplementary Table 4: Formula of the Master Mix

Component	Volume (µl)
EvaGreen qPCR System-ROX Free Master Mix	5
Forward Primer (10 µM)	0.6
Reverse Primer (10 µM)	0.6
Nuclease-free Water	2.8

Supplementary Table 5: qPCR Cycling Conditions for GAPDH and hTERT

	GAPDH	**hTERT**		
	Temperature (°C)	**Temperature (°C)**	**Duration**	**Cycles**
Pre-denaturation	**95**	95	10 min	1
Amplification	**95**	95	15 sec	45
	60*	62*	30 sec	
Melt Curve	**95**	95	10 sec	1
	65	65	1 min	
	97*	97*	Continuous (5 acquisitions per °C)	
Cool	**37**	37	30 sec	

*Fluorescent data was collected.

Supplementary Table 6: Recipe for Reaction and Control Master Mixes

Component	**Reaction Master Mix**	**Control Master Mix**
5X TRAPeze® RT Reaction Mix	2.5 µl	-
5X TRAPeze® RT Control Reaction Mix	-	2.5 µl
HotStart Taq Polymerase	0.2 µl	0.2 µl
Nuclease-free Water	8.8 µl	8.8 µl
Protein Sample	2 µl	2 µl

Supplementary Table 7: qPCR Cycling Conditions for Telomerase Activity

	Temperature	**Duration**	**Cycles**
Pre-Incubation	30 °C	30 min	1
	95 °C	2 min	
Amplification	94 °C	15 sec	45
	59 °C	60 sec	
	45 °C*	10 sec	

*Fluorescent data was collected.

Supplementary Table 8: siRNA Cocktail Mix of Dharmacon On-Target™ RPSA

Name	**Molecular Weight (g/mol)**	**Sequence**
ON-TARGETplus SMARTpool siRNA J-013303-05	13 414.9	CGACAUGAGUUGUACUUCU
ON-TARGETplus SMARTpool siRNA J-013303-06	13 384.9	GAUUGCAUAUCAAAGCAUA
ON-TARGETplus SMARTpool siRNA J-013303-07	13 444.8	GGUCAUGCCUGAUCUGUAC
ON-TARGETplus SMARTpool siRNA J-013303-08	13 384.8	UAUCAUAAAUCUCAAGAGG