



*Targeting LRP/LR for the prevention of tumour formation and metastasis in
lung cancer models*

by

Monique Bignoux

(678040)

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Supervisor: Prof Stefan FT Weiss

Co-supervisors: Dr. Eloise van der Merwe and Prof Mandeep Kaur

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Declaration

I, Monique Jeanette Bignoux, declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



20th day of April 2022 at Johannesburg

Abstract

The incidence and mortality rates of cancer are growing rapidly worldwide, with lung cancer being the most commonly occurring cancer in males. One of the most important considerations in the development of therapeutics for lung cancer is that of early detection and diagnosis, as thereafter, treatments become less effective and later malignant stages become fatal. However, in South Africa, the probability of early diagnosis is extremely low and thus the focal point is the need for effective mechanism-based targeted therapies for the treatment of lung cancer. Cancer is a multi-step process characterised by the hallmarks of cancer and their enabling characteristics. The 37 kDa /67 kDa laminin receptor (LRP/LR) is predominantly a cell surface receptor but also acts intracellularly and is known to be overexpressed in various cancer types. LRP/LR plays a role in numerous cancer hallmarks, including metastasis, angiogenesis, cell viability maintenance, apoptotic evasion and mediating telomerase activity. In this context, LRP/LR provides a putative target for the treatment of multiple cancer hallmarks. Furthermore, the knockdown of LRP/LR with LRP-specific siRNA technology significantly impedes adhesion and invasion, induces apoptosis and impedes telomerase activity *in vitro*. Therefore, this current study included an investigation into the effect of downregulating LRP/LR with LRP-specific siRNA on reducing cell viability, migration potential, telomerase activity as well as inducing apoptosis in A549 lung cancer cells. The A549 cells exhibited a significant decrease in cell viability, migration potential and telomerase activity, as well as a significant increase in apoptosis after downregulation of LRP/LR. Proteomics analysis further suggested the re-establishment of immune control over the lung cancer cells, a novel facet to LRP downregulation in cancer. This therefore indicates that the approach to target LRP/LR is a potentially powerful treatment against multiple cancer hallmarks. However, it is not feasible to use ‘naked’ siRNA molecules *in vivo* and therefore, an efficient delivery system is required. For this, siRNA can be encapsulated in polymeric polylactic-co-glycolic acid (PLGA), which enables the siRNA to more readily be taken up by cells. Therefore, this current study also included assessing PLGA nanoparticles as a potential delivery vehicle for LRP-specific siRNA. PLGA nanocapsule production was optimised for uniformity and size, thereafter HEK293 and A549 lung cancer cells were treated with varying concentrations of empty PLGA nanoparticles and showed no significant decrease in cell viability or LRP/LR levels when treated with 1-5% empty PLGA nanoparticles for 72 hours. Therefore, signifying that the PLGA nanocapsules are favourable for use as a delivery system.

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~Be fearless in the pursuit of what sets your soul on fire~

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Table of Contents

Declaration.....	2
Abstract.....	3
Acknowledgements.....	4
Financial Aid:.....	4
Research outputs	5
First Author publications (forming part of PhD thesis):.....	5
First and Co-author Publications (Not forming part of PhD thesis):	5
Co-authored Patents:	5
Conferences.....	6
List of Figures	10
List of Tables	11
List of Abbreviations	12
CHAPTER ONE - LITERATURE REVIEW.....	15
1.1 Cancer epidemiology and existing therapeutics.....	15
1.2 Hallmarks of cancer	16
1.2.1 Sustained proliferative signalling	17
1.2.2 Evading growth suppressors	19
1.2.3 Resisting cell death	20
1.2.4 Enabling replicative immortality	23
1.2.5 Inducing angiogenesis.....	26
1.2.6 Activating invasion and metastasis.....	27
1.2.7 Emerging hallmarks	29
1.2.8 Enabling characteristics	31
1.2.9 Current cancer therapeutics.....	32
1.3. The 37 kDa Laminin Receptor Precursor/ 67 kDa high affinity Laminin Receptor (LRP/LR) and its role in cancer	34
1.3.1. The role of LRP/LR in metastasis.....	34

1.3.2. The role of LRP/LR in angiogenesis	36
1.3.3. The role of LRP/LR in apoptosis	38
1.3.4 Alternative small molecule inhibitors targeting LRP/LR	40
1.4. Strategy for targeting LRP/LR to impede cancer hallmarks <i>in vivo</i>	41
1.4.1. Limitations to using siRNA as a therapeutic	41
1.4.2. Biodegradable polylactic-co-glycolic acid nanoparticles as a delivery system	41
1.5. Rationale and scope of the study	44
1.6. Aims and Objectives	45
1.6.1. Aim 1	45
1.6.2. Aim 2	45
CHAPTER TWO - EXPERIMENTAL TECHNIQUES	46
2.1. Cell culture	46
2.2. Poly-lactic-co-glycolic acid (PLGA) nanoparticle production and characterisation	47
2.2.1. PLGA nanoparticle synthesis: Double emulsification-solvent evaporation technique	47
2.2.2. PLGA nanoparticle purification and storage	48
2.2.3. PLGA nanoparticle characterisation	49
2.3. Treatments and transfection	49
2.3.1. Treatment of cells with empty PLGA nanoparticles	49
2.3.2. Transfection with LRP-specific siRNA	50
2.4. Protein extraction	51
2.5. Protein quantification: Bicinchoninic acid (BCA) assay	51
2.6. SDS-PAGE and western blotting	52
2.7. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) cell viability assay	53
2.8. Trypan Blue Exclusion assay	54
2.9. Cell morphology	54
2.10. APOPercentage™ Cell Apoptosis assay	54

2.11. Scratch Motility assay	55
2.12 Telomerase activity assay	56
2.13. Proteomic analysis	59
2.14. Statistical analysis.....	60
CHAPTER THREE - RESULTS.....	62
3.1. Determining the effect of siRNA-mediated downregulation of LRP/LR on multiple cancer hallmarks in an <i>in vitro</i> lung cancer (A549 cells) model	62
3.1.1. Optimising the downregulation of LRP/LR.....	62
3.1.2. siRNA-mediated downregulation of LRP/LR decreases cell viability and induces an apoptotic morphology	64
3.1.3. Downregulation of LRP/LR induces apoptosis	67
3.1.4. Downregulating LRP/LR decreases cell migration potential	69
3.1.5. Downregulating LRP/LR inhibits telomerase activity.....	71
3.1.6. Assessing the effect of downregulating LRP/LR on the proteome of A549 cells	72
3.2. Optimisation and assessment of PLGA nanoparticles as a delivery system.....	80
3.2.1. Optimisation of PLGA nanoparticle synthesis	81
3.2.2. Empty PLGA nanoparticles have no cytotoxic effect in non-tumourigenic cells	85
3.2.3. PLGA nanoparticles have no significant effect on LRP protein levels	86
CHAPTER FOUR - DISCUSSION.....	88
4.1. Determining the effect of siRNA-mediated downregulation of LRP/LR on multiple cancer hallmarks in an <i>in vitro</i> lung cancer (A549 cells) model	88
4.1.1. Downregulation of LRP/LR decreases cell viability by inducing apoptosis	89
4.1.2. LRP/LR downregulation decreases migration potential	91
4.1.3. Downregulation of LRP/LR inhibits telomerase activity	92
4.1.4. LRP/LR downregulation causes differential expression of numerous proteins.....	94
4.2. Optimisation and assessment of PLGA nanoparticles as a delivery system.....	102
CHAPTER FIVE – CONCLUSION.....	108
5.1. Future studies	109

Supplementary results	111
References.....	123
Appendix A – Supplementary information.....	139

List of Figures

Figure 1: Global Lung cancer incidence and mortality rates in 2018.....	15
Figure 2: Existing and emerging cancer hallmarks.....	17
Figure 3: The telomerase complex has two essential components, TERT and TERC	24
Figure 4: Blocking LRP/LR with IgG1-iS18 reduces the adhesive and invasive potential of cancer cells.....	35
Figure 5: Blocking LRP/LR with IgG1-iS18 reduces endothelial tube formation.....	37
Figure 6: The knockdown of LRP/LR induces apoptosis in cancer cells.....	39
Figure 7: Active and passive drug targeting strategies with nanoparticles.....	43
Figure 8: Modified Telomeric Repeat Amplification Protocol for real time quantification of telomerase activity.	58
Figure 9: Brief overview of the study design of Aim 1	61
Figure 10: Brief overview of the study design of Aim 2.	61
Figure 11: Transfection of A549 cells with LRP-targeting siRNA significantly decreases total LRP/LR levels.....	63
Figure 12: siRNA-mediated downregulation of LRP/LR significantly decreases cell viability in A549 lung cancer cells.	65
Figure 13: Downregulation of LRP/LR induces an apoptotic morphology in A549 cells.	66
Figure 14: siRNA-mediated downregulation of LRP/LR significantly increase apoptosis in A549 lung cancer cells.	68
Figure 15: Downregulation of LRP/LR significantly decreases A549 cellular migration.	70
Figure 16: Downregulation of LRP/LR significantly decreases telomerase activity in A549 cells.	72
Figure 17: All dysregulated proteins identified by proteomics analysis.....	74
Figure 18: Identification of interactions between all dysregulated proteins above a 2-fold change and analysed via STRING database.....	76

Figure 19: SEM characterisation of empty PLGA nanoparticles synthesized using poly(vinyl) alcohol as the stabilising agent.	81
Figure 20:SEM characterisation of empty PLGA nanoparticles centrifuged at various speeds for optimisation of purification.	83
Figure 21:Characterisation of optimised empty PLGA nanoparticles.	84
Figure 22:Assessing the cytotoxicity of empty PLGA nanoparticles on non-tumourigenic HEK293 and tumourigenic A549 cells.	86
Figure 23: Treatment of non-tumourigenic HEK293 and tumourigenic A549 cells with empty PLGA nanoparticles does not affect total LRP/LR levels.	87
Figure 24: The MHC class I antigen presentation pathway	97

List of Tables

Table 1: List of the dysregulated proteins above a 2-fold change and their function in biological pathways (Unconnected nodes, Transcription, Nucleotide metabolism, cytokine signalling, Adaptive immune system)	77
Table 2: All downregulated proteins identified via SWATH-MS after downregulation of LRP/LR.....	111
Table 3: All upregulated proteins identified via SWATH-MS after downregulation of LRP/LR.....	113
Table 4: Radioimmunoprecipitation assay (RIPA) lysis buffer	139
Table 5: SDS-PAGE gel (30% acrylamide, 2X resolving and 4X stacking).....	140
Table 6: Electrophoresis tank (Running) Buffer (pH 8.3)	141
Table 7: Transfer Buffer	141
Table 8: Phosphate Buffered Saline (PBS) (pH 7.2/7.4).....	142
Table 9: Western blot stripping buffer (pH 2.2)	142
Table 10: TRAP qPCR Mastermix	142

List of Abbreviations

µg	Microgram
µl	Microlitre
A	Amps
A549	Adenocarcinomic human alveolar basal epithelial cells
AD	Alzheimer's disease
Akt	Protein kinase B
AMPK	5' adenosine monophosphate-activated protein
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
Aβ	Amyloid Beta
BCA	Bicinchoninic acid assay
BSA	Bovine serum albumin
CHAPS	(3-((3-cholamidopropyl)dimethylammonio)-1-chromosome 10
CO ₂	Carbon dioxide
Cu	Copper
DAPI	4',6-diamidino-2-phenylindole
DDR	DNA damage response
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphates
DSB	Double stranded breaks
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol bis(2-aminoethyl)tetraacetic acid
EMT	Epithelial–mesenchymal transition
ERK	Extracellular-signal-regulated kinase
FAK	Focal adhesion kinase
FBS	Fetal bovine serum

g	Gram
gDNA	Genomic DNA
HEK293	Human Embryonic Kidney Cells
HSPG	Heparan sulfate proteoglycan
hTERT	Human Telomerase Reverse Transcription
IAC	Imaging and Analysis Centre, UCT
IL1 α/β	Interleukin 1 alpha/ beta
Kb	Kilobase
kDa	Kilo Dalton
LAMR1	Laminin receptor 1 also known as RPSA
LRP	Laminin receptor precursor protein
LRP/LR	37 kDa Laminin Receptor Precursor/67 kDa High
Affinity Laminin Receptor	
MAPK	Mitogen activated protein kinase
MDA MB231	M.D. Anderson Metastatic breast cancer cells 231
MgCl	Magnesium chloride
MHC	Major histocompatibility complex
ml	Millilitre
mM	Milli-molar
MMU	Microscopy and Microanalysis Unit, Wits University
MRC 5	Medical Research Council cell strain 5
mRNA	Messenger RNA
mtDNA	Mitochondrial Deoxyribonucleic Acid
mV	Milli-volt
NaCl	Sodium chloride
NAD ⁺	Nicotinamide adenine dinucleotide
NF κ B	Nuclear factor kappa-light-chain-enhancer of
ng	Nanogram
PAGE	Polyacrylamide Gel electrophoresis
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline tween
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde

Pol II	RNA polymerase II
PrP ^c	Cellular Prion Protein
PrP ^{sc}	Scrapie Prion Protein
PVDF	Polyvinylidene fluoride
qPCR	Quantitative Polymerase Chain Reaction
RB	Retinoblastoma
RNA	Ribonucleic Acid
RNase	Ribonuclease
ROS	Reactive Oxygen Species
Rpm	Revolutions per minute
RPSA	Ribosomal protein SA
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscopy
shRNA	Small Hairpin RNA
siRNA	Small Interfering RNA
TERC	Telomerase RNA Component
TERT	Telomerase reverse transcriptase
TGFβ	Transforming growth factor beta
t-loop	Telomere-loop
TNFα	Tumor necrosis factor alpha
TRAP	Telomere repeat amplification protocol
Tris-HCl	Tris hydrochloride
V	Volt
v/ v	Volume per volume
x g	Times gravity

CHAPTER ONE - LITERATURE REVIEW

1.1 Cancer epidemiology and existing therapeutics

Cancer is a global burden, and the incidence and mortality rates of cancer are growing rapidly worldwide. The most recent status report on the global cancer burden has reported that there were 18.1 million new cancer cases and 9.6 million cancer-related deaths, worldwide in 2018. [1]. In this report, it is noted that lung cancer is the most common cancer, accounting for 11.6% or 2.1 million reported cases, with lung cancer causing 1.8 million deaths, a staggering 18.4% of total cancer deaths. Thus, making lung cancer the leading cause of cancer deaths globally (Figure 1). In particular, lung cancer is the leading cause of cancer-related deaths in males and is the second leading cause of cancer death in females [1]. Furthermore, nonsmall cell lung cancers (NSCLCs) comprise approximately 85% of the disease and include adenocarcinoma, squamous cell carcinoma as well as large cell carcinoma. While there is a 50% 5-year post-operative patient survival rate when detected in the earlier stages (stage I/II) of NSCLC, more than 50% of patients are only diagnosed at later stages (stage IV) of NSCLC and this is coupled with an appalling 5-year survival rate of only 2% [2].

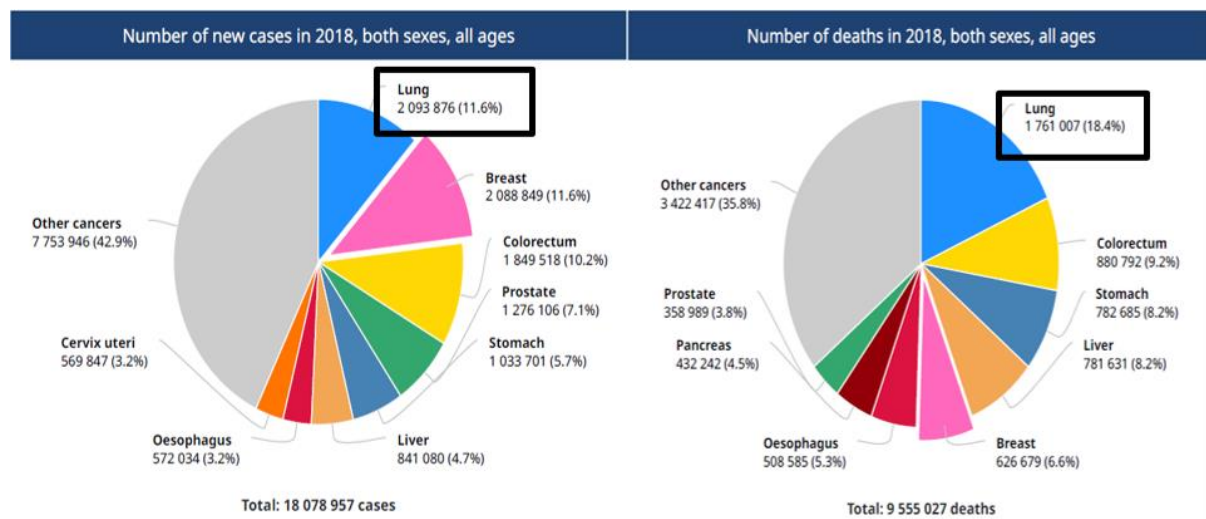


Figure 1: Global Lung cancer incidence and mortality rates in 2018.

Lung cancer accounted for 11.6% of all new cancer cases and 18.4% of all cancer-related deaths reported in 2018 (Adopted from GLOBOCAN, 2018).

Further to this, the global cancer incidence is projected to increase by 70% within the next 20 years. This includes both economically developing countries where cancer is the second leading cause of death, as well as economically developed countries, in which cancer is the

leading cause of death [1]. According to the latest available statistics from The Global Cancer Observatory (GLOBOCAN) for South Africa, there were 8 950 new cases reported and 7 730 lung cancer-related deaths in 2020 [3]. Unfortunately, it is clear that lung cancer statistics in South Africa are severely under-reported, and this is a dilemma faced by many institutions, including The Cancer Association of South Africa (CANSA) (cansa.org.za). The cancer incidence in South Africa is directly correlated with the high HIV/AIDS prevalence, due to the immune-compromised state of those affected [4]. Moreover, as South Africa is a low-middle income country and is therefore an economically developing country, there are many factors attributed to the ever-increasing rise in cancer cases, as well as the inaccurate statistics. These include low awareness, population growth, lack of education, access to only basic healthcare, as well as understaffed medical facilities and a lack of resources [5].

In the context of lung cancer, the most important consideration is that of early detection and diagnosis, as thereafter, treatments become less effective and later malignant stages become fatal [6]. However, in South Africa, the probability of early diagnosis is extremely low due to the aforementioned reasons and thus the focal point is the need for effective mechanism-based targeted therapies for the treatment of lung cancer.

1.2 Hallmarks of cancer

Cancer is a multi-step process, ultimately occurring as a result of the key biological processes, known as the hallmarks of cancer. These hallmarks include: sustained proliferative signalling, evasion of growth suppressors, resistance to cell death, replicative immortality, angiogenic induction and the activation of invasion and metastasis (Figure 2) [7]. The progress made in understanding these mechanisms of cancer pathogenesis has brought about the development of mechanism-based targeted therapeutics. In principle, this approach enables the development of hallmark-targeting cancer drugs that are directed toward particular molecular targets. This allows the drug to provide inhibitory effects on the related hallmark, with fewer off-target effects. However, developments targeting pathways relating to a single hallmark have had transitory responses, resulting in relapse. It is suggested that when there is an inability to depend on the capability of one specific hallmark, the cancer cells tend to depend more on another and this is an acquired form of drug resistance [8]. Therefore, it is of particular importance to produce multi-mechanism-targeting-based treatment strategies which deliver inhibitory effects on more than a single hallmark.

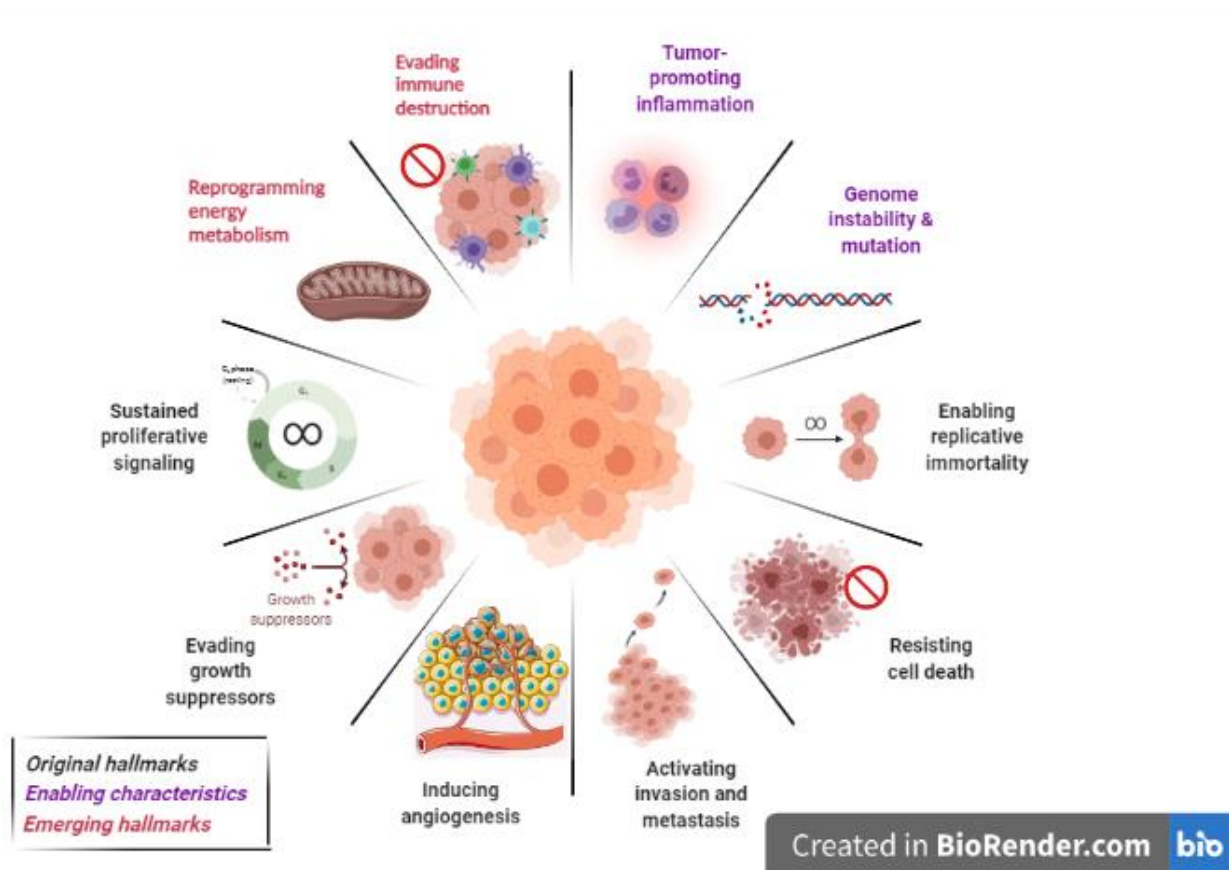


Figure 2: Existing and emerging cancer hallmarks.

A considerable amount of progress has been made in understanding the six original cancer hallmarks which has brought about the emergence of two additional hallmarks (Reprogramming energy metabolism and evading immune destruction) as well as enabling characteristics (Genome instability and mutation and Tumour-promoting inflammation) involved in the support of multiple hallmarks. The emerging hallmarks include the ability of some, if not all, cancers to modify cellular metabolism for the support of tumourigenic proliferation most effectively, as well as to evade destruction by the immune system for continued progression through the neoplastic process. Further to this, characteristics enabling the acquisition and support of multiple core and emerging hallmarks drive neoplastic progression through genome instability and mutation, as well as through the tumour-promoting consequences of inflammation caused by innate immune cells [7] (Created with BioRender.com).

1.2.1 Sustained proliferative signalling

Perhaps one of the most important characteristics of cancer cells is their ability to continuously proliferate. Under normal conditions, tissues produce and release signals promoting entry into the cell cycle in an exceptionally careful and controlled manner. This ensures the fundamental balance in cell number to maintain normal tissue function and structure. However, in cancer cells these signals are dysregulated, thereby enabling the cells to continuously progress through

the cell cycle [7], even in the presence of processes which would normally cause senescence or cell death. There are several different ways in which cancer cells gain the ability to maintain proliferative signalling. They are able to stimulate autocrine proliferative signals by producing growth factor ligands which then activate proliferative pathways by expressing cognate receptors on the same cell [7]. On the other hand, the cancer cells may be capable of stimulating paracrine proliferative signals from normal cells within the surrounding tissue. Such as when transforming growth factor β (TGF- β) is deleted in the cancer cells, they stimulate secretion of hepatocyte growth factor (HGF) to promote proliferative signalling and metastasis [9, 10]. Cancer cells are also capable of becoming hyper-responsive to limiting amounts of growth factor ligands by simply increasing cell surface receptor levels. Further to this, the cancer cells can be enabled to facilitate receptor activation, independently of cognate ligands, by simply modifying the structure of the receptor to be constitutively activated [7].

This independence of ligand-mediated receptor activation can be further acquired by the constitutive activation of downstream signalling pathways, counteracting the need for receptor activation [7]. Approximately 40% of human melanomas encompass mutations which affect the B-Raf protein structure, to result in constitutive activation of the Raf- mitogen activated protein kinase (MAPK) pathway [11]. In a similar manner, activating mutations have been detected in a multitude of different tumour types, whereby various isoforms of the catalytic subunit of the phosphoinositide 3-kinase (PI3K) have been found. In particular, mutations in PI3K have been observed to appear commonly in NSCLCs [12]. These activating mutations in PI3K then cause hyperactivation of the PI3K-Akt signalling cascades [13, 14].

Additionally, disruptions in negative-feedback mechanisms are able to enhance proliferative signalling, without the need for hyperactivation of the signalling abilities of a protein. One such example is the Ras oncoprotein, whereby the negative-feedback mechanism, which usually ensures that there is transient activation of signal transmission, is constitutively activated by oncogenic mutations in *ras* genes due to enhanced GTPase activity [15]. The *KRAS* isoform of the oncogene is one of the most commonly identified mutations in lung cancer [16] and is frequently identified as a co-mutational partner of TP53 (discussed in 1.2.2) [17]. Additionally, a comparable example of a defect in negative-feedback mechanisms includes mutations in PTEN phosphatase. Mutations which cause a loss-of-function in PTEN result in amplification in PI3K signalling due to a lack of degradation of phosphatidylinositol triphosphate (PIP₃),

thereby promoting the tumourigenic process [13, 14]. Altogether, indicating the role of these mutations in constitutive activation of growth, proliferation and survival pathways.

1.2.2 Evading growth suppressors

In addition to both the induction and maintenance of signals stimulating growth, cancer cells need to concurrently bypass growth suppressing signals. Tumour suppressor genes exist to do exactly as their name implies and restrict cell growth and proliferation, in numerous ways, to inhibit tumourigenesis. The quintessential examples are the retinoblastoma associated (RB) and TP53 proteins, which serve as the principal control centres of two vital integrative regulatory pathways that control whether cells continue to proliferate or alternatively enter senescence or apoptotic pathways [18]. Although these pathways are complex and context-dependent, the RB pathway is predominantly involved in transduction of extracellular signals, whereas TP53 is principally involved in receiving intracellular signals indicating stress or abnormalities [18]. Whilst RB focuses on the cell cycle regulation by transduction of signals for growth inhibition, TP53 can arrest cell-cycle progression until conditions have normalised when conditions are substandard, or trigger apoptosis when the damage is irreparable [18]. Under normal physiological conditions, p53 is modulated by mdm2, whereby the activation of p53 by cellular stresses eventually induces transcription of mdm2, which then binds to p53 to promote its degradation. However, during the tumourigenic process, cellular responses to p53 are dysregulated due to loss of p53 function (either through mutations or alteration of p53 inhibitors) [19]. A protein that has been seen to be involved in the accumulation of p53 and pRb, is the promyelocytic leukaemia protein (PML). PML mediates nuclear transport of these proteins and is therefore crucial in their apoptotic activity. In particular, PML is able to protect p53 from mdm2-mediated degradation and inhibition [20]. Cancer cells with irregularities in either of these pathways, therefore, lack critical components of growth suppression. Indeed, it has been observed that Rb is inactivated in numerous cancer types [21, 22], including lung cancer, where it is associated with a very poor prognosis [23]. Additionally, TP53 has been found to be mutated in more than 50% of tumours that have been sequenced [24], with these mutations being described as one of the most significant events in lung cancers resulting in a poor prognosis, as well as chemotherapeutic resistance [25].

Further to this, the evasion of contact inhibition is an additional way in which cancer cells manage to evade growth suppression mechanisms. The phenomenon of contact inhibition

exists such that normal tissue homeostasis is maintained. Contact inhibition exists in two different modes, contact inhibition of locomotion (CIL) and contact inhibition of proliferation (CIP). Contact inhibition is the process whereby non-tumourigenic cells grown *in vitro* migrate until the cells encounter one another. Once the cells have come into contact with each other, the cells either stop their migratory process (CIL), or stop proliferating (CIP), depending on the context of the collision [26]. Although this has been studied quite extensively *in vitro*, there is little information on the mechanism *in vivo*, it is simply expressed that the modelled mechanisms of contact inhibition *in vitro* serve as a substitute of a mechanism which exists *in vivo*. This is clear, in that cells excised from a tumour and grown *in vitro*, do not exhibit this phenomenon and instead continue to grow as if no contact had been made [27].

It has more recently been established however, that this process occurs as Merlin, the product of the neurofibromatosis type 2 (*NF2*) tumour suppressor gene, is involved in mediating the connection between adhesion molecules on the cell surface and receptor tyrosine kinases in the transmembrane region. An example of this, is the coupling of E-cadherin to the EGF receptor, whereby Merlin strengthens the adhesion of the cell-cell attachments mediated by cadherins and then further limits the ability of growth factor receptors to send mitogenic signals [28, 29]. An additional example is the LKB1 protein, which is involved in epithelial cell polarity, whereby it plays a role in epithelial structure organisation and maintaining tissue integrity. When LKB1 is upregulated in senescent epithelial structures, the proliferative abilities of the Myc oncogene are counteracted. However, when LKB1 is downregulated, the integrity of epithelial structures is weakened, and the cells become vulnerable to transformation induced by Myc [30, 31]. Interestingly, there is a high rate of inactivating mutations in LKB1 found in NSCLCs, which often co-exist with activating mutations in *ras* [32, 33]. This therefore shows how these mechanisms of creating and maintaining complex tissues are vital ways in which the cells suppress and counterbalance improper proliferative signals.

1.2.3 Resisting cell death

Apoptosis

Once the tumour has progressed beyond constitutive proliferative signalling and evasion of growth suppressors, the cells become largely genetically unstable as checkpoints have been avoided and important regulators have been lost. Under normal circumstances, the cells would undergo stress induced programmed cell death (apoptosis), however, many tumours that have

reached this point of malignancy are then able to evade the apoptotic process altogether and continue to a higher-grade malignancy. Therefore, the intricate balance between cell proliferation and cell death is disrupted in cancer, and normal tissue homeostasis is not maintained [34]. There are two key apoptotic pathways, the death receptor (extrinsic) and mitochondrial (intrinsic) pathways, which converge at the same point. The extrinsic pathway involves caspase-8 activation, while the intrinsic pathway involves caspase-9 activation. The extrinsic signalling pathways involve ligands (eg. FASL and TNF- α) binding to transmembrane death receptors (eg. FASR and TNFR1), which contain cytoplasmic death domains that transmit the death signal from the cell surface to intracellular signalling pathways [35]. These extracellular signals result in the recruitment of FADD, which associates to pro-caspase-8 to induce formation of a death-inducing signalling complex (DISC), ultimately resulting in the activation of the initiator caspase-8, to trigger apoptosis [36]. Whereas the intrinsic signalling pathways involve a diverse array of non-receptor-mediated, intracellular signals that cause loss of mitochondrial membrane potential, resulting in the release of pro-apoptotic proteins (eg. Cytochrome c and Smac/DIABLO) into the cytoplasm [37]. One example of this includes release of Cytochrome c, which can then bind to and activate Apaf-1 and pro-caspase-9 to form an apoptosome, leading to activation of the initiator caspase-9 [38]. However, both of the key pathways both lead to activation of the execution pathway, which is initiated when either pathway leads to caspase-3 activation [39]. Considered to be the most important of the executioner caspases, caspase-3 induces disintegration of the cell into apoptotic bodies and activates the CAD endonuclease, which can then degrade DNA and cause chromatin condensation [39]. On a biochemical level, cells undergoing apoptosis then exhibit protein cross-linking and cleavage, breakdown of DNA and cell surface presentation for phagocytic recognition. On a morphological level, early apoptotic cells present cell shrinkage, with chromatin condensation, while late apoptotic cells then present with membrane blebbing and cell fragmentation into apoptotic bodies. The apoptotic bodies are then phagocytosed so as to avoid inflammatory responses. Furthermore, the lack of the inflammatory response is because the apoptotic cell constituents are not released into the surrounding tissue, but rather quickly undergo phagocytosis with no inflammatory cytokines being released by the engulfing cells [40, 41]. Importantly, the immune system plays a key role in inducing apoptosis, whereby CD8⁺ cytotoxic T lymphocytes can trigger the extrinsic pathway [42] as well as induce apoptosis in virus infected cells and tumour cells via a caspase-independent (perforin/granzyme) pathway [43].

One common example of cancer gaining the ability to evade apoptotic responses, is by the inactivation of the TP53 tumour suppressor, through various mutations. TP53 serves as a DNA damage sensor, whereby extensive DNA damage and chromosomal aberrations cause TP53 to upregulate expression of pro-apoptotic proteins, such as the BH3 only proteins (eg. BAX and BAK) within the BCL-2 family. Thus, when TP53 is inactivated, intrinsic apoptosis is not triggered [44]. Additionally, cancer cells are able to circumvent apoptotic processes through downregulating pro-apoptotic factors such as Bax, by upregulating expression of anti-apoptotic proteins such as Bcl-2, or through the increased expression of pro-survival factors such as insulin-like growth factor 1/2 [45]. The abundance of mechanisms used by cancer cells to avoid apoptosis has shown how malignancy can evolve in a plethora of ways and further illustrates the complexity of the malignant state.

Autophagy

Apoptosis, however, is only one mode of cell death and does not include other aspects such as autophagy and necrosis. Autophagy is a catabolic degradation process, an additional means by which tissues maintain cellular homeostasis. Paradoxically to apoptosis, however, autophagy appears to play a much larger role in cancer cell survival, rather than being tumour suppressive in nature [46]. The process of autophagy enables stressed cells or organelles to be broken down and recycled. Cellular organelles are enveloped in autophagosomes, which can then fuse with lysosomes, to break the vesicle contents down into lower molecular weight catabolites. These are then used by cells to support their survival when they are stressed as well as in nutrient deficient environments [47]. This process can serve as a tumour suppressive mechanism by eliminating damaged organelles, oncogenic proteins and misfolded proteins. However, once a tumour has been established, autophagy tends to increase and plays a role in promoting cancers, whereby the process supplies those catabolites for metabolism to maintain mitochondrial function, therefore, indicating how autophagy can be used to mediate cancer cell survival (reviewed in [48]). One protein known to be involved in the tumour suppression function of autophagy is Beclin 1, an important protein in phagophore formation that has been found to be downregulated in various cancers [49]. This loss of Beclin 1 leads to a reduction in autophagy and increased cell proliferation, indicating its tumour suppressor function [50]. However, as a regulator of tumour promotion, it has been shown that autophagy is increased in various cancer cells, including colon, pancreatic and lung, containing RAS activating mutations to enhance tumour growth and survival [51]. This indicates that autophagy is a vital component involved

in tumour inhibition, and that dysregulation in autophagy is a crucial mechanism in tumour promotion.

Necrosis

In contrast to autophagy and apoptosis, necrotic cell death causes the cells to burst, thereby releasing the cell contents into the surrounding microenvironment in the tissue, rather than the cell doing so in a precisely organised manner so as not to stress neighbouring cells. This then causes the release of pro-inflammatory signals into the local microenvironment, which results in the recruitment of inflammatory cells from the immune system (such as macrophages) [52]. This consequently allows those cells to examine the degree of damage caused and eliminate necrotic debris. Although necrosis does appear to be an indiscriminate process, there are regulatory factors which influence this process under certain circumstances [53]. This regulated form of necrosis has been termed necroptosis [53, 54]. In particular, necroptosis is involved in both embryonic development and maintaining adult tissue homeostasis and can be induced via activation of certain receptors in the plasma membrane, in addition to regulation by genetic factors [55]. Furthermore, necroptosis can occur when caspases become inactivated, thereby shifting the cells from apoptosis, either into a mixed necrotic and apoptotic morphology or into a complete necrotic modality [53]. In this way, tumour cells are able to switch from apoptosis and use this immune activating process in a way which promotes tumour progression. Moreover, the immune inflammatory cells have the ability to promote the angiogenic (*see 1.2.5 below*) and invasive (*see 1.2.6 below*) processes as well as enhance cancer cell proliferation. In this case, the necrotic cells are capable of stimulating surrounding cells to proliferate, by recruiting the inflammatory cells which subsequently release growth-stimulating factors, such as IL-1 α , thereby facilitating the progression of the neoplasm [52]. Therefore, although it may seem apparent that necrosis would be a means by which to counteract the over proliferation of cancer cells, tolerating necrosis, or switching from apoptosis to necrosis, seems to be more advantageous to invasive tumours by the resultant recruitment of factors which stimulate growth of the surviving cells.

1.2.4 Enabling replicative immortality

Although not a form of cell death, senescence is an additional mechanism of defence against cellular stressors and is intrinsically linked to the shortening of telomeric structures, whereby the cell permanently exits the cell cycle and is unable to divide but remains viable. Evading

senescence is therefore another means by which cells are capable of transforming into a hyperproliferative, immortalised state [56, 57]. This state of unlimited replicative potential consequently allows the cancer cells to produce tumours of a macroscopic nature and is facilitated by an upregulation of telomerase activity [58]. Telomerase is a ribonucleoprotein with reverse transcriptase activity and is principally responsible for elongating telomeres, which are tandemly repeated TTAGGG repeats found capping the ends of chromosomes to aid in preventing chromosomal degradation and the loss of coding DNA [59]. Telomerase is made up of two essential components, namely, in humans: the reverse transcriptase catalytic component, hTERT, which is responsible for the addition of telomeric repeats; and the functional RNA component, hTERC, which serves as the telomeric template (Figure 3). Telomerase activity becomes undetectable in most human somatic cells during early embryonic development, which is attributable to transcriptional repression of hTERT [60]. However, in cancer cells telomerase activity is upregulated to maintain replicative immortality [56].

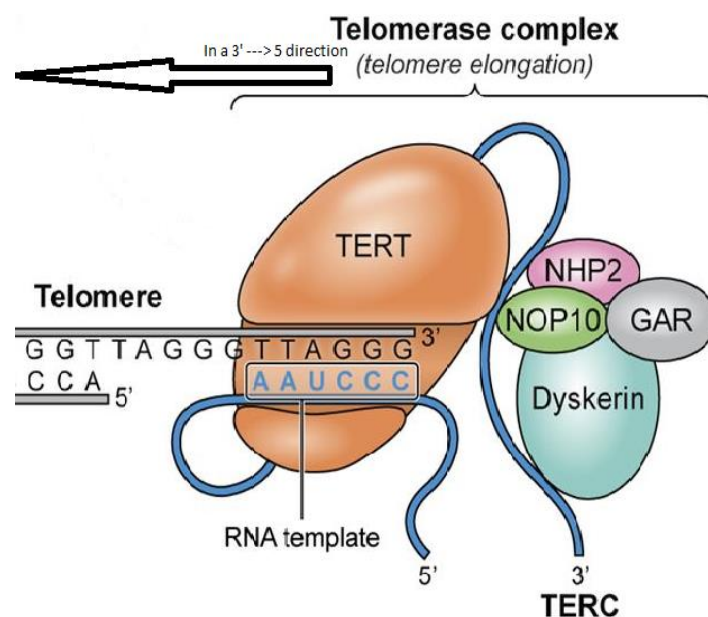


Figure 3: The telomerase complex has two essential components, TERT and TERC

Telomere extension is facilitated by TERT and TERC, which catalyse the addition of the telomeric TTAGGG repeats to the chromosome ends. TERT serves as the reverse transcriptase catalytic component, which adds telomeric repeats by reading the RNA template, TERC. These are displayed in a complex with NHP2, GAR, NOP10 and Dyskerin, which are partly involved in stabilising the complex. (Adapted from [61]).

The role of telomerase in senescence and immortalization

Telomeres have an associated protein complex, known as the shelterin complex, which forms distinct t-loop-like structures to prevent chromosome ends from being recognised as double-stranded DNA breaks [62]. This therefore protects chromosomes from degradation and end-to-end fusions, which would otherwise result in unstable dicentric chromosomes that promote tumourigenesis. Chromosome ends, or telomeres, are predisposed to shorten with each successive cell division, through a process well-known as the “end replication problem”, due to the semi-conservative mechanism of DNA polymerase [63]. To this effect, when telomeres shorten to a critical length in non-immortalized cells, the dysfunctional telomere is recognised as damaged DNA and cellular quiescence is triggered by a DNA damage signal, subsequently causing the cells to go into a state of growth arrest [64]. Cells can remain in this senescent state for years provided no other changes occur and as such, this is considered to be a type of tumour suppressor mechanism in humans.

Conversely, human carcinomas circumvent inhibitory pathways induced by DNA damage, as well as senescence, through the upregulation of telomerase activity [57]. The initiation and maintenance of the senescent state involves cell cycle checkpoint proteins, including p53/p21 and pRB/p16. When these proteins are inactivated, cells can bypass senescence and subsequently proliferate at the expense of genetic stability, leading to cell death. However, cells can survive this by activating telomerase, whereby the cells are then able to maintain the length of these unstable telomeres, after which malignancy occurs [65]. Interestingly however, it has been shown that cancer cells which have escaped quiescence, can be reversed to the senescent state by restoring the tumour suppressor, p53 [66]. Therefore, it is generally believed that telomeres and telomerase are the connection between cellular senescence and immortalization [67]. However, it is important to illustrate that the common trend in neoplastic progression infers the acquisition of telomerase activity in malignant cells is delayed initially, to allow the cells to acquire mutations which promote the tumourigenic process. Subsequent activation of telomerase in the more progressed state of malignancy then allows for stabilisation of the mutated genome and provides the ability to enable replicative immortality [68, 69].

The role of telomerase in apoptosis

There is an increasing amount of evidence to suggest that telomerase is correspondingly implicated in cancer and ageing by actions beyond its function in maintaining telomeres [58]. It is known that the inhibition of telomerase results in apoptosis through the resultant loss of

telomere maintenance. Where, the loss of telomerase activity causes progressive telomere shortening and the telomere capping function of the shelterin complex becomes inactivated, eventually causing cell death [70]. However, the catalytic subunit of telomerase, hTERT, has been found to play a role in regulating apoptosis, independently of telomere maintenance and telomerase activity. It has been demonstrated both *in vitro* and *in vivo*, that there is a direct association between an overexpression of TERT and an increased resistance to apoptosis [71]. Furthermore, this is indicated in that telomerase expression did not prevent stress-induced senescence in normal human fibroblasts, but rather protected the cells from apoptosis [72]. Moreover, it has also been shown that downregulating hTERT, with siRNA specific to hTERT, activates Bax which triggers a mitochondrial cell death pathway. Further, this occurred without apparent telomere erosion as a result of telomerase inhibition [73]. In addition, it has been shown that telomerase suppression induces activation of p53 via PML (interaction described above in 1.2.2), resulting in cell cycle arrest and apoptosis both *in vitro* and *in vivo* [74]. Additional non-canonical functions of TERT include a role in enhancing cell proliferation [75] and DNA damage repair [76].

Telomerase has been detected in 85-90% of malignant human tumours [65], therefore, telomerase has become an attractive target for potential cancer therapeutics. Further to the role in cancer progression, it has even been shown that elevated levels of telomere dysfunction and telomerase activity are associated with cancer recurrence and chemotherapeutic resistance [77]. Moreover, what makes the inhibition of telomerase particularly appealing, is that because most somatic cells do not possess detectable telomerase activity, inhibiting telomerase should have limited off-target effects.

1.2.5 Inducing angiogenesis

Another key cancer hallmark is the process of angiogenic induction by tumour cells [7]. Angiogenesis refers to the process by which new blood vessels form from existing ones. Much like the requirement of normal tissues for oxygen and nutrients, this too is a necessity for tumours. Under normal circumstances, the development of vasculature occurs principally during embryogenesis, after which it enters a predominantly quiescent state. Angiogenesis does occur in adults during wound healing and menstrual cycles; however, this is transient and is switched off when these processes are not occurring. Contrarily, during tumour formation, this angiogenic switch is almost constitutively activated, thus resulting in the growth of new blood

vessels to aid in maintaining the developing tumours [78]. Common angiogenic regulators include the inducer, vascular endothelial growth factor-A (VEGFA) as well as the inhibitor, thrombospondin-1 (TSP-1). Vascular endothelial cells display stimulatory or inhibitory receptors on their cell surface, to which these signalling proteins bind. The VEGF gene codes for multiple ligands involved in angiogenesis, including postnatal and embryonic development, as well as in pathological and physiological circumstances in adults. VEGF signalling can subsequently be activated by hypoxic conditions, as well as oncogenic signals [79]. Alternative pro-angiogenic factors, including family members of the fibroblast growth factor (FGF), are also capable of being overexpressed for the purpose of maintaining tumour angiogenesis [80]. TSP-1 however, provides an important compensatory mechanism, whereby it binds to the inhibitory receptors in the transmembrane to suppress the pro-angiogenic signals, effectively turning the angiogenic switch off [81]. Therefore, if TSP-1 is not present at sufficient levels, or is mutated to be ineffective, it is not able to adequately inhibit the angiogenic process.

Vasculature observed in tumours is generally abnormal, whereby there are complex and excessive branching networks of blood vessels with advanced capillary sprouting, distortions and enlargements in vessels, irregular blood flow, leaking and microhaemorrhages as well as aberrant cell proliferation and apoptosis of the endothelial cells [82]. Although intuitively this process would seem to only be important for established tumours, it has been shown that angiogenesis is induced much earlier on in neoplastic progression than expected and occurs in pre-malignant phases of tumour development [83]. Therefore, solidifying the integral status of angiogenesis as a hallmark of cancer.

1.2.6 Activating invasion and metastasis

The metastatic process is perhaps the most detrimental of all the hallmarks as it signifies the later stages of tumourigenesis that are specifically complex to treat and is often coupled with a very poor prognosis. The invasion-metastasis cascade is the multi-step process of invasion and metastasis, whereby there are successive cell-biological changes, which begin with local invasion and ultimately result in the growth of secondary macroscopic tumours at a site distant to the original tumour. The process starts with local invasion through the basal lamina into the nearby circulatory system (intravasation), where the cancer cells subsequently escape the lumina of the blood vessels into the parenchyma of distant tissues (extravasation). The cells are then able to form micro-metastatic lesions that grow into macroscopic tumours [84]. A key

aspect of metastasis is that the morphology of the cancer cells tends to transform quite apparently, due to changes in their attachment to the ECM, as well as to other cells. A frequently observed alteration is the loss of E-cadherin expression. E-cadherin is responsible for forming epithelial cell sheets, as well as for maintaining the senescent state of the cells in the sheets, and is therefore a key suppressor of the invasive and metastatic process [85]. Furthermore, the epithelial-mesenchymal transition (EMT) is the process by which transformed epithelial cells adopt the processes normally used during embryonic development and wound healing. Thereby, allowing the cells to acquire the ability to resist apoptosis, invade, as well as disseminate to distant sites. Therefore, enabling the invasive and metastatic phenotype. The EMT is multi-faceted and quite complex in itself, as it can be activated perpetually or transiently and to varying degrees during the invasive and metastatic process [86]. It can therefore be seen how the invasion and metastatic processes are considered to be a distinct hallmark.

Colonisation and micrometastatic dormancy

Once the process of extravasation has occurred and the cancer cells have disseminated to distant tissues, they then must sufficiently adapt to their new microenvironment in order to effectively colonise or grow into macroscopic tumours. This then imposes further complexity to the metastatic process, as the cancer cells must then adapt to a completely foreign environment where they must undergo a myriad of challenges for this to happen and there are many ways in which this can be achieved. In some cancer types, it is possible for the primary tumour to release suppressing factors systemically, thereby rendering the micrometastases dormant until such a case that the primary tumour is removed, revealing explosive metastatic growth soon after [87]. In other types of cancer, such as melanoma and breast cancer, there have been cases where, decades after the resection of the primary growth, there is an eruption of macroscopic metastases. Therefore, reflecting the presence of dormant metastases which have managed to solve the complexity of tissue colonisation, after much trial and error [88]. Furthermore, the activation of tumour angiogenesis is another hurdle in which to overcome, as it has been shown that some dormant micrometastases were incapable of forming macroscopic tumours due to their inability to activate angiogenesis [89, 90]. Additional mechanisms of micrometastatic dormancy may include the induction of autophagy to circumvent nutrient starvation. This occurs until such a time that the microenvironment changes to permit growth and proliferation or alternatively, until such a time that the cells adapt to the nutrients that are available, such as when the micrometastases convert to expressing a phenotype closer to that

of the surrounding stroma [90]. Moreover, dormancy of the micrometastases can be caused by the immune system exerting a tumour suppressor function [91], as well as anti-growth signals disseminating from the surrounding normal tissue ECM [88].

It can thus be seen that once the cancer cells have successfully managed to invade, disseminate and colonize distant sites, they have undergone multiple complex adaptations such that they are considered to be biologically different. Whereby, they no longer resemble the primary tumour and therefore, do not respond in the same way to treatment. Accordingly, it is therefore clear why the invasion-metastasis cascade is classified as a hallmark on its own, and why there is a great need for early detection and diagnosis, as well as treatments which target metastatic processes more specifically.

1.2.7 Emerging hallmarks

Reprogramming energy metabolism

Alterations in cancer cell energy metabolism has emerged as an additional core hallmark in recent years, as this trait has proved to be as widespread in various cancers as the other aforementioned traits that are considered cancer hallmarks [7]. Indeed, the ability of cancer cells to convert from using mitochondrial oxidative phosphorylation to anaerobic glycolysis allows for diverting the resultant glycolytic intermediates to be used in numerous biosynthetic pathways, including the generation of amino acids and nucleosides. Therefore, facilitating the biosynthesis of organelles and macromolecules for assembly of new cells, despite the poor efficiency of ATP generation during glycolysis, relative to that of normal oxidative phosphorylation in the mitochondria [92]. In particular, normal cells tend to process glucose to pyruvate by glycolysis in the cytoplasm, after which the mitochondria convert this to carbon dioxide under aerobic conditions. However, under anaerobic conditions, the glycolytic pathway is favoured, where relatively small amounts of pyruvate are sent to the mitochondria. Cancer cells, however, tend to reprogram their energy production through glucose metabolism to favour glycolysis, even in the presence of oxygen, which is considered aerobic glycolysis and is referred to as the “Warburg-effect” [93]. To compensate for the reduction in ATP afforded by glycolysis, cancer cells tend to upregulate glucose transporters such as GLUT1, which greatly increases import of glucose into the cytoplasm. Not only does this allow the cancer cells to meet the biosynthetic demands of increased cell proliferation, but it furthermore

allows the cancer cells to survive when their nutrient and oxygen demand exceeds that of the abilities of the existing vasculature to deliver said metabolites [94].

A key aspect of this process appears to be an association with activated oncogenes, such as *myc* and *ras*, as well as mutations in tumour suppressors, such as the abovementioned TP53 [94, 95]. Specifically, *myc* is involved in inducing genes associated with the metabolic reprogramming of cancer cells [96]. Where Ras is specifically involved in inducing GLUT1 transporters, to support glycolytic metabolism [97]. Furthermore, glycolytic pathway reliance can be further enhanced by hypoxic conditions often observed in tumours, whereby the hypoxia response system acts simultaneously to upregulate glucose transporters and other enzymes involved in glycolysis [94, 95]. Therefore, it is possible for both hypoxia and the Ras oncoprotein to independently increase the levels of transcription factors such as HIF-1 α and HIF-2 α , to ultimately upregulate the glycolytic pathway [98, 99]. It can thus be seen how cancer cells reprogramme their energy metabolism and therefore why this is suggested to be an additional, emerging hallmark of cancer.

Evading immune destruction

Another emerging hallmark is that of immunoevasion by tumours. A vital component of tumour surveillance is the ability of various factors in the immune system to detect and eradicate cancerous cells before tumours can form. Indeed, it has been observed that a deficiency in function or development of CD8⁺ cytotoxic T lymphocytes, natural killer cells and CD4⁺ T helper cells shows demonstrable increases in the incidence of tumours in mice. Accordingly, both the innate and adaptive components of the immune system play a role in tumour surveillance and therefore, eradication of tumours [100, 101]. Furthermore, there is increasing evidence to suggest this, one such example is that there have been cases of immunosuppressed recipients of organ transplants, which develop cancers derived from the apparently tumour-free donor. This suggests that in the donor where there was a fully functioning immune system, the cancer cells were kept in check by being held in a dormant state [102]. Moreover, cancer cells are able to evade the action of cytotoxic T lymphocytes and natural killer cells in numerous ways, including secretion of immunosuppressor factors (such as TGF β and PDGF), recruiting immunosuppressive inflammatory cells (such as myeloid derived suppressor cells - MDSCs) [103], and losing major histocompatibility class (MHC) I antigen presentation machinery [104].

It can therefore be seen how the immune system serves as a significant barrier to the formation and progression of tumours, and thus how the formation of tumours is associated with evasion of the immune system. Ultimately showing why cancer immunoediting is now considered to be an additional, emerging cancer hallmark.

1.2.8 Enabling characteristics

Genome instability and mutation

There is a clear causal association between genome instability and the acquisition of hallmark characteristics in cancer, this is thus considered to be an enabling characteristic underlying the hallmarks of cancer [7]. Studies have revealed a distinct pattern in DNA mutations relating to specific tumour types [105]. In addition, there is a clear selective advantage conferred by certain mutant genotypes, which enable the growth and consequent dominance of subclones of cells in a local tissue environment. Consequently, the process of multi-step tumour progression can be depicted as successive clonal expansions, each of which is generated by the acquisition of a mutant genotype, which enables one or multiple of the known hallmarks. Indeed, this can occur by chance mutations, epigenetic modifications [106], as well as by an increased rate of mutation orchestrated by the cancer cells themselves [107]. In particular, the enhanced accumulation of mutations is accomplished by the cancer cells through increasing sensitivity to mutagenic agents, as the cells can breakdown the components involved in detecting and resolving defects in DNA (genomic maintenance). The cancer cells can then further exacerbate this mutability by damaging systems that normally monitor the genomic integrity of cells, thereby inhibiting the processes which force genetically damaged cells to either undergo apoptosis or enter a quiescent state [108]. It can then be seen how the role of TP53 (briefly discussed in 1.2.3) is vital to this process. Although not classified as tumour suppressor genes, the genes involved in DNA maintenance machinery can be considered “caretaker” genes. There are 3 key facets to these caretaker genes, which are often lost during cancer progression. In summation, this involves: detection of DNA damage and activation of repair machinery, repairing DNA damage and inactivating mutagens before the DNA is damaged [109]. It is thus clear how genome instability is considered to be an enabling characteristic of the hallmarks of cancer.

Tumour-promoting inflammation

Furthermore, another characteristic involved in promoting tumourigenesis, and therefore acting as an enabling characteristic of the cancer hallmarks, is tumour-associated inflammatory responses [7]. Inflammation contributes towards numerous cancer hallmark capabilities as it is able to supply growth, survival and proangiogenic factors, ECM-modifying enzymes to facilitate invasive and metastatic processes, as well as induce signals which activate EMT [52, 110, 111]. Furthermore, in some cases, inflammation is apparent at earlier stages of tumourigenesis and is clearly capable of advancing nascent neoplasms into developed cancers [112, 113]. In addition, inflammatory cells are capable of releasing reactive oxygen species (ROS), which are actively mutagenic for the surrounding cancer cells, thereby advancing their genetic transformation and enhancing the malignant state [52]. Other molecules released by inflammatory cells include, but are not limited to, signalling molecules such as EGF, VEGF and FGF, as well as cytokines (such as IL-1 and TNF- α) which enhance the inflammatory state [112, 114]. Moreover, as these immune inflammatory cells exist in the surrounding stroma of the tumour, the tumour microenvironment therefore adds an additional level of complexity to tumourigenesis. It can therefore be observed how the tumour-promoting abilities of the inflammatory tumour microenvironment serve as an enabling characteristic of the hallmark capabilities of cancer. Due to the exceptionally complex nature of neoplastic progression, it is thus imperative that current and future cancer therapeutics take into consideration the complexities and specificities that arise from each individual, as well as the individual cancer types.

1.2.9 Current cancer therapeutics

The most commonly used treatment approach for cancer is surgery, however, once the cancer has developed to later stages then additional treatments including radiotherapy and chemotherapy, or a combination of both is often required. Unfortunately, these treatment strategies tend to have severe side-effects and are often not effective enough, resulting in relapse. Therefore, prompting the exploration into alternative therapeutic strategies such as targeted therapy and hormone therapy [115]. Targeted therapies generally include delivering agents to specific molecular targets involved in cancer cell survival and these therapeutics can be natural or drug-based. In this context, monoclonal antibodies and small interfering RNAs (siRNAs) are often explored in their abilities to target tumours, whilst anti-angiogenic drugs are used for targeting angiogenic processes in the tumours [116]. Monoclonal antibodies act by

blocking interactions on the cell surface, which then affect the related signal transduction pathways. Several antibodies have been approved by the FDA for treatment of haematological cancers and solid tumours. Some well-known antibodies include Erbitux®, Iressa®, Herceptin®, Kadcylla® and particularly for NSCLC lung cancer, Avastin®, which targets VEGF. Whilst monoclonal antibodies have the advantage of direct and rapid action, as well as a long half-life, they do also have the disadvantage of being costly to produce, as well as typically only being able to act on the cell surface [115].

RNA interference (RNAi) is becoming increasingly popular for the downregulation or silencing of genes that are involved in tumour development. Double stranded small interfering RNA, or siRNAs, are made to be complementary to the mRNA of the target genes, which are then able to target the mRNA for degradation, subsequently preventing protein expression of the molecular target, ultimately resulting in its downregulation [117]. Therefore, current studies exploring the use of siRNAs as a therapeutic generally aim to target metastatic and angiogenic processes. However, as siRNA technology is a relatively new concept for cancer treatment, most siRNAs being explored as potential therapeutics are still only at the clinical testing stages and thus have yet to reach the market. This is predominantly as a result of the difficulty in finding an effective delivery system for *in vivo* siRNA treatment. Therefore, the struggle is not only in finding an effective siRNA to target molecular pathways underlying the cancer, but also in finding an efficient and minimally toxic delivery system.

Every individual cancer type and the metastatic disease that arises, behaves differently and is biologically different, which highlights the exceptionally complex mechanisms underlying cancer. Without a doubt, the proposed hallmarks of cancer, devised and originally described by Hanahan and Weinberg, have provided a solid foundation on which to base a logical understanding of neoplasticism. Although these distinct hallmarks have been categorised as such, it is vital to understand that while there are some distinct features, for the most part the mentioned hallmarks do not act independently. There is co-dependence and complementarity which exists amongst the hallmarks [118] and as such, it is vital that any further progression in understanding the basis to neoplastic change as a whole, as well as on a cell-type specific level, needs to take this into account. Therefore, the hallmarks need to be targeted as a whole, rather than individually, for the prospective treatment of cancer.

1.3. The 37 kDa Laminin Receptor Precursor/ 67 kDa high affinity Laminin Receptor (LRP/LR) and its role in cancer

LRP/LR, otherwise known as LAMR1, RPSA, p40 and MGr1-Ag/37LRP, is a non-integrin laminin receptor. This receptor has various physiological functions in the cellular environment. LRP/LR is known to reside in the nucleus, where it interacts with histones and thus, has a function in the maintenance of nuclear structures [119], however, it is also found in the cytoplasm. Here, LRP/LR is known to be homologous to p40, the protein fundamental to the processing of 20s rRNA for the assembly of 18s rRNA and the maturation of the 40s ribosome [120]. Furthermore, LRP/LR is found in plasma membrane lipid raft regions, where the C-terminus serves as an extracellular receptor for numerous extracellular matrix (ECM) proteins, bacteria, viruses, prion proteins (including both the cellular prion protein, PrP^c, and the infectious prion protein, PrP^{Sc}) [121], as well as amyloid β (A β) [122]. More recently, LRP/LR has been observed to interact with telomerase [123]. On account of these interactions, LRP/LR has been implicated in the ageing process, as well as in an assortment of diseases, these include: bacterial and viral infections, transmissible spongiform encephalopathies, Alzheimer's disease and importantly for this study, cancer (for review see [124, 125]). Indeed, the significance of this receptor was first discovered by the observation of elevated LRP/LR levels in cancer cells [126] and the consequential association of this receptor in various malignant processes (reviewed in [124, 125]). Specifically, LRP/LR plays a role in metastasis, angiogenesis, cell viability maintenance, apoptotic evasion [124, 127], and mediating telomerase activity [123, 128-131]. In this context, the 37 kDa laminin receptor precursor/67 kDa laminin receptor (LRP/LR) provides a putative target for the treatment of multiple cancer hallmarks.

1.3.1. The role of LRP/LR in metastasis

Metastatic disease is the leading cause of cancer-related deaths, with an estimated 90% of patients dying as a consequence of the metastases. Inherently, metastasis is extremely complex due to the intrinsically distinct ECM and cell compositions of the secondary tumours, thus making the metastatic tumours biologically different to the originating tumour. Importantly, standard cancer drug discovery and development processes for immunotherapies and molecular targets commonly overlook the abilities of the potential therapeutics to inhibit the metastatic process [132]. It is therefore crucial for current studies to test putative therapies in their abilities to inhibit metastasis. LRP/LR is vital to the process of metastasis, which occurs as a consequence of the overexpression of this receptor on cancer cells, thus resulting in an

enhancement of the adhesive and invasive potential of these cells [133]. In addition, LRP/LR enhances cellular proliferation and contributes towards maintaining cellular viability [134]. As aforementioned, metastasis is a multi-step process which allows cells of a malignant nature to disseminate from a primary tumour site, migrate to a distant site and adapt to the new microenvironment. Thus allowing for exploitation at this new site, to evade nutritional depletion [135]. This process is mediated by the nature of these cells, which allows them to adhere to and invade the basement membrane [135, 136]. The process of adhesion of a metastatic cell is mediated by the interaction between LRP/LR and the ECM molecule, laminin-1. This interaction results in the stimulation of type IV collagenolytic activity, thus causing degradation of the basal lamina, after which these tumorigenic cells invade and migrate through the blood stream [137, 138], via the process of intravasation [139]. Post-circulation, the cancerous cells invade a site, distant from the primary site, and proliferate to form a secondary tumour. This occurs through the process of extravasation, where these cells adhere to plasma membranes of mesenchymal tissue at the target site, by translocating to the connected microvessels [140]. LRP/LR is known to be overexpressed in many different cancerous cells including but not limited to: prostate [141, 142], gastric [143], ovarian [144], colon [142, 145], cervical [142, 146], lung, oesophageal and breast cancer cells [142]. These elevated levels enhance the interaction between LRP/LR and laminin-1 and as a result, enhance the aggressiveness of tumours and increase their metastatic potential.

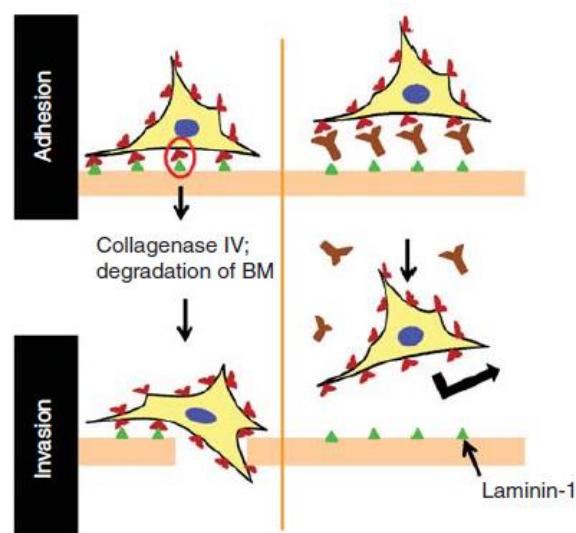


Figure 4: Blocking LRP/LR with IgG1-iS18 reduces the adhesive and invasive potential of cancer cells.

(Adapted from [124])

It has been extensively shown that the anti-LRP/LR specific antibody, IgG1-iS18, blocks the interaction between LRP/LR and laminin-1 and this interference significantly impedes the adhesive and invasive potential of metastatic lung, cervical, colon, prostate [142], breast, oesophageal [147], liver [148], pancreatic, neuroblastoma [149], colorectal [150] and melanoma [151] cells (Figure 4). These cell lines all exhibited either high intracellular or extracellular levels of LRP/LR, compared to poorly invasive MCF-7 cells, thus correlating LRP/LR levels to the adhesive and invasive potential of the cancerous cells and ultimately, to the aggressiveness of tumours. This, therefore, confirms the vital role of the interaction between LRP/LR and laminin-1, in the steps critical to initiating the process of invasion. Furthermore, triple negative MDA-MB231 cells (do not express oestrogen, progesterone and human epidermal growth factor receptors) [152] exhibited very high total levels of LRP/LR, correlating with a very high invasive potential [147]. This suggests LRP/LR could be playing an important role in this type of cancer. LRP/LR could be compensating for this, by providing an alternate for these receptors to aid in growth and tumour progression [124]. In addition, it has been shown that small interfering RNA (siRNA), directed against LRP mRNA, significantly reduced the invasive potential of the tumorigenic fibrosarcoma HT1080 cell line [153]. Therefore, technologies employed to target LRP/LR, including the anti-LRP/LR specific antibody, IgG1-iS18, as well as LRP-targeting siRNA, both of which diminish the interaction between LRP/LR and laminin-1, provide the possibility for therapeutic intervention against metastatic cancer. Moreover, it has been observed that LRP/LR further facilitates the progression of tumours through the induction of tumour angiogenesis, as well as through increasing cell viability [134, 154].

1.3.2. The role of LRP/LR in angiogenesis

Under normal physiological conditions, vessel formation occurs when stimulatory factors, such as VEGF and FGF, stimulate vascular endothelial cells to migrate and proliferate, as mentioned above [155, 156]. This occurs in a multi-step process, by initial degradation of surrounding ECM, through the secretion and activation of metalloproteases allowing for migration, proliferation and differentiation of endothelial cells, towards the stimulatory signals [157, 158].

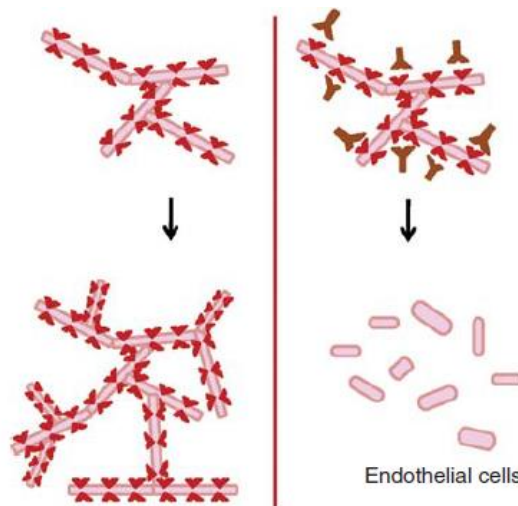


Figure 5: Blocking LRP/LR with IgG1-iS18 reduces endothelial tube formation.

(Adopted from [124])

LRP/LR has been proposed to play an important role in tumour angiogenesis, due to the aforementioned interaction between LRP/LR and laminin-1, which promotes the degradation of the basal lamina through the activation of the matrix metalloprotease, type IV collagenase [138]. This step is considered to be the rate-limiting step in the process of angiogenesis [138]. Moreover, the matrix metalloproteases assist in endothelial cell migration towards angiogenic stimuli, by facilitating the release of pro-angiogenic factors (such as VEGF, FGF and PDGF) [138]. Furthermore, the 67 kDa LR is essential in the angiogenic process for its role in neovascularization and haematopoietic stem cell mobilization, processes producing new endothelial cells and therefore, stimulating angiogenesis [159, 160]. Additionally, it has been found that human umbilical vein endothelial (HUVE) cells abundantly express LRP/LR [154]. Further, it was observed that tubular formation was impeded when these HUVE cells were treated with an anti-LRP/LR specific antibody (Figure 5) [124]. Therefore, conceding the possibility that blockade of the interaction between laminin-1 and LRP/LR decreases the invasive potential of HUVE cells. This reduction in invasive potential, in turn, has a regulatory effect on the rate-limiting degradation of the basal lamina, the step vital for allowing migration, differentiation and tubular formation. With this in mind, halting angiogenesis by targeting LRP/LR, to block and reduce the interaction between LRP/LR and laminin-1 with anti-LRP/LR specific tools, such as IgG1-iS18 or siRNA, respectively, offers the possibility of therapeutic options for treating cancer.

1.3.3. The role of LRP/LR in apoptosis

Another vital role of LRP/LR, is its role in evading apoptosis. The three core biochemical changes that occur during apoptosis include, caspase activation, breakdown of DNA and protein as well as changes in cell membranes, which cause phagocytosis [161]. Caspases are proteases that serve to both initiate and execute apoptosis and can be activated by two key pathways, namely the mitochondrial/ intrinsic pathway and the death receptor/extrinsic pathway [34]. As previously mentioned, apoptotic pathways are tightly regulated under physiological conditions, however, when these pathways become dysregulated, numerous pathologies, including cancer, can arise [162]. LRP/LR is critical in apoptosis and it has been observed that downregulation of LRP/LR induces apoptotic processes [163]. It has been shown that once LRP/LR has bound to laminin, LRP/LR can then interact with focal adhesion kinase and this interaction then activates survival pathways, such as PI3-kinase/AKT and MEK/ERK1/2 [164]. Subsequently, the anti-apoptotic protein, Bcl-2, a downstream effector of the death receptor and mitochondrial apoptotic pathways, is upregulated and consequently inhibits apoptosis [164, 165].

It has been shown that downregulation of LRP/LR with the use of siRNA technology, directed against LRP mRNA, caused a reduction in lung and cervical cancer cell viability. Furthermore, this study revealed that the siRNA-mediated knockdown of LRP/LR resulted in increased levels of caspase-3 activity in these cells (Figure 6), therefore suggesting a vital role of LRP/LR in maintaining cell viability and evading apoptosis in cancerous cells [134]. In addition, a recent study has shown that siRNA-mediated downregulation of LRP/LR induced apoptosis in early and late stage malignant melanoma cells via caspase 8 (extrinsic pathway) and caspase 9 (intrinsic pathway), respectively [166]. In a similar study, downregulation of LRP/LR via the same siRNA technology significantly hampered anti-apoptotic pathways in both early and late-stage colorectal carcinoma cells via activation of both caspase 8 and 9 [167].

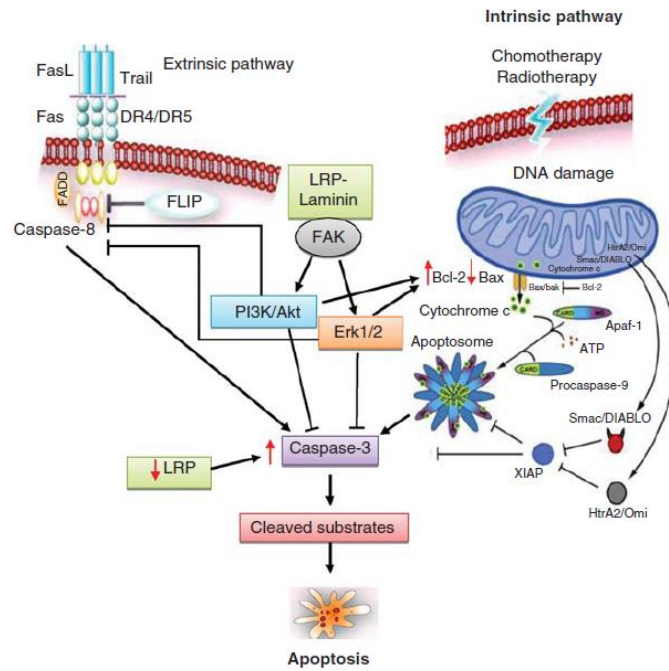


Figure 6: The knockdown of LRP/LR induces apoptosis in cancer cells.

The downregulation of LRP/LR reduces activation of the PI3K/Akt and Erk1/2 pathways, ultimately resulting in the activation of caspase-3, which induces apoptosis (Adopted from Jovanovic et al, 2015).

Interestingly, the abovementioned studies all show the critical role of LRP/LR in the tumourigenic setting. Particularly, it is the key role that LRP/LR plays in metastatic processes, through its role in mediating adhesion and invasion as well as the enhancement of angiogenic processes, the maintenance of cell viability and the evasion of apoptosis [124]. This, therefore, strongly suggests that targeting LRP/LR on the cell surface, with anti-LRP/LR specific antibodies, such as IgG1-iS18, blocks the interaction between LRP/LR and laminin-1, thereby significantly impeding metastatic processes. Further, siRNA technology directed against the mRNA of LRP, mediates the downregulation of LRP/LR, which inhibits angiogenesis, reduces cell viability and induces apoptosis over and above decreasing the adhesive and invasive potential of cells [124, 127]. Thus, employing LRP/R targeting siRNA technology is a possibly powerful therapeutic approach for the treatment of cancer.

It has also been shown that in addition to its various functions and interactions in cancer and neurodegenerative disorders, LRP/LR plays a role in mediating telomerase activity in breast cancer cells [123]. In addition, it was shown that overexpressing LRP/LR enhances telomerase activity [128, 131]. Furthermore, the knockdown of LRP/LR with LRP-specific siRNA

significantly reduced telomerase activity [123]). This is, therefore, another way in which LRP/LR can be targeted as a potential therapeutic intervention for cancer.

1.3.4 Alternative small molecule inhibitors targeting LRP/LR

Although there have been various studies testing the efficacy of IgG1-iS18, as well as LRP-targeting siRNAs for the blockage and downregulation of LRP/LR in numerous cancer types *in vitro*, alternative small molecule inhibitors have also been identified. Particularly, a synthetic molecule, known as NSC47924, was identified to selectively inhibit the adhesion between LRP/LR and laminin, with subsequent reduction in adhesive and invasive potential of breast cancer (MDA-MB-231) and fibrosarcoma (HT1080) cells. Consequently, an inhibition of LRP-LR-mediated cell invasion and migration was observed. However, apoptosis was not induced as the mechanism behind the decreased invasiveness appeared to be as a result of 67 kDa LR internalisation by NSC47924, therefore resulting in a reduction of the interaction between LRP/LR and laminin-1 [168]. Furthermore, an additional study showed that a synthetic lysyl-tRNA synthetase (KRS) targeting molecule, known as BC-K-YH16899, is able to inhibit the interaction between KRS and LRP/LR with subsequent suppression of breast cancer metastasis *in vivo*. KRS is an enzyme which interacts with LRP/LR and is seen to be overexpressed in cancer cells, where BC-K-YH16899 is involved in preventing the translocation of KRS to the cell surface, thereby disrupting the interaction between KRS and LRP/LR [169].

Apart from these identified synthetic molecules, a natural green tea polyphenol known as EGCG has been identified to impede cancer progression. EGCG is known for its role in inducing apoptosis, inhibiting proliferation, as well as containing properties to prevent matrix metalloproteinases [170, 171]. Further analysis revealed a specific binding site for EGCG on LRP/LR, which allowed for EGCG to exert the observed anti-tumourigenic effects [172]. Indeed, a study performed in A549 lung cancer cells shows the role of LRP/LR in mediating EGCG growth inhibition [173]. Additionally, the natural molecule known as Coptisine has been observed to activate apoptosis in hepatocellular carcinoma, via its interaction with the 67 kDa LR on the cell surface. In this specific context, the cell surface LRP/LR acts as a death receptor in those cells that overexpress it [174]. Remarkably, both EGCG and Coptisine appear to act via the same pathway, which suggests they both bind to similar sites on LRP/LR, thereby indicating that this site may specifically be involved in cell-death signalling and therefore, could be another avenue targeted for therapeutic design [127].

Bearing in mind the many ways in which LRP/LR is involved in carcinogenesis as well as the various ways in which it has been targeted by various research groups, there is no doubt that targeting LRP/LR has great potential as a therapeutic for the treatment of cancer. However, because of its numerous roles in the tumourigenic process, the most effective therapeutic is going to be one which takes this into account, and which aims to target more than one, if not all, of the cancer-promoting roles of LRP/LR. In this context, the most efficacious means of targeting the various roles of LRP/LR in tumourigenesis, is to downregulate the overexpressed LRP protein with LRP mRNA- targeting siRNA.

1.4. Strategy for targeting LRP/LR to impede cancer hallmarks *in vivo*

1.4.1. Limitations to using siRNA as a therapeutic

Numerous studies, as mentioned above, have shown that the use of siRNA, particularly to downregulate LRP/LR *in vitro*, impedes the process of tumourigenesis. Therefore, it is essential to assess the effect of LRP/LR knockdown *in vivo*. Since siRNAs are sensitive to degradation in the circulatory system, the use of ‘naked’ siRNA molecules has several limitations, including a short half-life, low efficiency and poor stability. These make for difficulty in targeting particular sites in the cellular environment, as the siRNA is mostly degraded by the time it reaches these sites [175]. Therefore, the major limitation currently facing the prospect of using siRNAs as a therapeutic strategy for cancer, is the efficiency of the delivery system. For this, viral delivery mechanisms, such as lenti- and adenoviral vectors have been used. Although these do provide efficient delivery systems, there are several factors which limit the clinical applicability of viral vectors. These include: a high mutagenic potential, limitation on loading capacity, expensive cost of production and most significantly, the safety risks because of the immunogenic and inflammatory effects caused by the introduction of a virus [175]. Therefore, it is necessary to pursue non-viral alternatives that are able to both protect the siRNA and transport the siRNA to the target tissue, but which induce nominal toxicity and immunogenic response [176].

1.4.2. Biodegradable polylactic-co-glycolic acid nanoparticles as a delivery system

In this context, a promising alternative is the use of biodegradable polymeric nanoparticles, which can serve as nanocarriers of macromolecular drugs, as a result of their advantages over regular drug delivery systems such as viral vectors [177, 178]. There are four key advantages of these biodegradable nanoparticles. The first, is the protection offered against enzymatic and

chemical degradation, therefore increasing the *in vivo* half-life [179]. The second, is that the size of the nanoparticles can allow for bypassing cell membranes and the blood-brain barrier [180, 181]. The third, is the fact that the particles are biodegradable, allowing for sustained and controlled release of contents [182], and the fourth advantage is that these particles can be surface modified in many different ways to allow for organ, tissue and cell specificity (Figure 7), such as with cell-type specific ligands. Therefore reducing the required administrated dose, as well as reducing side-effects [183]. Thus, a potential option for the encapsulation of the LRP-targeting siRNA, are the biodegradable polymer matrix nanoparticles made up of polylactic-co-glycolic acid (PLGA). These encompass all of the advantages of biodegradable nanoparticles as mentioned above as they are highly stable, exhibit low toxicity and allow for controlled release of the contents [184]. PLGA was first employed for use in clinical sutures since they can be completely absorbed by the body. Furthermore, PLGA has been approved by the FDA to be used as carriers for controlled drug delivery, as well as for tissue scaffolds, due to their excellent safety profile and biocompatibility [185]. However, PLGA nanoparticles can be synthesised in various ways for numerous different applications.

Important factors that need to be taken into consideration when planning to use PLGA nanoparticles as a delivery system for drugs are the surface morphology and particle size range, since these factors play a role in drug release, as well as the foreign body response *in vivo*. Previous studies have shown that microparticles (5-10 μm) are too large to be phagocytosed and therefore, oral administration resulted in a foreign body response. However, in the circulatory system, nanoparticles smaller than 1 μm are not tagged and removed by the mononuclear phagocyte system [186]. Furthermore, for the administration of nanoparticles without risking the blockage of blood vessels, the PLGA nanoparticles need to be in the 250-500 nm size range. This size range allows for the particles to be delivered orally, rectally, transdermally, ocularly, nasally, subcutaneously, intraperitoneally, intramuscularly, as well as directly into the systemic circulatory system [187-189]. Moreover, further consideration needs to be taken into the role of particle size as a barrier to drug release. Importantly, the larger the particle, the longer the diffusion path length and therefore, the longer it takes for the drug to be released from the particle. Specifically, there is an exponential relationship between the nanoparticle radius and the diffusion of the drug [190]. Additionally, consideration needs to be taken into the morphology of the nanoparticles and in this case, particles with smooth, spherical characteristics allows them to be endocytosed more readily by cells [191], without damaging them. In addition, although it has been observed that solid polymeric PLGA

nanocapsules have a high encapsulation efficiency, these nanoparticles trap the siRNA resulting in a very low drug release efficiency, resulting in a slow release time with reduced therapeutic outcome [192]. In particular, Raicher et al, 2015 showed that hollow nanocapsules with the siRNA encapsulated in an aqueous core exhibited a much-improved release profile [193]. Ultimately, the prime characteristics for using PLGA nanoparticles as a delivery vehicle for LRP-targeting siRNA would require the nanoparticles to be smooth, round, hollow and within the 250 -500 nm size range.

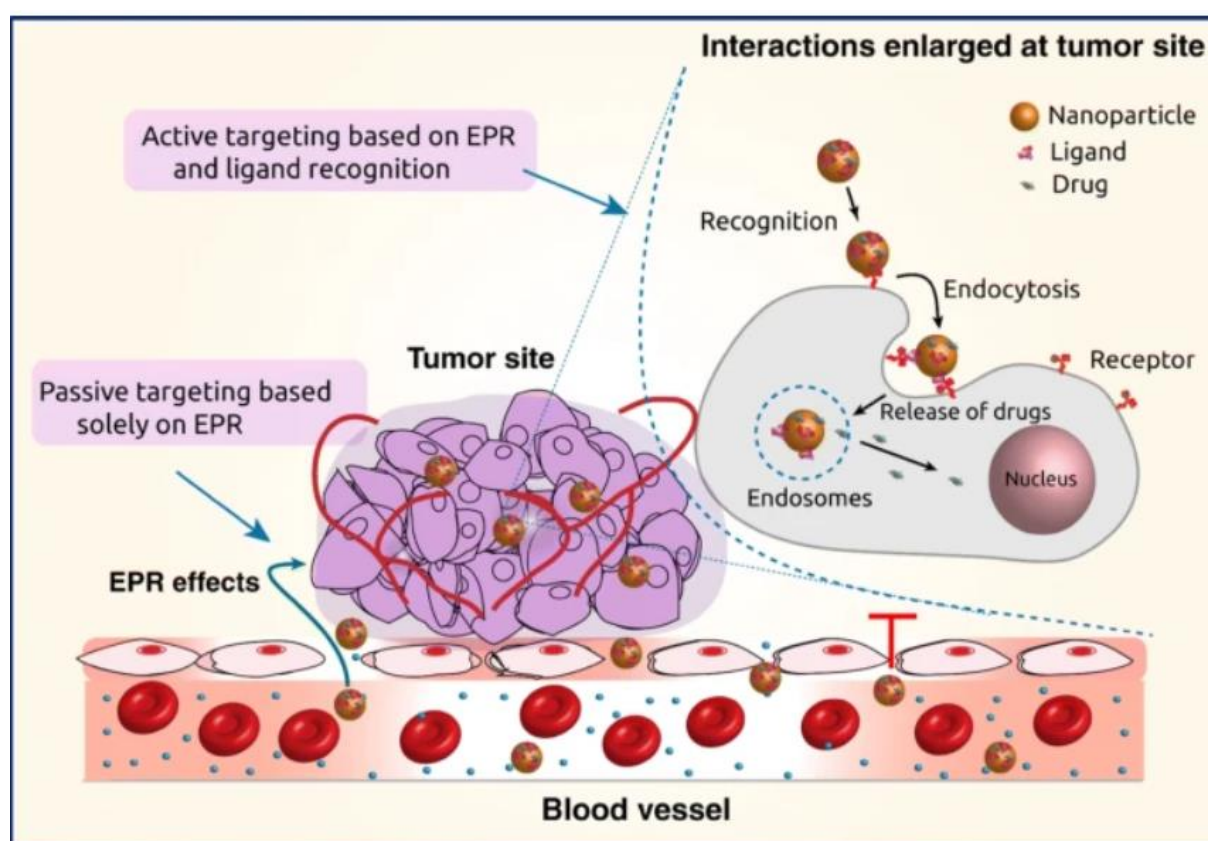


Figure 7: Active and passive drug targeting strategies with nanoparticles

Graphical illustration of passive and active nanoparticle-drug targeting approaches. In passive targeting, the nanoparticles are able to pass through the leaking walls and collect at the site of the tumour via the enhanced permeability and retention (EPR) effect. Active targeting can be performed by surface modification of the nanoparticles with specific ligands that bind to receptors on the cancer cells. Adopted from [194].

1.5. Rationale and scope of the study

The incidence and mortality rates of cancer are growing rapidly worldwide. Lung cancer is the most commonly occurring cancer and the leading cause of cancer-related deaths. Cancer is a multi-step process, which occurs as a result of genome instability, the underlying cause of the hallmarks of cancer. LRP/LR has been shown to have a very large and vital role in cancer. Particularly, LRP/LR plays a role in metastasis, by mediating adhesion and invasion as well as enhancing angiogenesis, maintaining cell viability and evading apoptosis. In addition, LRP/LR plays a role in mediating telomerase activity. The loss of telomerase activity causes progressive telomere shortening, eventually causing cell death. Human carcinomas circumvent inhibitory pathways induced by DNA damage, as well as senescence, through the upregulation of telomerase activity. Telomerase has been detected in 85-90% of malignant human tumours and, therefore, has become an attractive target for potential cancer therapeutics. Furthermore, the knockdown of LRP/LR with an LRP-specific siRNA significantly impedes adhesion and invasion, induces apoptosis, impedes angiogenesis and impedes telomerase activity in various *in vitro* cancer types. Thus, this strategy to target LRP/LR has a four-fold function in reducing cancer development. However, we have yet to assess the effect of downregulating LRP/LR in lung cancer cells and it is not feasible to use naked siRNA molecules *in vivo* and therefore, an efficient delivery system is required. For this, siRNA can be encapsulated in polymeric PLGA, which enables the siRNA to more readily be taken up by cells.

Therefore, the current study contained two parts, whereby the first involved an investigation into the effect of ‘naked’ (not encapsulated in PLGA nanoparticles) LRP-specific siRNA-mediated downregulation in A549 lung adenocarcinoma cells. This included optimising the downregulation of LRP in the A549 cells with LRP-targeting (RPSA) siRNA, which was followed by an assessment of the effect of LRP downregulation on cell viability and morphology, apoptosis, migration potential and telomerase activity. Moreover, proteomic analysis was performed to provide insight into the pathways involved after LRP is downregulated. Thereafter, the second part of this study investigated the use of PLGA nanoparticles as a potential delivery system for the LRP-targeting siRNA. This involved first optimising the nanoparticle synthesis, with subsequent assessment of the empty nanocapsules on cytotoxicity and LRP levels in non-tumourigenic HEK293 cells and tumourigenic A549 lung cancer cells.

1.6. Aims and Objectives

1.6.1. Aim 1

To determine the effect of siRNA-mediated downregulation of LRP/LR on multiple cancer hallmarks including cell viability, apoptosis, migration potential and telomerase activity in a lung cancer cell culture model.

Objectives for aim 1

1. To optimise the downregulation of LRP/LR using LRP-specific naked siRNA in A549 lung cancer cells.
2. To investigate the effect of LRP/LR downregulation on cell viability in A549 lung cancer cells using the MTT and Trypan Blue dye Exclusion assays.
3. To assess the effect of siRNA-mediated downregulation of LRP/LR on cell morphology.
4. To investigate the effect of LRP/LR downregulation on apoptosis in A549 lung cancer cells using the APOPercentage™ assay.
5. To investigate the effect of LRP/LR knockdown on the invasive potential of lung (A549) cancer cells using the modified scratch motility assay.
6. To investigate the effect of LRP/LR downregulation on telomerase activity in A549 lung cancer cells via qPCR.
7. To assess the effect of LRP/LR downregulation by performing proteomics analysis.

1.6.2. Aim 2

To optimise the production of PLGA nanocapsules and to assess the effect of empty PLGA nanocapsules on cytotoxicity and LRP/LR protein levels.

Objectives for aim 2

8. To optimise the production of PLGA nanoparticles.
9. To assess the cytotoxic effect of empty PLGA nanoparticles on non-tumourigenic HEK293 cells and A549 lung cancer cells.
10. To assess the effect of empty PLGA nanoparticles on LRP/LR protein levels in non-tumourigenic HEK293 cells and A549 lung cancer cells.

CHAPTER TWO - EXPERIMENTAL TECHNIQUES

2.1. Cell culture

Non-tumorigenic, Human Embryonic Kidney cells (HEK293) obtained from ATCC (USA) and tumourigenic, A549 human alveolar adenocarcinoma lung cancer cells kindly provided by Dr. Clement Penny, WITS Health Sciences, South Africa were used in this study. Biosafety review board clearance number: 20150350Lab

Both cell lines were cultured under the same physiological conditions including 5% CO₂ at 37 °C in a humidified atmosphere (standard conditions). These cells were cultured in sterile 25-75 cm³ cell culture flasks (NeST Technology, Virginia, USA), and were maintained in the same growth medium namely, Dulbecco's modified Eagle's medium (DMEM) with high glucose (4.5 g/l) (Hyclone, GE Life Sciences, Massachusetts, USA) containing 4 mM L-Glutamine, supplemented with 10 % foetal bovine serum (FBS) (Biowest, Nuaille, France) for additional nutrients and 1% penicillin/streptomycin (Thermo Fisher Scientific, Massachusetts, USA) for bacterial contamination control. Both cell lines were passaged when a confluency of approximately 70-80% was reached. Briefly, cells were passaged by firstly performing a wash with 2-5 ml of 1X phosphate buffered saline solution (PBS) (Table 6, Appendix A). After discarding the wash, the cells were incubated in 1-2 ml of trypsin/EDTA (Biowest, Nuaille, France) at 37 °C for 5 minutes to allow for the detachment of the cells. Thereafter, 2-5 ml of fresh complete growth medium was added to the cells to inhibit the trypsin/EDTA, after which the cells were counted and diluted appropriately for either treatments or for propagation. The sub-cultured cells were then maintained in a final volume of 5-10ml fresh complete growth medium. Additionally, cryopreserved stocks of the A549 and HEK293 cell lines were made in case of unforeseen circumstances such as contamination and equipment failure. The procedure follows as described above, however, after pelleting, the cells were then resuspended in 1 ml of a 10% (v/v) dimethyl sulfoxide (DMSO): 90% (v/v) complete growth medium solution and transferred into cryovials. The stocks were then stored at -80 °C. When cell stocks were required, the stocks were allowed to thaw 90% at room temperature, after which 1 ml of pre-warmed completed growth medium was added in a drop-wise manner. The 2 ml was then transferred to Eppendorf tubes and centrifuged at 1 000 xg for 2 minutes to remove the DMSO and old medium. The cell pellet was gently resuspended in 1 ml fresh complete growth medium and added to a 25 cm³ flask containing 4 mls of complete growth medium.

2.2. Poly-lactic-co-glycolic acid (PLGA) nanoparticle production and characterisation

2.2.1. PLGA nanoparticle synthesis: Double emulsification-solvent evaporation technique

The Poly (lactic-co-glycolic acid) (PLGA) nanoparticles were synthesized making use of the double emulsification-solvent evaporation technique as previously described [195], with minor changes. Due to the lack of previous exposure to this technique, there were a few steps that had to be optimised before the following protocol was used for further experimental analysis. The water-oil-water (double) emulsion is a technique by which PLGA is used to encapsulate both hydrophilic and hydrophobic drugs in nano-scale form. Briefly, the PLGA gets dissolved into an organic phase, such as dichloromethane (DCM), which serves as the oil phase, and this is then emulsified with a stabiliser such as Vitamin E- D- α -Tocopherol polyethylene glycol succinate (TPGS), which serves as the water phase. Vitamin E- TPGS was used as the emulsifying agent for its advantages, which include improved emulsification and encapsulation efficiency, over other stabilizing agents such as poly (vinyl alcohol), PVA). Since siRNA is anionic and therefore, hydrophilic, the siRNA would be emulsified with the polymer solution preceding nanoparticle formation. Hydrophobic drugs would be added directly to the oil phase. Small polymer droplets (nanocapsules) are then formed by sonicating the solution using high intensity bursts. The emulsion which results from this is then added to a large quantity of Vitamin E-TPGS and stirred for a number of hours to both allow the formed nanoparticles to harden, as well as to allow the DCM to evaporate. The raw nanoparticle solution that is produced is subsequently centrifuged to collect the nanocapsules and further washed to remove the Vitamin E-TPGS.

In detail, 100 mg of PLGA polymer was weighed out into a 13 mm x 100 mm glass test tube, after which 1 – 2 ml (to test 100 mg/ml and 50 mg/ml initial dilution) of either PVA or DCM was added to the polymer in the test tube. The top of the tube was subsequently covered with aluminium foil, and the foil then tightly sealed with Parafilm to ensure as little evaporation of the PVA or DCM as possible. The level of solvent was then marked on the outside of the test tube and the polymer was allowed to dissolve overnight at room temperature, with the addition of solvent the following day if evaporation had occurred. The polymer solution was then added dropwise to the centre of 4 ml 0.3% (w/v) Vitamin E-TPGS in a new 50 ml Falcon tube, while vortexing. Once all of the polymer solution was added, the emulsion was further vortexed for

15 seconds and then immediately sonicated on ice 3X for 10 seconds each at 40% amplitude using a 700 W probe sonicator. In a 100 ml beaker, 45 ml of the 0.3% Vitamin E-TPGS was left to stir, 2 ml of this solution was added to thin the now sonicated emulsion which was then poured into the remaining 43 ml stirring Vitamin E-TPGS. This was partially covered with foil and further stirred for 3 hours to allow the nanoparticles to harden and the DCM to evaporate, resulting in the 'raw' sample.

2.2.2. PLGA nanoparticle purification and storage

To separate out the larger nanoparticles that were synthesized, various centrifugation speeds were tested to determine which speed would provide the most uniformly sized nanoparticles with minimal sample loss. To test the various centrifugation speeds, 1 ml aliquots of the raw samples were centrifuged separately for 1 minute at 500 xg, 8 000 xg, 10 000 xg, 12 000 xg and 14 000 xg and consequently characterised by Scanning Electron Microscopy (SEM). However, for all subsequent experimentation the following protocol was used. Briefly, once the samples were centrifuged at 500 xg for 1 minute, the supernatants were then transferred to new Eppendorf tubes and further centrifuged at 17 000 xg for 15 minutes to pellet out the remaining nanoparticles. The supernatant was subsequently discarded, and the pellet washed to remove the Vitamin E-TPGS solution. The wash step was repeated for a total of three washes and included resuspending the pellets in 1 ml dH₂O using a water bath sonicator, centrifuging the solution at 17 000 xg for 15 minutes and discarding the supernatant. Each pellet produced was then resuspended in dH₂O or 1X sterile PBS to 10% of the original aliquot volume, and the suspensions then collected to a total volume of 5 ml. For long term storage, this was then placed into one 50 ml Falcon tube in preparation for lyophilisation. The solution was further frozen with the tube lying at an angle to increase the surface area in contact with air, at – 80 °C. Once frozen, the lid of the tube was removed, and the tube opening covered with Parafilm. Small holes were created in the Parafilm to allow for water sublimation during the lyophilisation process, and the tube was then placed into the lyophiliser, with the lyophilisation process being carried out for 72 hours. Lyophilised nanoparticles were then stored at -80 °C until the samples were used for downstream applications. However, for short term storage and treatments, the 5ml final volume of purified nanoparticles in 1X PBS was used for treatments as described (2.3.1).

2.2.3. PLGA nanoparticle characterisation

To characterise the nanoparticles in terms of morphology and size, Scanning Electron Microscopy (SEM) was used, together with dynamic light scattering (DLS). To prepare the samples for characterisation by SEM, 10 µl of each of the nanoparticle samples were placed on to strips of double-sided carbon tape stuck on to stainless-steel specimen stubs and allowed to dry overnight in a laminar flow hood. The stubs containing the samples were subsequently sputter coated with a single coat of Gold Palladium and one coat of Carbon, each for 30-120 sec. The nanoparticles were subsequently imaged using the FEI Quanta 400 FEG-SEM at the Wits University, Microscopy and Microanalysis Unit (MMU) at an acceleration voltage (high voltage-HV). However, when this was unavailable due to repairs, the TESCAN MIRA3 FEG-SEM at the University of Cape Town, Imaging and Analysis Centre (IAC) was used. Particles were visualised under a beam strength of 2 (MIRA3) – 30 kV (Quanta), at a working distance of 5- 15 mm and a spot size of 1. A minimum of three images were captured per batch of nanoparticles to attain a representative sample of the size and morphology of the particles. DLS was further performed to measure the hydrodynamic size of the particles as well as the polydispersity index (PDI) of the raw samples, the samples centrifuged at 500 xg un-purified (still in Vitamin E-TPGS), as well as the purified samples. The PDI is a means of describing the extent of non-uniformity of particle size distribution whereby, values above 0.7 indicate a very broad particle size distribution and values below 0.05 are considered extremely monodisperse [196].

2.3. Treatments and transfection

2.3.1. Treatment of cells with empty PLGA nanoparticles

The HEK293 and A549 cells were treated with 1-10% (v/v) of the empty PLGA nanoparticles, to assess how the nanoparticles themselves, might affect cellular viability, as well as LRP levels on both non- and tumourigenic cell lines. This was to determine the viability of using the PLGA nanoparticles as a delivery mechanism for LRP-targeting siRNA.

Briefly, the HEK293 and A549 cells were seeded into a 24-well plate and incubated overnight under standard conditions to reach a confluency of 60-70%. The cells were then treated with 1, 5 and 10% (v/v) of the purified empty PLGA nanoparticles in a final volume of 500 µl/well for 48-72 hours under standard conditions. Media changes were performed the following day to remove nanoparticles not taken up by the cells and to replenish with 500 µl/well (for 48-

hour incubations) or 2 ml/well (for 72-hour incubations) fresh complete growth medium to ensure sufficient nutrients for the cells to survive the remainder of the incubation. The treated cells were then either used to extract protein for western blots or assayed for cell viability.

2.3.2. Transfection with LRP-specific siRNA

The A549 cells were transfected with LRP-targeting siRNA to assess whether the downregulation of LRP/LR is a potential therapeutic strategy for targeting multiple cancer hallmarks. Therefore, all subsequent assays were performed post-transfection of the A549 cells with the LRP-specific siRNA (MISSION® esiRNA-RPSA, Sigma Aldrich), as well as with a non-targeting control (NTC) siRNA (MISSION® siRNA Universal Negative Control #, Sigma Aldrich). During the process to optimise the downregulation of LRP/LR, the MISSION® esiRNA-RLUC siRNA was initially used as the negative control siRNA, however this had off-target effects, whereby it caused a decrease/downregulation of LRP/LR. The MISSION® siRNA Universal Negative Control #1 was then employed as the NTC and caused no off-target effects and was therefore used for all further experimentation. Transfections were performed using the X-tremeGENE siRNA Transfection Reagent (Roche) and the protocol was followed as per instructions.

Briefly, the A549 cells were seeded into a 24-well plate and incubated overnight under standard conditions to reach a confluency of 60-70%. The RPSA and NTC siRNA were then separately combined with pre-warmed Opti-MEM® 1 serum-free culture medium at 0.5 µg/well, while the transfection reagent was simultaneously combined with pre-warmed Opti-MEM at an equal volume to that of the respective siRNA (2.5 µl siRNA: 2.5 µl X-tremeGENE transfection reagent). The transfection reagent mixtures were then combined with the respective siRNA mixtures and incubated for 10 minutes at room temperature to allow lipid nanoparticle complexes to form. The siRNA/transfection reagent mixtures were then added to the corresponding wells at 100 µl/well in a final volume of 500 µl complete growth medium and incubated for 24-72 hours under standard conditions. Media changes were performed the following day to remove transfection reagent containing medium and replenished with 500 µl/well (for 48-hour incubations) or 2 ml/well (for 72-hour incubations) fresh complete growth medium to ensure sufficient nutrients for the cells to survive the remainder of the incubation. Mock transfections, containing the transfection reagent in the serum-free medium only (and no siRNA), were also performed as above to ensure the transfection reagent was not causing any

off-target effects. The transfected cells were then either imaged using light microscopy, used to extract protein for western blots, or assayed for cell viability, apoptosis, migration potential, telomerase activity and proteomic analysis.

2.4. Protein extraction

Cell lysates were prepared by first harvesting cells. Growth media was removed from the culture plates containing the relevant cell lines, cells were then washed with 1 ml 1X PBS. All culture media and washes were harvested into 15 ml Falcon tubes. Thereafter, cells were detached by the addition of 500 µl/well Trypsin/EDTA with incubation at 37 °C for 5 minutes. Culture media was then added at 1 ml/well to inactivate the Trypsin/EDTA and the cell suspension was transferred to the relevant 15 ml Falcon tubes and centrifuged at 4900 rpm for 10 minutes. The supernatant was removed and 100-200 µl 2X RIPA lysis buffer (Table 2, Appendix A) was added dependent on the cell pellet size. The cells were resuspended in the lysis buffer, transferred to 1.5 ml Eppendorf tubes, vortexed briefly and then incubated at 4 °C for 15 minutes. The lysates were then centrifuged at 5000 xg for 10 minutes to pellet out cell debris. The protein-containing supernatant was then transferred to new 1.5 ml Eppendorf tubes and a BCA assay was then performed to determine the total protein concentration for western blot analysis.

2.5. Protein quantification: Bicinchoninic acid (BCA) assay

The BCA assay allows for protein quantification to ensure accurate and equal loading of proteins when performing a western blot. This assay is based on an observable colorimetric change of which the absorbance can then be measured using an absorbance spectrophotometer. The reaction is based on temperature and the peptide bonds which exist within the proteins. These peptide bonds reduce Cu^{2+} in the copper sulphate reagent, which is added, to Cu^{1+} . The bicinchoninic acid chelates the Cu^{1+} which then forms a purple product [197]. Therefore, the amount of Cu^{2+} which is reduced is in direct proportion to the number of peptide bonds present and therefore, allows for direct quantification of the concentration of protein present.

Briefly, a set of BSA protein standards of known concentrations (0, 0.2, 0.4, 0.6, 0.8 and 1 mg/ml) were prepared and 10 µl/well of each standard was loaded into a 96-well plate in triplicate. Samples of unknown concentration were added in triplicate, at 3 µl/ well and made

up to 10 μ l with MilliQ water. BCA solution was then made up to 1:30 Copper Sulphate: Bicinchoninic acid. Thereafter, 200 μ l of this BCA solution was added to each well, including controls, such that the final ratio was 1 part sample to 20 parts BCA solution. The plate was then incubated at 37 °C for 30-40 minutes until the colour change had sufficiently developed. The absorbance was then measured at 562 nm using the VICTOR® Nivo™ Multimode Microplate Reader (PerkinElmer). A linear regression curve was then calculated for the standards and the concentration of the samples calculated using the equation of the curve. Samples were then made up for western blot analysis.

2.6. SDS-PAGE and western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting (WB) were used to detect protein expression levels. This technique was used to measure total protein levels of LRP and β -actin in the A549 and HEK293 cells.

The lysates were prepared and quantified as outlined (3.3). The protein samples were standardised to 1-2 mg/ml and heated for 5 min at 95°C in Blue Loading Buffer (New England Biolabs) containing 40 mM dithiothreitol. A total of 10 μ g (β -actin and LRP) of protein, together with PageRuler Prestained Protein Ladder (Thermo Fisher) was then separated on a 12% Polyacrylamide gel (Table 3, Appendix A) in 1X Electrophoresis Tank Buffer (Table 4, Appendix A) for 60-90 minutes at 120 V using the Mini Protean gel system (Bio- Rad, California, USA). Proteins were resolved until the dye front reached 0.5 cm above the bottom of the gel. Filter papers were pre-soaked in 1X Transfer Buffer (Table 5, Appendix A) and PVDF membrane was cut to size and activated in methanol for 5 minutes, then further equilibrated in the transfer buffer prior to transfer. The gels were then cut to size and positioned on top of the pre-cut, equilibrated membranes, sandwiched between 6 layers (3 on top and 3 underneath) of pre-soaked filter paper. Transfer was performed using the Trans-Blot® Turbo™ Transfer system (Bio- Rad, California, USA) where transfer was performed at 25 V – 1.0 A for 30 min. Once transfer was complete, the membranes were blocked in 5% milk powder in 1X PBS and 0.1% Tween 20 (PBST) for 1 hour. After blocking, primary antibody (1:10 000 primary labelled β -actin and Hu IgG1-iS18) diluted in 3% BSA in 1X PBST was added to the blots and incubated overnight with gentle shaking at 4 °C. The membranes were subsequently washed 3 times in 1X PBST for 10 minutes each with shaking to remove any unbound antibody. Secondary antibody (Anti-Hu -HRP) diluted in 5% milk powder in 1X PBST was added, and

the blots incubated for 1 hour in the dark at room temperature with shaking. The membranes were then washed 3 times as outlined above. The proteins were visualized with Clarity™ Western ECL Blotting Substrate (Biorad) and the ChemiDoc™ Imaging System (Biorad). Densitometric analysis was performed with Image Lab 5.1 software (Biorad), whereby all values were further normalised against the β -actin loading control.

2.7. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) cell viability assay

The MTT assay was used to assess cell viability in the A549 and HEK293 cells. This was performed once downregulation of LRP/LR was optimized and confirmed in the A549 cells. Additionally, cell viability was assessed in the A549 and HEK293 cells post-treatment with 1-10% of the empty PLGA nanoparticles. This assay allowed the determination of whether downregulating LRP influences cell viability, as well as whether the empty PLGA nanoparticles are cytotoxic.

This assay is based on a colorimetric effect produced as a result of NAD(P)H –dependent oxidoreductases being present in the cell and thus indicating metabolic activity as a direct reflection of cell viability [198]. Mitochondrial Succinate Dehydrogenase, which is present in viable cells, is able to reduce the tetrazolium dye, MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) to its insoluble form, causing the formation of purple formazan crystals. The quantity of crystals formed directly indicates cellular viability and therefore, delivers a means of quantifying cell viability in relation to an untreated sample.

Briefly, A549 and HEK293 cells were seeded into a 24-well plate and incubated overnight under normal conditions and further treated as described (3.3). Protocatechuic acid (8 mM PCA) was used as a positive control, while the untreated cells served as the negative control. After incubation with the respective treatment, 100 μ l of 1 mg/ml MTT was added to each well and incubated for 2 hours at 37 °C. The resultant formazan crystals were dissolved in 200 μ l DMSO/well and resuspended, thereafter the samples were plated into a 96-well plate in duplicates of 100 μ l each. The optical density was subsequently determined using the VICTOR® Nivo™ Multimode Microplate Reader (PerkinElmer) at 570 nm. The percentage cell viability was then established by calculating the treated values as a percentage of the corresponding untreated values for each biological repeat, with the untreated set to 100%.

2.8. Trypan Blue Exclusion assay

The dye exclusion assay was performed to confirm the decrease in cell viability of the A549 cells post- downregulation of LRP/LR, as observed via the MTT assay. This assay is used to determine the number of viable cells in a suspension of cells. The basis of this assays relies on the fact that dead cells do not have intact membranes and therefore, take up the Trypan blue dye, whereas live cells with undamaged membranes do not [199]. Briefly, cells were seeded and transfected as described (3.3.2). After a 72-hour transfection, the culture medium was removed, and the cells were washed with 500 µl/well 1xPBS. The cells were then incubated in 100 µl/well Trypsin/EDTA for 5 minutes at 37 °C. Once the cells had detached, they were resuspended in 1 ml complete growth medium and 20 µl of this cell suspension was transferred to a new Eppendorf tube. The cells were then resuspended in 20 µl Trypan blue dye (1:1) and inserted into a cell counting slide (Biorad, California, USA), which was subsequently inserted into the TC 20™ automated cell counter. The cell counter provided the percentage cell viability for each sample which was recorded and analysed statistically.

2.9. Cell morphology

The A549 cells were imaged via light microscopy after downregulation of LRP/LR to assess for cellular morphological changes. Briefly, the A549 cells were transfected as described (3.3.2) in 24- well plates, and imaged 48- and 72 hours post-transfection using the Zeiss Primovert light microscope (Zeiss, Oberkochen, Germany) at 40X magnification. Images were then analysed visually for signs of morphological changes indicating apoptosis, including the appearance of cell shrinkage and membrane blebbing.

2.10. APOPercentage™ Cell Apoptosis assay

The APOPercentage assay was performed to determine the effect of LRP/LR downregulation on apoptosis in the A549 cells post-transfection. The Cell-APOPercentage™ Apoptosis Assay kit (biocolor, UK) was used, and the protocol was followed as per kit instructions. This assay makes use of a dye that selectively stains cells undergoing apoptosis, as the membrane-flip of the phosphatidylserine during the apoptotic process allows uptake of the APOPercentage dye. Whereas necrotic cells are not stained as they cannot retain the dye.

Briefly, cells were seeded and transfected in 24-well plates as described (3.3.2). The medium was carefully removed from each well, 30 minutes prior to the end of the incubation period, and replaced with 500 µl fresh complete growth medium containing 5% v/v of the APOPercentage™ dye. This was subsequently incubated for 30 minutes at 37 °C. The dye containing medium was then removed and the cells were washed with 500 µl/well 1X PBS. The wash was discarded, and the cells were then left in 100 µl/well 1X PBS for visualization and imaging. The cells were imaged using the Zeiss Primovert light microscope (Zeiss, Oberkochen, Germany) at 20X magnification. Once images had been captured, the PBS was discarded, and the cells were incubated in 50 µl/well Trypsin/EDTA for 5 minutes at 37 °C to detach the cells. APOPercentage™ Dye Release Agent was then added to the detached cells at 200 µl/well and the plate was shaken for 10 minutes to release the intracellularly accumulated dye. The contents of each well (200 µl/well) were then transferred to a flat-bottomed 96-well plate and the absorbance was subsequently read at 550 nm with the VICTOR® Nivo™ Multimode Microplate Reader (PerkinElmer). All necessary controls were incorporated, including 8 mM PCA treatment as an apoptosis positive control, untreated/uninduced cells as the negative control, as well as a reagent blank to account for background absorption. The fold increase was then calculated, where the untreated was set to 1 and the treatments and controls were calculated as a fold increase in apoptosis, compared to the untreated cells. Although images were captured, direct analysis of these images was not performed, and all data analysis was performed colorimetrically.

2.11. Scratch Motility assay

The scratch motility assay was performed to determine the effect of downregulating LRP/LR on the migration potential of the A549 cells. The A549 cells were transfected as described (3.3.2) and allowed to reach 90-95% confluency to ensure a confluent monolayer was created (approximately 24 hours post-transfection). A vertical scratch was made across the cell layer with a P2 pipette tip, using a ruler as a guide. Markings were then made along the scratch, on the underside of the plate to ensure consistency in the subsequent imaging process. A medium change was then performed to replace with 500 µl/well fresh complete growth medium, containing 5 µg/ml Mitomycin C to inhibit cell proliferation, thereby ensuring that the cells observed in the scratch are due to migration and not proliferation. The wells were then imaged with the Zeiss Primovert light microscope (0 hour) and were further imaged 24- and 48 hours post-scratch. Image analysis of the scratch was then performed using ImageJ (National

Institutes of Health and LOCI, University of Wisconsin) and presented in terms of percentage wound closure.

2.12 Telomerase activity assay

Telomerase activity in the A549 cells was quantified after downregulation of LRP/LR with the use of a modified Telomeric Repeat Amplification Protocol. This protocol provides a highly sensitive assay for fluorometric detection and allows real time quantification of telomerase activity. The principle of the assay is based on the telomeric repeat amplification protocol (TRAP) as initially defined by Piatyszek *et al.*, (1995). However, this protocol makes use of fluorescent dsDNA binding dyes which provide a way in which to detect and quantify telomerase activity by directly measuring real time fluorescence emission via qPCR, rather than by electrophoresis. The initial stage of the reaction allows the telomerase enzyme in an extracted sample to add telomeric repeats to the 3' end of a Telomeric Substrate (TS) (extension). Hereafter, the extended products are amplified by Taq polymerase, utilizing upstream (TS) and downstream (ACX) primers (amplification). The ACX primer serves as a reverse primer and comprises a 6-bp 'anchor', which acts to cap the 3' end of the telomerase product after the first PCR cycle. Therefore, preventing any further elongation of the telomerase products. Additionally, this ACX primer prevents self-amplification, assisting in reducing primer-dimer formation[200]. The final stage of the assay consists of detecting fluorescent signals at the end of the taq extension step of each amplification cycle (detection). This occurs as a fluorescent dsDNA binding dye, such as SYBR Green, is incorporated into the reaction mixture. The amount of TRAP products produced are then directly proportional to the fluorescence emission produced (Figure 8) This protocol, therefore, takes into consideration the real time measurement of fluorescence produced which directly allows for relative quantification of telomerase activity through the addition of telomeric repeats.

Briefly, cell pellets were resuspended in 200 µl of CHAPS Lysis Buffer/ 10^5 - 10^6 cells. The suspensions were incubated on ice for 30 minutes, after which the samples were centrifuged in a microcentrifuge at max speed ($12\,000 \times g$) for 20 minutes at 4 °C to pellet cell debris. The supernatants were then transferred to clean 1.5 ml Eppendorf tubes, 10 µl was aliquoted for protein quantification and the remaining supernatants were snap frozen in liquid nitrogen and stored at -80°C. The protein was then quantified with the NanoDrop™ One (Thermo Fisher Scientific, Massachusetts, USA) and standardized to 500 ng/µl for all experimental and control

reactions. The TRAP reaction mix was prepared and loaded into individual wells on a 96-well plate, together with 1µg/µl protein samples to a final volume of 12.5 µl. The reaction Mastermix consisted of Luna® Universal qPCR Mastermix (New England Biolabs, Massachusetts, USA), EGTA (to inhibit RNases), TS and ACX primers, as well as Nuclease free (PCR grade) water (Table 8, Appendix A). All samples were analysed via qPCR with the CFX96™ Touch qPCR System (Bio- Rad, California, USA) using the following cycling parameters: One cycle of 37°C for 30 minutes (telomere extension step), 95°C for 2 minutes and 45 cycles of 95°C for 15 seconds, 59°C for 60 seconds and 45°C for 10 seconds (where fluorescent reads were taken). Telomerase activity was thereafter calculated as a function of the untreated controls set to 100%. Negative controls were included in the plate set up and included: a minus telomerase control, consisting of only CHAPS Lysis Buffer to ensure the buffer had not been contaminated and had no telomerase activity; a no template control consisting of only Nuclease free water to normalise against primer dimer formation in the absence of telomerase activity. Since telomerase is a heat-sensitive enzyme, a heat-treated telomerase negative control was also included to evaluate each sample for heat sensitivity, whereby a second stock of 500 ng/µl of each sample was incubated at 95 °C for 20 minutes prior to detection, to inactivate telomerase. A telomerase positive cell extract was included as a positive control. The Cq values generated were then analysed using the Bio-Rad CFX Maestro software. All values were standardised to a percentage of the untreated controls which were set to 100%, after background subtraction of the heat-treated samples.

The diagram illustrates the Telomerase Assay (TA) process. On the left, a pipette icon is shown next to the text "Whole cell extract containing Telomerase". Below this, a list of components is provided: "Tag pol", "dNTPs", "Primers", and "SYBR green". An arrow points from these components to the "Telomerase Substrate" (TS) region of a DNA strand. The TS region is represented by a horizontal line with a series of vertical bars (telomeric repeats) extending from it. The 5' end of the TS is labeled "5' -". The 3' end of the TS is labeled "- 3'". The TS is composed of a sequence of telomeric repeats (GGTTAG) followed by a non-telomeric sequence (AATCCGTCGAGCAGGTTT). The text "Telomerase enzyme adds telomeric repeats (GGTTAG) onto the 3' end of the Telomeric Substrate oligonucleotide" is written below the TS. To the right of the TS, the "Telomeric repeats" are shown as a series of blue vertical bars. The 3' end of the TS is labeled "- 3'". The diagram shows the addition of telomeric repeats (GGTTAG) onto the 3' end of the TS, resulting in a longer TS with more repeats. The final product is shown as a TS with a longer sequence of telomeric repeats, labeled "TS - Telomerase Product".

The diagram illustrates the principle of PCR-based telomere length measurement using ACX primers. It shows a DNA strand with a telomeric repeat sequence (AATCCGTTAG) being amplified by PCR. The top part shows the TS upstream/forward primer binding to the A-rich strand. The bottom part shows the ACX downstream/reverse primer binding to the T-rich strand. The ACX primer prevents 3' elongation of the product after the first PCR cycle, ensuring that the length of the products accurately indicates telomerase activity in that sample.

Products are amplified by PCR using specific primers (TS upstream/forward primer and ACX downstream primer)

ACX primer prevents 3' elongation of telomerase products by capping the 3' end of the product after the first PCR cycle, therefore length of products accurately indicates telomerase activity in that sample

The diagram illustrates the mechanism of real-time PCR. On the left, a double-stranded DNA (dsDNA) template is shown with 5' and 3' ends. Three green circles, labeled 'F', represent fluorescent dyes (SYBR, Luna) binding to the dsDNA. An arrow points to the right, leading to a graph of Fluorescence versus Copies per reaction (Ct). The graph shows a sigmoidal amplification curve. A horizontal dashed line indicates the 'Threshold'. The region above the threshold is labeled 'Amplification', and the region below is labeled 'No amplification'.

Created in **BioRender.com**

This protocol makes use of fluorescent dsDNA binding dyes which allows for detection and quantification of telomerase activity by directly measuring real time fluorescence emission via qPCR. Step 1 (**Extension**) of the reaction allows the telomerase enzyme in an extracted sample to add telomeric repeats to the 3' end of a Telomeric Substrate (TS). The whole cell extract containing the Telomerase enzyme is added to a Mastermix containing all reagents required for the reaction in a 96-well plate including Taq polymerase, dNTPs, the upstream (TS) and downstream (ACX) primers as well as a fluorescent dye (such as SYBR green). Step 2 (**Amplification**) is where the extended products are amplified by Taq polymerase, utilising the upstream (TS) and downstream (ACX) primers. The ACX primer is the reverse primer and consists of a 6-bp 'anchor', which caps the 3' end of the telomerase product after the first PCR cycle to prevent further elongation of the telomerase products. Additionally,

this ACX primer prevents self-amplification, to assist in reducing primer-dimer formation. Step 3 (Detection) of the protocol includes the detection of fluorescent signals produced by the incorporation of the fluorescent dye into the amplification products and occurs at the end of the extension step of each amplification cycle. The amount of TRAP products produced are then directly proportional to the fluorescence emission produced. (Created with BioRender.com).

2.13. Proteomic analysis

Proteomic analysis is an established method for gaining a broader, cohesive understanding of biological relationships and pathways involving numerous proteins, which can be examined in a single analysis. In particular, Sequential Window Acquisition of All Theoretical Mass Spectra (SWATH-MS) is a proteomic platform centred around a data independent acquisition (DIA) theory, which includes concurrent identification and quantification of proteins [201]. Briefly, SWATH-MS measurements involve trypsin digestion of unlabelled protein samples, whereafter the peptides resulting from this are analysed using liquid chromatography connected to a tandem mass spectrometer (LC-MS) [202]. During this process, the mass spectrometer runs in a data-independent acquisition mode, which means that fragmentation of ionized proteins within the sample that exist within a particular specified mass range is performed using an unbiased and systematic approach [202]. SWATH-MS provides a significant advantage, in that it accurately delivers quantitative analyses of peptides, including thousands of proteins. Additionally, this technique is appropriate for samples that necessitate precise and reproducible quantification for the major fraction of the expressed proteome in a given sample[202]. Furthermore, various studies showed that single-shot analyses exhibited proteome coverages of roughly 50% of the MS-measurable proteomes in complex mammalian samples [203, 204]. Therefore, employing protein expression proteomics followed by functional analysis of the dysregulated proteins, provides the ability to screen the numerous proteins involved after the downregulation of LRP/LR, which allows for a broader view into the mechanism of the receptor, as well as to gain insight into using siRNA-mediated downregulation of LRP/LR as a potential therapeutic.

The A549 cells were seeded, transfected, and harvested as previously described (3.3.2). Cell pellets were snap frozen in liquid nitrogen and stored at -80 °C. All sample and data processing were performed at The Council for Scientific and Industrial Research (CSIR) centre. Only proteins with a minimum fold change of ≥ 2 and a maximum adjusted p-value of ≤ 0.01 were

considered to be differentially expressed. Functional analysis was then further performed personally using the Search Tool for Retrieval of Interacting Genes/Proteins (STRING) database (v11.5) and the Reactome pathway browser. The STRING database is dedicated to wide-scope protein interactions, whereby it integrates primary and predicted interactions between proteins, this includes annotated pathway knowledge and text-mining results [205]. When a set of data is put into STRING, the provided protein networks go through a scoring process. This occurs whereby all evidence STRING finds for the interactions contributing to a given network is scored and benchmarked and a final combined score between zero and one gives an approximation of the confidence in whether the proposed association is biologically meaningful or not [206]. A medium confidence (0.4) interaction score and all active interaction sources (text-mining, experiments, databases, co-expression, neighbourhood, gene fusion and co-occurrence) were applied for all shown STRING networks. Reactome provides a stable, high-performance service for visualising, interpreting and analysing pathways [207]. Furthermore, the Reactome pathway analysis tools are accurate and reliable and exist to handle Omics data obtained from high-throughput technologies, such as in this case for the proteomics data obtained through SWATH-MS. Reactome also makes use of external information and cross-references other sources within the database, in addition to curated data [207].

2.14. Statistical analysis

Statistical analyses were performed using Microsoft Excel 365 (Microsoft Corporation) and GraphPad Prism Software. All experiments were performed with a minimum of three biological repeats with error bars representing standard deviation. A one-way ANOVA analysis was performed for all *in vitro* assays, followed by pairwise comparisons using a two-tailed student's *t*-test, both at a 95% confidence interval; where *p* values < 0.05 were considered statistically significant (**p* < 0.05, ***p* < 0.01 and ****p* < 0.001).

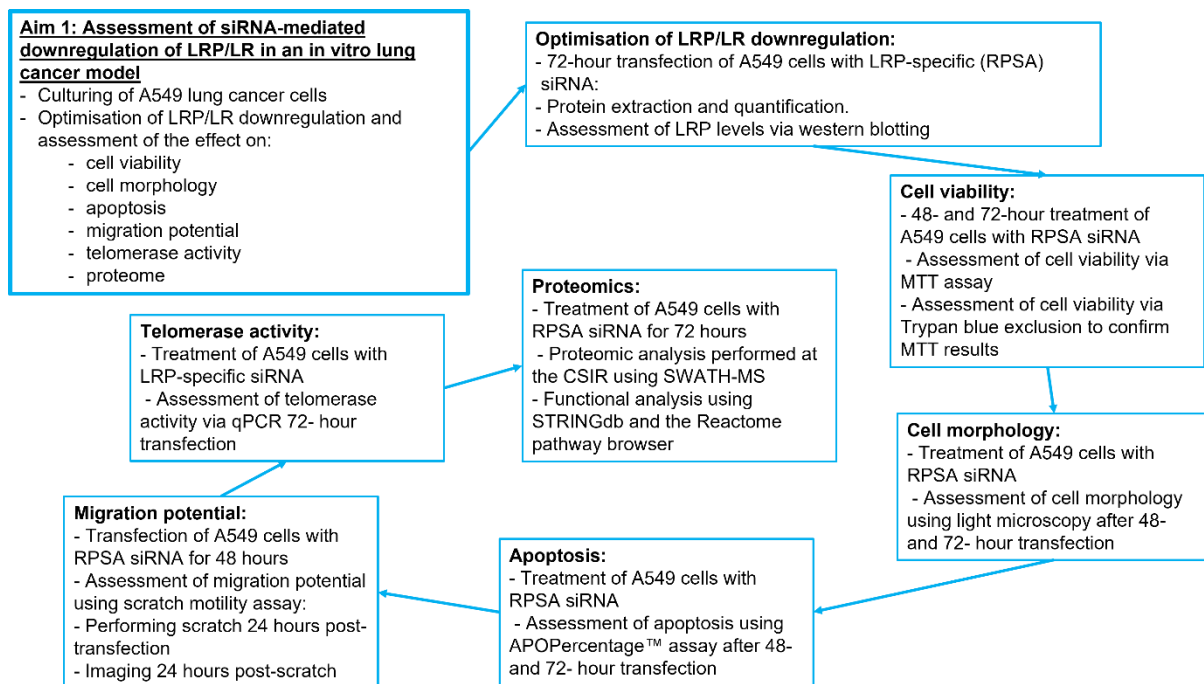


Figure 9: Brief overview of the study design of Aim 1

A brief outline of the experimental procedures performed to assess the effect of downregulating LRP/LR in the A549 lung cancer cells.

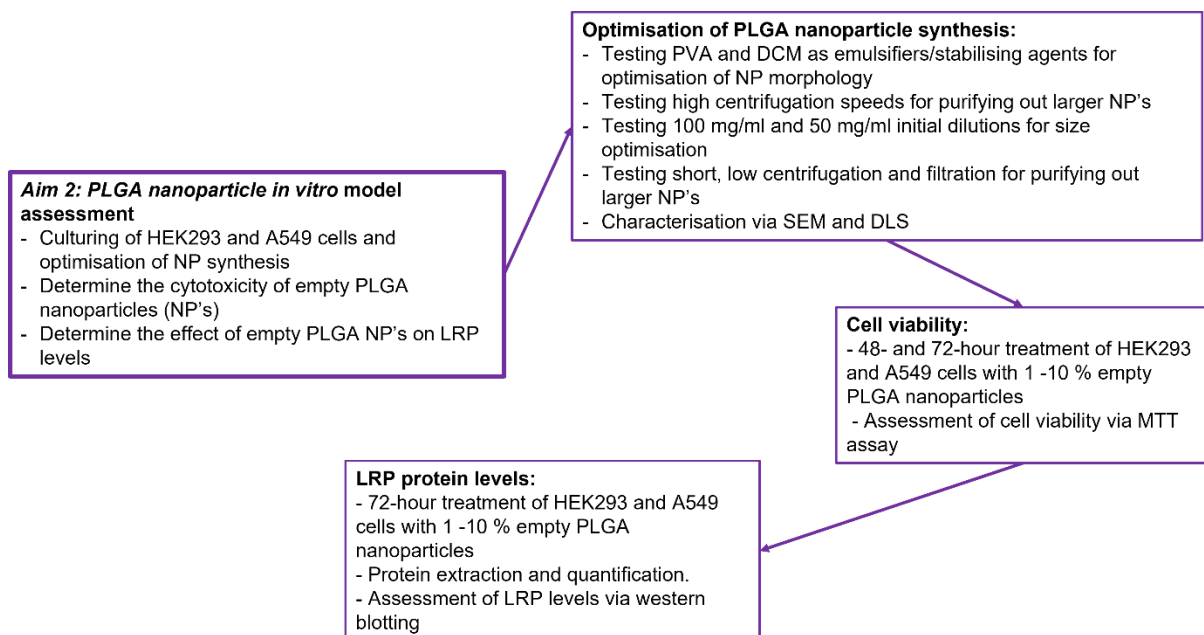


Figure 10: Brief overview of the study design of Aim 2.

A brief outline of the experimental procedures performed to assess the use of PLGA nanoparticles by investigating the effect of the empty nanoparticles in non- and tumourigenic cells.

CHAPTER THREE - RESULTS

The first part of this study focussed on assessing the effect of downregulating LRP/LR in A549 lung cancer cells, to determine whether targeting LRP/LR for downregulation would offer a novel and potential therapeutic strategy for the treatment of lung cancer. Thereafter, the aim was to assess whether PLGA nanoparticles would provide an effective method for the delivery of LRP-targeting siRNA. All figures containing SEM micrographs can be found enlarged in the Appendix.

3.1. Determining the effect of siRNA-mediated downregulation of LRP/LR on multiple cancer hallmarks in an *in vitro* lung cancer (A549 cells) model

Since downregulating LRP/LR with LRP mRNA-targeting siRNA has been shown to have a significant therapeutic effect against various cancer types *in vitro*, the first part of this study included performing a transient transfection with LRP-specific siRNA (RPSA siRNA) and subsequently evaluating the effect of the downregulation of LRP/LR on cell viability, as well as on apoptosis, migration potential and telomerase activity. Further to this, a full proteomic analysis was performed to gain more insight into the role of LRP/LR in lung cancer, as well as an improved understanding into the pathways involved when LRP/LR is downregulated.

3.1.1. Optimising the downregulation of LRP/LR

Since we had yet to assess the effect of targeting LRP for downregulation using the MISSION® esiRNA-RPSA in the A549 lung cancer cells, it was thus imperative to optimise this process, prior to any experimentation being performed with the purpose of determining the downstream effects of downregulating LRP/LR.

To optimise the downregulation of LRP levels in the A549 lung cancer cells, transfection with LRP-specific siRNA (RPSA) as well as with a universal negative control siRNA (RLUC) was performed and the cells incubated for 72 hours. Thereafter, LRP protein levels were assessed by western blotting with subsequent densitometric analysis. The downregulation was successful in that the RPSA siRNA transfected cells exhibited a 40% decrease in LRP levels, however the cells transfected with RLUC siRNA also displayed a significant 10% decrease in LRP levels (Figure 11. A). A significant downregulation of LRP protein levels by at least 60% in the RPSA siRNA transfected cells was desired, in order to observe significant effects in downstream experimentation and therefore, the siRNA amounts were increased. Since a change

in LRP levels in the negative control siRNA transfected cells implies off-target effects, a different negative control siRNA (a non-targeting control – NTC siRNA) was assessed. Additionally, a mock transfection control (transfection reagent only) was included to account for any effects possibly caused by the transfection reagent. There were no apparent effects on LRP levels in either the NTC siRNA or mock transfected cells, when compared to the non-transfected cells. However, LRP levels in the RPSA siRNA transfected cells indicated a significant downregulation of 73%, as was desired (Figure 11. B). These conditions were therefore carried through for all downstream experimentation.

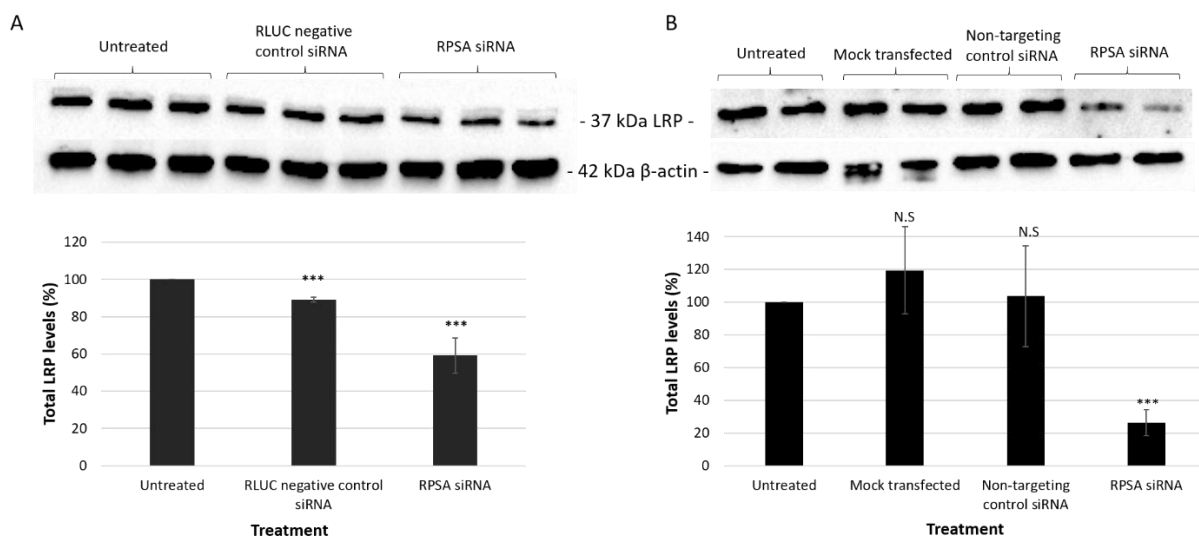


Figure 11: Transfection of A549 cells with LRP-targeting siRNA significantly decreases total LRP/LR levels.

Western blotting with subsequent densitometric analysis showed a significant decrease in LRP levels after a 72-hour transient transfection of A549 cells with the LRP-specific siRNA (RPSA) however, the negative control siRNA (RLUC) likewise caused a significant decrease in LRP levels. The negative control should not affect protein levels; therefore, a different negative control siRNA was tested. Indeed, the non-targeting control (NTC) siRNA and mock transfections did not significantly affect LRP levels. (A) LRP levels were significantly decreased by 40% in the RPSA siRNA transfected cells, with a significant decrease of 12% in LRP levels observed in the RLUC transfected cells. (B) One-way ANOVA analysis showed a significant (** $p = 0.0084$) impact of the transfection on LRP levels, which were decreased by 74% in the RPSA siRNA transfected cells, with no significant effect on LRP levels observed in the NTC and mock transfected cells. β -actin was used as the loading control. Untreated is set to 100%, with all samples expressed as a percentage of the untreated samples. This data is representative of the mean $\pm$ SD, $n=3$ biological repeats. N.S = nonsignificant, * $\leq p$, 0.05, ** $\leq p$, 0.01, *** $\leq p$, 0.001; Student's t -test.

3.1.2. siRNA-mediated downregulation of LRP/LR decreases cell viability and induces an apoptotic morphology

Resistance to cell death, including the evasion of apoptosis, is another crucial way in which cancer cells continue to survive, even when conditions are unfavourable for non-cancerous cells. This is therefore a vital hallmark of cancer, which needs to be targeted as part of the search for cancer therapeutics. The overexpression of LRP/LR in cancer cells significantly contributes towards the viability and inhibition of apoptotic pathways in cancer cells [164]. Consequently, downregulating LRP/LR should therefore decrease cell viability, and increase apoptosis.

Since a significant decrease in LRP protein levels was observed after transfection of the A549 cells with RPSA siRNA, cell viability was subsequently assessed via MTT and Trypan blue exclusion assays. A significant decrease in cell viability was observed in the A549 cells transfected with RPSA siRNA after both 48- and 72 hours, whereas no significant decrease in cell viability was observed in the NTC siRNA and mock transfected controls when compared to the untreated cells (Figure 12). In particular, when assessed via MTT assay, the cells transfected with RPSA siRNA for 48- and 72 hours showed a significant 11.65% and 70.45% decrease in cell viability, respectively (Figure 12. A). Furthermore, the cells treated with 8 mM PCA as the positive control exhibited a significant decrease in cell viability as expected, when compared to the untreated cells. Additionally, when assessed via the Trypan blue exclusion assay for confirmation, the cells transfected with RPSA siRNA for 72 hours showed that only 33.33% of the cells remained viable, while again, the NTC siRNA transfected cells showed no significant change in cell viability. (Figure 12. B). Therefore, confirming that indeed, the downregulation of LRP/LR significantly reduces cell viability.

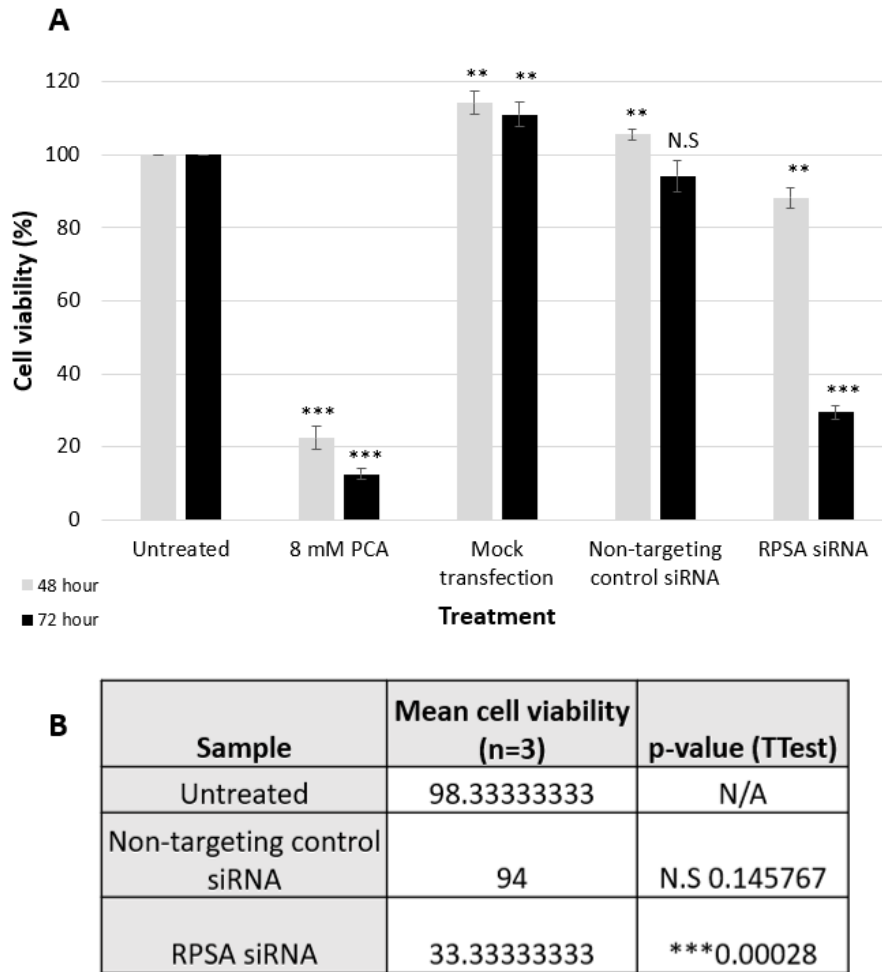


Figure 12: siRNA-mediated downregulation of LRP/LR significantly decreases cell viability in A549 lung cancer cells.

Cellular viability of A549 cells as determined by (A) 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) and (B) Trypan blue exclusion assays after downregulation of LRP/LR with RPSA siRNA. (A) Cell viability was assessed via MTT assay 48- and 72 hours post-transfection, with the untreated set to 100%. One-way ANOVA analyses showed a significant impact of downregulating LRP/LR on decreasing cell viability by 11.65% and 70.45% after 48- (** $p = 3.21E-11$) and 72 hours (** $p = 8.86E-12$), respectively. Protocatechuic acid (PCA) was used as a positive control. (B) Cell viability was assessed via Trypan blue exclusion 72 hours post-transfection; one-way ANOVA analysis indicates a significant ($p = 1.39E-05$) effect of downregulating LRP/LR on cell viability, with a decrease of 67% observed. The mock transfected and cells transfected with non-targeting control (NTC) siRNA present no significant decrease in cell viability. All values indicate the mean $\pm$ STD. Error bars represent standard deviation. $n=3$ biological repeats. N.S = nonsignificant, $* \leq p, 0.05$, $** \leq p, 0.01$, $*** \leq p, 0.001$; Student's t -test.

Considering the observed decrease in cell viability, the cellular morphology was then assessed via light microscopy to obtain an indication of the mode of cell death occurring. The A549 cells were therefore transfected as mentioned above, for 48- and 72 hours. Evident cell shrinkage and membrane blebbing, characteristic of apoptosis [208, 209], was observed after LRP/LR downregulation, thus indicating the induction of apoptosis in these transfected A549 cells (Figure 13). Untreated cells and cells transfected with NTC siRNA exhibited normal morphology with no indication of cell death observed both 48-and 72 hours post-transfection (Figure 13). Therefore, portraying that the downregulation of LRP/LR significantly decreases A549 lung cancer cell viability and induces an apoptotic morphology.

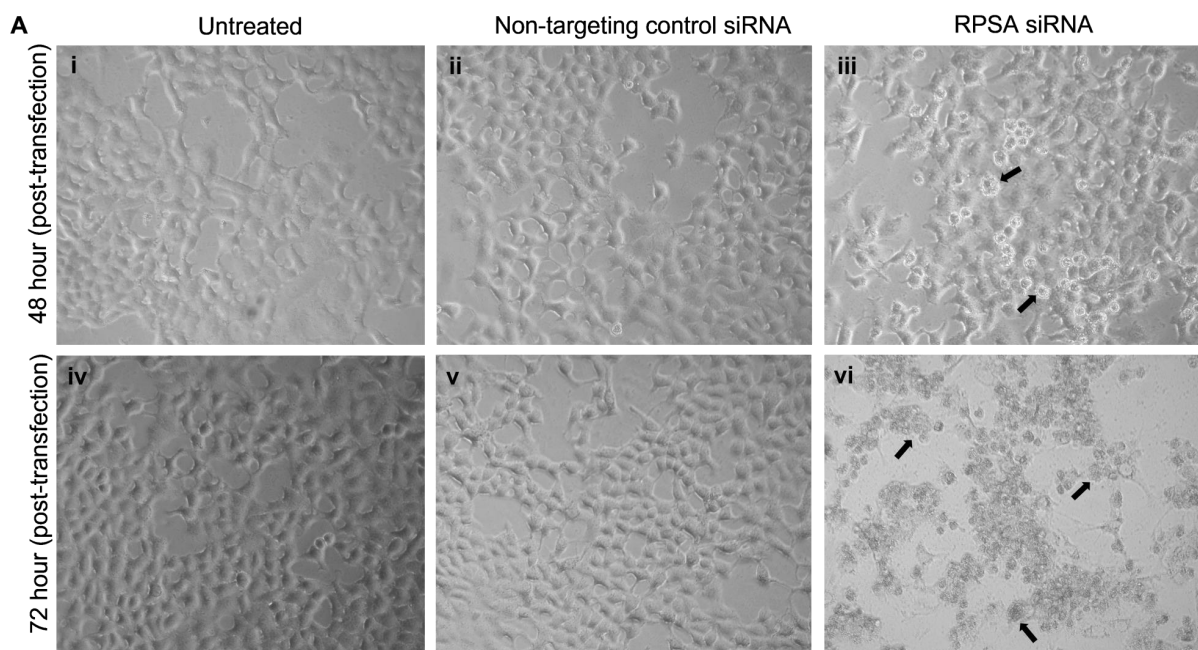


Figure 13: Downregulation of LRP/LR induces an apoptotic morphology in A549 cells.

Light microscopy was used to assess cellular morphological changes which occurred after downregulation of LRP/LR. Representative phase-contrast images. (i-iii) A549 cells that were (i) untreated, (ii) transfected with the non-targeting control (NTC) siRNA and (iii) transfected with RPSA siRNA and imaged 48 hours post-transfection. (iv-vi) A549 cells that were (iv) untreated, (v) transfected with the NTC siRNA and (vi) transfected with RPSA siRNA and imaged 72 hours post-transfection. (i-ii & iv-v) Indicate the normal morphological characteristics of epithelial cells growing adherently in a monolayer, demonstrating that the NTC siRNA and transfection reagent have no effect on the morphology of the cells. (iii) A549 cells start to exhibit cell-shrinkage and membrane blebbing suggestive of apoptosis after 48- hours. (vi) After 72 hours, most of the A549 cells are displaying apoptotic morphology, including cell-shrinkage and membrane blebbing. Black arrows point to

apoptotic cells and all images were taken at 40 X magnification. Enlarged images can be found in Appendix B.

3.1.3. Downregulation of LRP/LR induces apoptosis

LRP/LR plays a large role in apoptosis and it has been shown that once LRP/LR has bound to laminin, LRP/LR can then interact with focal adhesion kinase and this interaction then activates survival pathways, subsequently, anti-apoptotic proteins are upregulated, which consequently inhibits apoptosis [164]. Therefore, the upregulation of LRP/LR, which enhances activation of survival pathways, through its interaction with laminin, inhibits apoptosis. Thus, downregulation of LRP/LR should reduce this interaction with laminin and subsequently prevent the activation of survival pathways, thereby allowing for the activation of apoptosis.

Since the downregulation of LRP/LR reduced cell viability and the cells exhibited an apparent apoptotic morphology, an APOPercentage™ assay was performed to determine whether the mode of cell death induced was indeed apoptosis. Stained cells were imaged using light microscopy, whereafter the stain was quantified spectrophotometrically. When the NTC siRNA and mock transfected cells were analysed via light microscopy, there were no apparent stained cells and no apparent apoptotic morphological characteristics, therefore providing no visible indication of apoptosis occurring in these cells (Figure 14. B). However, when determined spectrophotometrically, the mock and NTC siRNA transfected cells showed a slight, but significant increase of 0.53- and 0.41-fold in apoptosis, respectively, after the 72-hour transfection (Figure 14. C). Furthermore, the other assays performed did not indicate any off-target effects caused by the mock and NTC siRNA transfections. Therefore, it is possible that these wells were not sufficiently washed after staining, resulting in some background absorbance being quantified when measured with UV/VIS spectrophotometry. Conversely, the A549 cells transfected with RPSA siRNA after 48- and 72 hours were clearly stained (Figure 14. A) and exhibited a significant 1.65- and 2.51-fold increase in apoptosis, respectively (Figure 14. C). This therefore indicates that indeed the decrease in cell viability observed after downregulation of LRP/LR can be attributed to apoptosis.

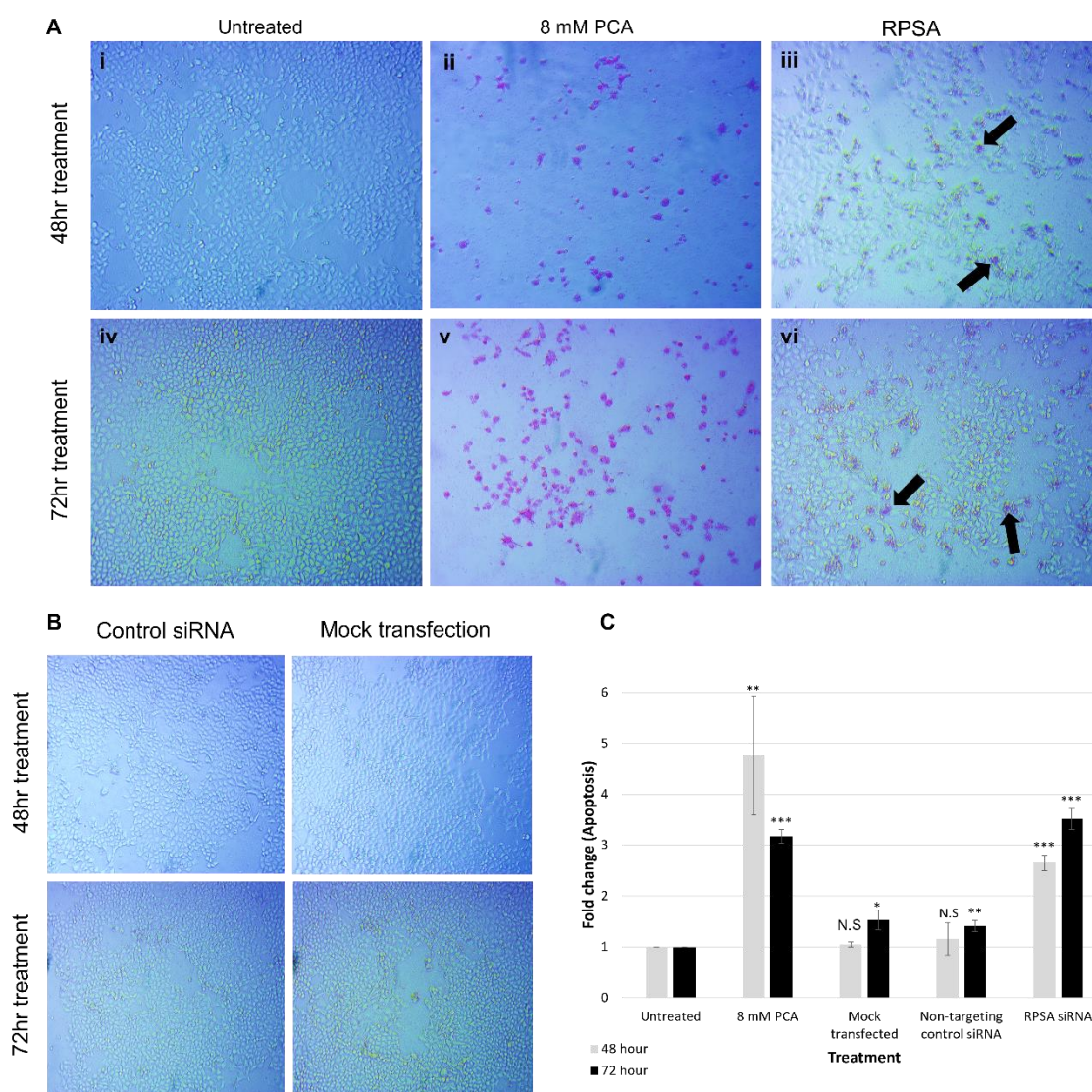


Figure 14: siRNA-mediated downregulation of LRP/LR significantly increase apoptosis in A549 lung cancer cells.

Apoptosis in A549 cells as determined by Cell-APOPercentage™ assay after downregulation of LRP/LR with RPSA siRNA. This assay makes use of a dye that gets imported selectively into cells undergoing apoptosis, whereas necrotic cells are unable to retain the dye and remain unstained. After imaging, the dye is released into solution and the concentration obtained using a microplate reader. (A) Representative phase-contrast images of the A549 cells 48- and 72 hours post-transfection, with pink cells stained with the APOPercentage™ dye undergoing apoptosis indicated by black arrows. (i-iii) A549 cells (i) untreated, (ii) treated with 8 mM PCA and (iii) transfected with RPSA siRNA for 48 hours. (iv-vi) A549 cells (iv) untreated, (v) treated with 8 mM PCA and (vi) transfected with RPSA siRNA for 72 hours. (B) Representative phase-contrast images of the mock and NTC transfected A549 cells 48- and 72 hours post-transfection, indicating no apoptosis. (C) Colorimetric analysis of the fold change in apoptosis was performed 48- and 72 hours post-transfection. One way ANOVA analysis showed a significant effect of the treatments on apoptosis after 48 (***) and 72 (***) hours.

2.47E-08) hours with a 1.65- and 2.51-fold increase observed, respectively. The untreated was set to 1 and 8 mM protocatechuic acid (PCA) was used as a positive control. All values indicate the mean $\pm$ STD. n=3 biological repeats. N.S = nonsignificant, * $\leq p$, 0.05, ** $\leq p$, 0.01, *** $\leq p$, 0.001; Student's *t*-test. Enlarged images can be found in Appendix B.

3.1.4. Downregulating LRP/LR decreases cell migration potential

The activation of invasion and metastasis is one of the key cancer hallmarks that are currently known [7], and as mentioned previously, is a characteristic which is often ignored during standard methods for discovery and development of cancer drugs [132]. One of the processes of adhesion of a metastatic cell is mediated by the interaction between LRP/LR and laminin-1. Since the overexpression of LRP/LR on cancer cells increases this interaction, causing enhanced invasion and migration of the tumorigenic cells [137, 138], then downregulating LRP/LR should decrease this interaction, and therefore the metastatic processes which follow.

Cell migration is a key process in metastasis, therefore, the A549 cells were transfected with the RPSA siRNA for 48 hours, during which a scratch assay was performed to assess cell migration potential (Figure 15). After 24 hours of transfection the scratch was made, and the cells imaged immediately (0 hour) (Figure 15. A. i-iii). The cells were subsequently imaged again, 24 hours post-scratch, thus 48 hours after transfection (Figure 15. A. iv-vi). The cells transfected with the RPSA siRNA exhibited only 10.17% wound closure, compared to the untreated cells exhibiting 42.29% wound closure, therefore indicating a significant decrease in wound closure in the cells transfected with RPSA siRNA (Figure 15. B). No significant difference in wound closure was observed in the NTC siRNA transfected cells, when compared to the untreated cells. Since the anti-proliferative agent, Mitomycin C, was included during the Scratch assay, it can confidently be concluded that the wound closure observed was due to cell migration, rather than cell proliferation. Thus, the downregulation of LRP/LR therefore results in a significant 32.12% decrease in the migration potential of A549 cells.

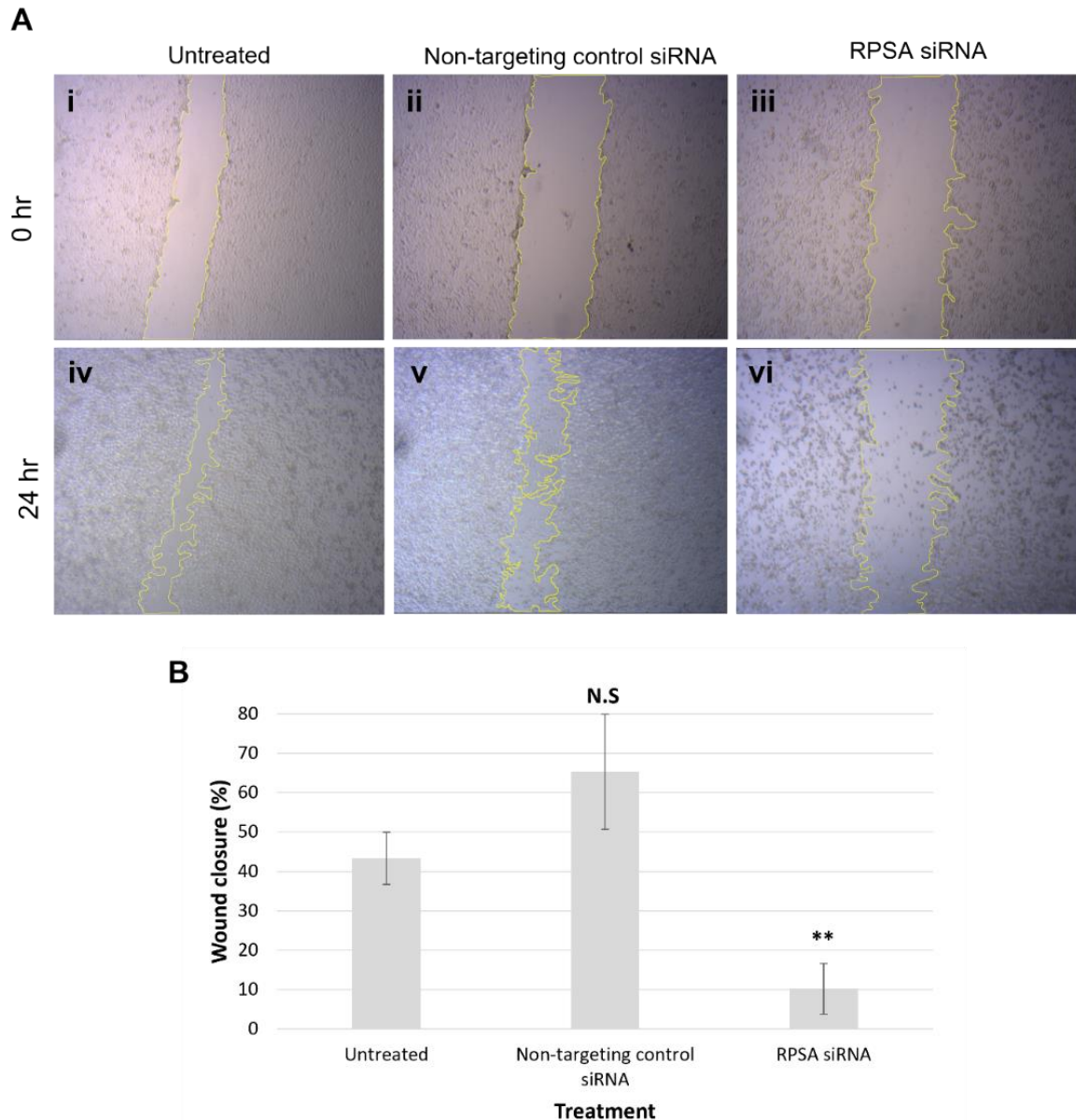


Figure 15: Downregulation of LRP/LR significantly decreases A549 cellular migration.

A scratch motility assay shows a significant decrease in the percentage wound closure, and therefore, a significant decrease in the migration potential of A549 cells after 48-hour siRNA-mediated downregulation (RPSA siRNA) of LRP/LR. A549 cells were transfected 24 hours prior to the scratch assay being performed, with images taken 24 hours (0 hour, when the scratch was made) and 48 hours (24 hours post-scratch) post transfection. **(A)** Representative phase-contrast images show the decrease in cell migration, whereby the yellow outlines indicate the area of the wound. **(i-iii)** A549 cells **(i)** untreated, **(ii)** transfected with non-targeting control (NTC) siRNA and **(iii)** transfected with RPSA siRNA for 24 hours, with the scratch performed 24 hours post-transfection (0 hour) showing no cell migration has occurred. **(iv-vi)** A549 cells **(iv)** untreated, **(v)** transfected with NTC siRNA and **(vi)** transfected with RPSA siRNA for 48 hours, showing varying degrees of cell migration has occurred. **(B)** One-way ANOVA analysis showed a significant (** $p=0.0015$) impact of the transfection on the

migration potential. Analysis of the difference between the area of the scratch at 0 hour and at 24 hours post-scratch indicates a significant 33% decrease in wound closure, and therefore migration potential in the RPSA transfected A549 cells, while the non-targeting control (NTC) siRNA has no significant effect on migration potential. This data is representative of the mean $\pm$ SD, n=3 biological repeats. N.S = nonsignificant, * $\leq p$, 0.05, ** $\leq p$, 0.01, *** $\leq p$, 0.001; Student's t-test. Enlarged images are in Appendix B.

3.1.5. Downregulating LRP/LR inhibits telomerase activity

The upregulation of telomerase activity in cancer cells is an additional way in which cancer cells tend to override inhibitory signals, which further allows the cells to replicate in the presence of genomic instability, rather than undergoing senescence or cell death [57]. We have previously shown that LRP levels have a direct correlation with telomerase activity. Whereby, the overexpression of LRP significantly increases telomerase activity [128, 131] and the downregulation of LRP/LR significantly decreases telomerase activity [123, 165]. Therefore, this study included investigating whether the downregulation of LRP/LR would affect telomerase activity in the A549 lung cancer cells.

Therefore, the A549 cells were transfected with RPSA siRNA for 72 hours, after which the modified TRAP assay was performed via qPCR to detect telomerase activity levels. A highly significant decrease in telomerase activity was observed, whereby the cells transfected with RPSA siRNA exhibited a 99.19% decrease in telomerase activity, when compared to the untreated sample which was set to 100% (Figure 16). The NTC siRNA transfected samples showed no significant change in telomerase activity, as was expected, when compared to the untreated sample. Therefore, the downregulation of LRP/LR causes an almost complete inhibition of telomerase activity in the A549 lung cancer cells and as such, the cell's immortality and replicative ability are impeded, thus targeting an additional cancer hallmark.

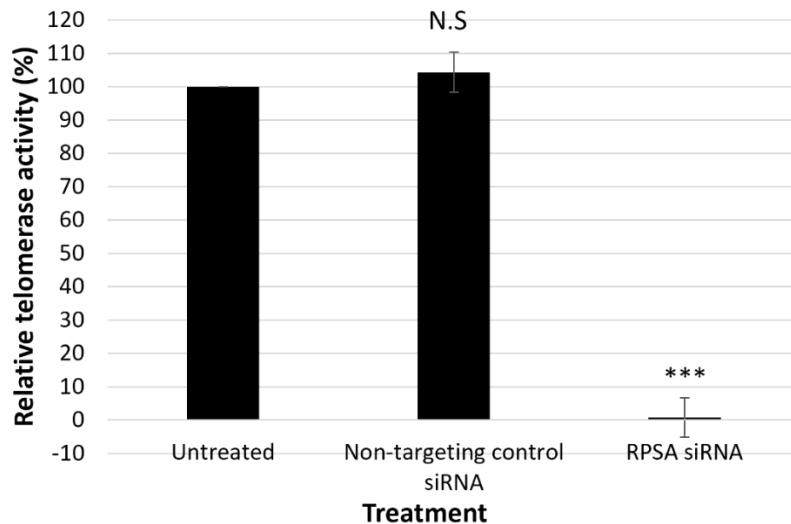


Figure 16: Downregulation of LRP/LR significantly decreases telomerase activity in A549 cells.

Relative telomerase activity, as assessed by qPCR using the modified TRAP assay, is significantly ($***p= 1.23E-04$) affected in A549 cells after 72-hour transfection, as shown by one-way ANOVA analysis. All values were normalized against the negative telomerase activity/heat-treated controls. LRP-specific (RPSA) siRNA downregulation of LRP/LR caused a significant 99.19% decrease in telomerase activity. The cells transfected with the NTC siRNA exhibited no significant change in telomerase activity. Untreated is set to 100%, with all samples expressed as a percentage of the untreated samples. This data is representative of the mean $\pm$ SD, $n=3$ biological repeats. N.S = nonsignificant, $* \leq p, 0.05$, $** \leq p, 0.01$, $*** \leq p, 0.001$; Student's *t*-test.

3.1.6. Assessing the effect of downregulating LRP/LR on the proteome of A549 cells

In order to get a broader overview of the mechanistic role of LRP/LR downregulation, SWATH-MS was performed after a 72-hour transfection with RPSA-siRNA, with subsequent protein set enrichment analysis using STRING database and the Reactome pathway browser.

All identified dysregulated proteins were analysed initially using STRINGdb in order to gain some insight into the protein-protein interactions occurring within the dataset (Figure 17. B). Within this dataset, it was revealed that 22 proteins were downregulated, whilst 52 proteins were found to be upregulated after LRP downregulation. The fold change within this dataset ranged between -3.71974 and 7.2337 (Figure 17. A). Furthermore, since four replicates were used per treatment, standard guidelines suggest that reporting data analysis should only include proteins with a minimum 2-fold change, whereas for reporting on proteins with a 1.5-fold

change, 10 replicates would be needed [210]. Therefore, only the proteins with a fold change of 2 and above underwent further enrichment analysis and have been reported on.

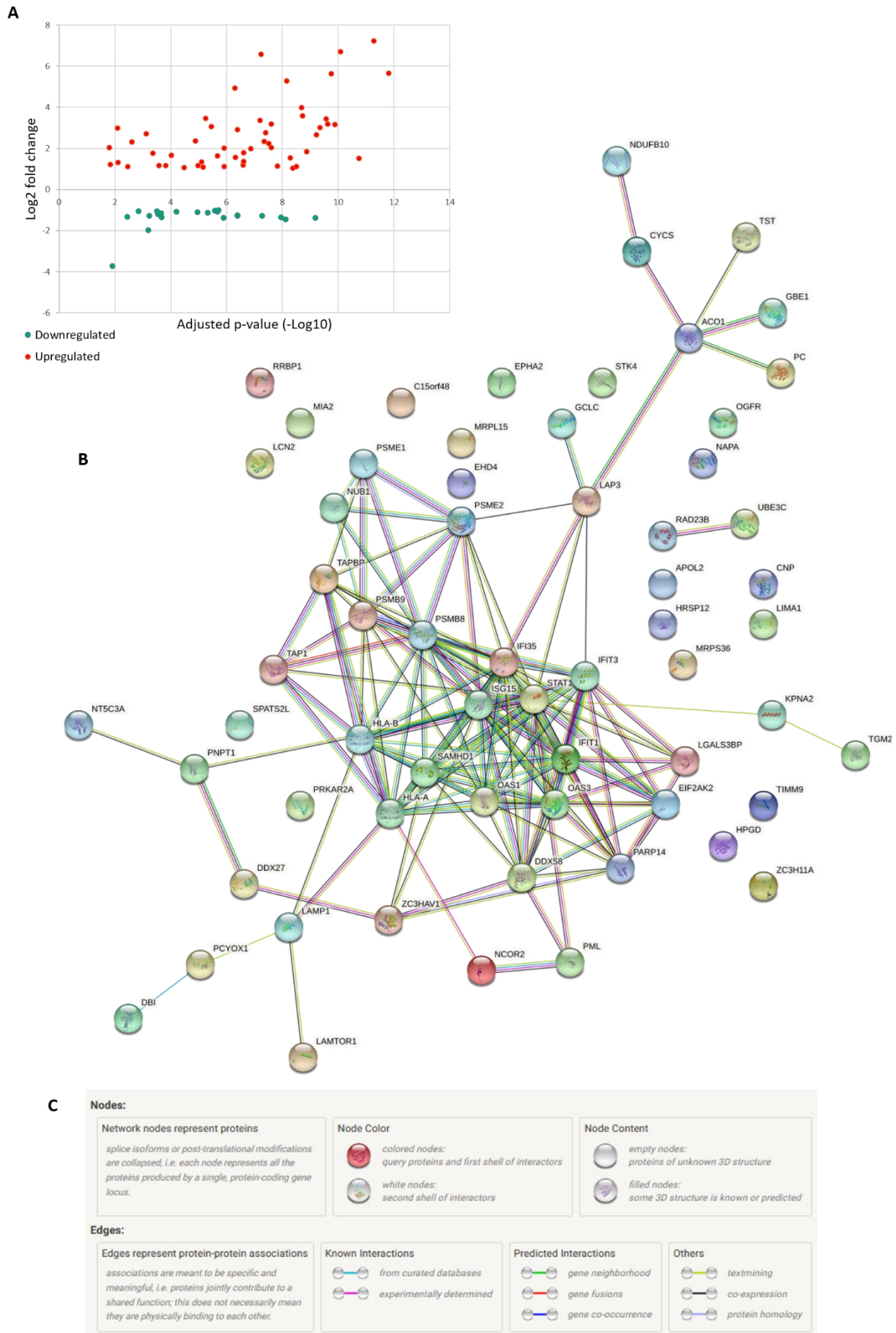


Figure 17: All dysregulated proteins identified by proteomics analysis.

Protein-protein interaction network of all dysregulated proteins associated with downregulation of LRP/LR after a 72-hour transfection. (A) A scatter plot indicating the dysregulated proteins identified via SWATH-MS; 22 proteins were seen to be downregulated and 52 proteins were observed to be upregulated. (B) The STRING database was used to generate the network, where potential interactions of proteins with medium confidence are shown. (C) The circular bubbles are referred to as 'nodes' and indicate the query proteins. The colours of the nodes indicate a different protein and is not representative of up- or downregulation. The lines between the nodes indicate 'edges' which represent the protein-protein interactions. These interactions are not necessarily indicative of physical binding interactions between the proteins but show a biologically meaningful shared interaction. The colours of the edges represent different types of evidence for each interaction.

Therefore, 32 dysregulated proteins were identified with a 2-fold change and above and further, four distinct protein-protein interaction networks were clustered according to common shared pathways (Figure 18). These include the main cluster (green) involved in cytokine signalling in the immune system, a cluster of 6 proteins (pink) involved in the adaptive immune system, a cluster of 3 proteins (black) involved in nucleotide metabolism, a cluster of 2 peptides (blue) involved in transcription, as well as a cluster of 6 unconnected proteins (red) which indicate a lack of interaction (currently known) with any of the other query proteins (Figure 18). The protein-protein interaction enrichment p-value was calculated by STRINGdb to be less than $1.0E-16$, which indicates that this group of proteins are at least moderately biologically associated, therefore inferring a common relationship amongst the query proteins. The full list of pathways identified by Reactome for each of these proteins can be found in Table 1.

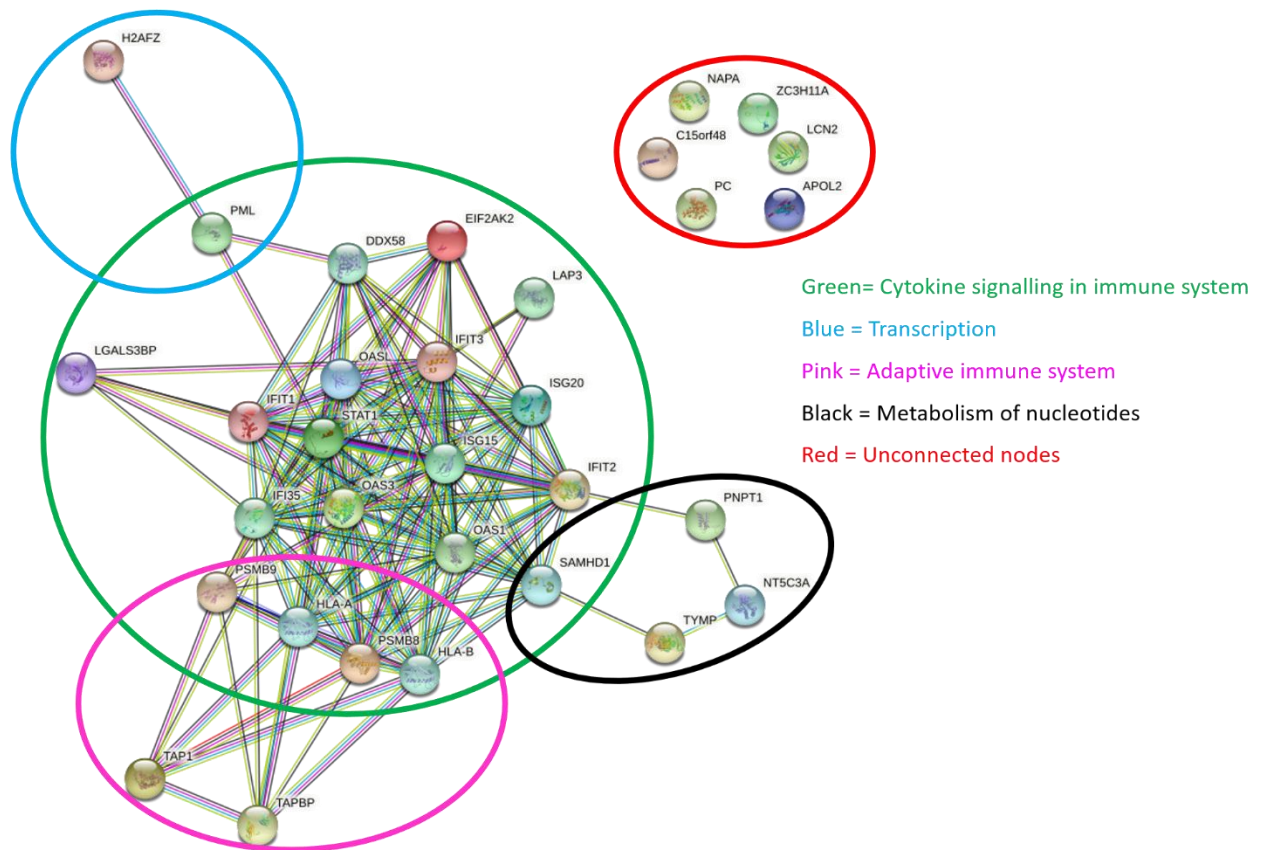


Figure 18: Identification of interactions between all dysregulated proteins above a 2-fold change and analysed via STRING database.

Protein-protein network of all dysregulated proteins with a fold change of 2 or above, four distinct protein-protein interaction networks are clustered according to common shared pathways. These include the main cluster (green) involved in cytokine signalling in the immune system, a cluster of 6 proteins (pink) involved in the adaptive immune system, a cluster of 4 proteins (black) involved in nucleotide metabolism, a cluster of 2 peptides (blue) involved in transcription, as well as a cluster of 6 unconnected proteins (red) which indicate a lack of interaction with any of the other query proteins. The circular bubbles are referred to as 'nodes' and indicate the query proteins. The colours of the nodes indicate a different protein and is not representative of up- or downregulation. The lines between the nodes indicate 'edges' which represent the protein-protein interactions. These interactions are not necessarily indicative of physical binding interactions between the proteins but show a biologically meaningful shared interaction. The colours of the edges represent different types of evidence for each interaction. PPI enrichment p-value: $<1.0E-16$.

Table 1: List of the dysregulated proteins above a 2-fold change and their function in biological pathways (Unconnected nodes, Transcription, Nucleotide metabolism, cytokine signalling, Adaptive immune system)

Fold change	Gene/STRING identifier (UniProt ID)	Protein Name	Pathway/function ^a
-3.7197	ZC3H11A (O75152)	Zinc finger CCCH domain-containing protein 11A	Transcription, metabolism of RNA, meiosis
-1.9631	H2AFZ (P0C0S5)	Histone H2A.Z	Cell cycle, cellular response to stress, chromatin modification, activation of HOX genes, disease of programmed cell death, base excision repair, DNA replication pre-initiation, transcription, protein metabolism, NOTCH + Wnt + RHO + nuclear receptor signal transduction
1.9839	C15orf48 (Q9C002)	Normal mucosa of esophagus-specific gene 1 protein	Unclassified
2.0217	APOL2 (Q9BQE5)	Apolipoprotein L2	Unclassified [211]
2.0361	LCN2 (P80188)	Neutrophil gelatinase-associated lipocalin	Innate immune system – cytokine signalling, iron uptake and transport
2.0493	NAPA (P54920)	Alpha-soluble NSF attachment protein	Post-translational protein modification, vesicle-mediated transport - membrane trafficking
2.2511	PNPT1 (Q8TCS8)	Polyribonucleotide nucleotidyltransferase 1, mitochondrial	Nucleotidyl group transfer [212]
2.3045	PC (P11498)	Pyruvate carboxylase, mitochondrial	Disease of metabolism, metabolism of carbohydrates + vitamins and cofactors
2.3345	EIF2AK2 (P19525)	Interferon-induced, double-stranded RNA-activated protein kinase	Infectious disease, cytokine signalling
2.3584	PSMB8 ^b (P28062)	Proteasome subunit beta type-8	Cell cycle, cellular response to stimuli, developmental biology, disease of signal transduction and transmembrane transporters, infectious disease, DNA replication pre-initiation - DNA synthesis, adaptive and innate immune system – cytokine signalling, post-translational protein modification, regulation of mRNA stability, metabolism of amino acids. Apoptosis, NOTCH signalling, Pol II

			transcription, ABC-family protein transport
2.6578	PSMB9 (P28065)	Proteasome subunit beta type-9	Cell cycle, cellular response to stimuli, developmental biology, disease of signal transduction and transmembrane transporters, infectious disease, DNA replication pre-initiation – DNA synthesis, adaptive and innate immune system – cytokine signalling, post-translational protein modification, regulation of mRNA stability, metabolism of amino acids, apoptosis, NOTCH signalling, Pol II transcription, ABC-family protein transport
2.7058	PML (P29590)	Promyelocytic Leukaemia Protein	Infectious disease, cytokine signalling, Post- translational protein modification, Pol II transcription
2.7725	TAP1 (Q03518)	Antigen peptide transporter 1	Adaptive immune system
2.9067	LGALS3BP (Q08380)	Galectin-3-binding protein	Haemostasis -platelet activation, signalling and aggregation
2.9820	IFIT2 (P09913)	Interferon-induced protein with tetratricopeptide repeats 2	Cytokine signalling
3.0249	TYMP (P19971)	Thymidine phosphorylase	Metabolism of nucleotides
3.0590	ISG20 (Q96AZ6)	Interferon-stimulated gene 20 kDa protein	Cytokine signalling
3.1678	NT5C3A (Q9H0P0)	Cytosolic 5'-nucleotidase 3A	Metabolism of nucleotides
3.1814	LAP3 (P28838)	Cytosol aminopeptidase	Lysosomal proteolytic degradation [213]
3.1882	TAPBP (O15533)	Tapasin	Adaptive immune system
3.3612	SAMHD1 (Q9Y3Z3)	Deoxynucleoside triphosphate triphosphohydrolase	Cytokine signalling, metabolism of nucleotides
3.4444	IFI35 (P80217)	Interferon-induced 35 kDa protein	Cytokine signalling
3.4587	OAS3 (Q9Y6K5)	2'-5'-oligoadenylate synthase 3	Cytokine signalling
3.5885	STAT1 (P42224)	Signal transducer and activator of transcription 1	Disease of signal transduction, cytokine signalling, RTK and NOTCH signalling, Pol II transcription
3.9890	HLA-A (P04439)	HLA class I histocompatibility antigen, A-3 alpha chain	Adaptive immune system – cytokine signalling, post-translational protein modification

4.9251	HLA-B (P01889)	HLA class I histocompatibility antigen, B-7 alpha chain	Adaptive and innate immune system – cytokine signalling
5.2837	DDX58 (O95786)	Probable ATP-dependent RNA helicase	Innate immune system – cytokine signalling, post-translational protein modification
5.6279	OAS1 (P00973)	2'-5'-oligoadenylate synthase 1	Cytokine signalling
5.6556	IFIT3 (O14879)	Interferon-induced protein with tetratricopeptide repeats 3	Cytokine signalling
6.5920	OASL (Q15646)	2'-5'-oligoadenylate synthase-like protein	Cytokine signalling
6.7055	ISG15 (P05161)	Ubiquitin-like protein ISG15	Infectious disease, DNA repair- DNA damage bypass, innate immune system – cytokine signalling
7.2337	IFIT1 (P09914)	Interferon-induced protein with tetratricopeptide repeats 1	Cytokine signalling

^a Pathway/function of the proteins presented were identified using publicly available databases, such as UniProt (<http://www.uniprot.org>), STRING database (<https://string-db.org>) and Reactome pathway browser (<https://reactome.org/PathwayBrowser/#TOOL=AT>).

Query proteins are coloured according to the clustering observed in Figure 18.

^b Proteins with identifiers in a different colour indicate involvement in an additional pathway and are coloured according to the shared cluster.

The UniProt identifiers of these 32 proteins were then analysed using the Reactome pathway browser tool and 28 out of 32 identifiers in the query sample were found in Reactome, where 320 pathways were hit by at least one of the query proteins. The four proteins not identified as part of a pathway in Reactome include, apolipoprotein L2 (APOL2/Q9BQE5), Polyribonucleotide nucleotidyltransferase 1 (PNPT1/Q8TCS8), Cytosol aminopeptidase (LAP3/P28838) and Normal mucosa of esophagus-specific gene 1 protein (C15orf48/Q9C002). These four proteins were identified as unconnected nodes in STRINGdb and were therefore, not identified as interacting with the other query proteins. This, together with the inability for these proteins to be identified as contributing to a pathway in Reactome, indicates that there is not currently enough knowledge and evidence to suggest an interaction either amongst these proteins or between these and the other query proteins at this point.

However, of the 28 proteins identified as contributing to a known pathway in Reactome, 21 of the submitted identifiers (UniProt id's) were found to play a role in the immune system. Of these, 21 were shown to be involved in cytokine signalling in the immune system, 6 were

indicated in the innate immune system and 6 were observed to be involved in the adaptive immune system. At least 1 of each of the remaining 5 proteins were found to play a role in various other pathways including transcription, nucleotide and protein metabolism, cell cycle checkpoints, apoptosis, signal transduction and transport of small molecules via vesicle-mediated transport. The two proteins, proteasome subunit beta type-8 and type-9 (PSMB8/P28065 and PSMB9/P29590) were indicated by Reactome to play a role in almost all the identified pathways as can be seen in Table 1 and this correlates to a role in targeting small molecules for removal via the proteasomal degradation pathway.

The proteomics analysis showed that LRP downregulation caused differential expression of proteins involved in re-establishing the immune response (STAT 1, ISG15, ISG20, PSMB8, PSMB9, TAP, TAPBP, NAPA, HLA-A and HLA-B), inhibiting cell proliferation and inducing apoptosis (ISG15, ISG20, PML, OAS1, OAS3, OASL, DDX58, EIF2AK2, STAT1, SAMHD1 and H2A.z) and improving therapeutic response (IFIT1, IFIT2, IFIT3, IFI35, TYMP and NT5C3A) in cancer cells. Furthermore, LGALS3BP, PC, LCN2 and ZC3H11A had fold changes of 2.91, 2.3, 2.04 and -3.72 respectively. However, there is little evidence to suggest a role of these proteins in the lung cancer cells. Additionally, their functions in cancer appear to be controversial at present.

Therefore, altogether it has been shown that siRNA-mediated downregulation of LRP/LR in the A549 lung cancer cells ultimately leads to a reduction in cell viability via apoptosis, a decrease in migration potential, an almost complete suppression of telomerase activity as well as an apparent restoration of immune control over the cells.

3.2. Optimisation and assessment of PLGA nanoparticles as a delivery system

LRP-targeting siRNA has proven to be an effective treatment for numerous cancer types *in vitro*. However, for this to be tested further *in vivo*, an effective, non-toxic delivery system needs to be employed to overcome the many barriers that arise when attempting to systemically administer ‘naked’ siRNA for *in vivo* testing. Therefore, the second part of this study involved optimising the synthesis of PLGA nanocapsules and subsequently testing the empty nanoparticles for cytotoxicity, as well as determining whether the nanoparticles themselves

would have any off-target effects on LRP/LR levels, in both non-tumourigenic HEK293 cells, as well as tumourigenic A549 lung cancer cells.

3.2.1. Optimisation of PLGA nanoparticle synthesis

In order to reduce the cost of the study as well as time, PLGA nanoparticle synthesis was optimised on empty nanoparticles, to produce uniformly sized, spherical nanocapsules (hollow) predominantly in the 200-500nm size range. Initially, poly(vinyl) alcohol (PVA) was tested as the stabilising agent/emulsifier, however, we were unable to attain a sample with the above specified characteristics. Indeed, SEM characterisation showed that three different batches of nanoparticles produced using PVA as the emulsifier exhibited primarily cuboid/sheet-like polymer formations which greatly varied in size and formed very large aggregates (Figure 19). Further, we could not confirm whether or not the nanoparticles were hollow. Consequently, we then attempted producing the nanoparticles using Vitamin E-TPGS as the emulsifier/stabilising agent and found only spherical nanoparticles were synthesized (Figure 20).

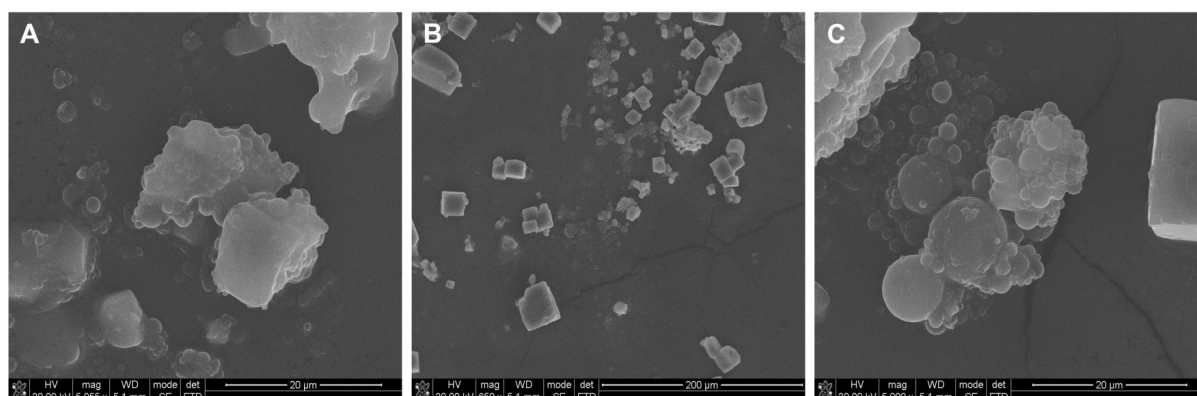


Figure 19: SEM characterisation of empty PLGA nanoparticles synthesized using poly(vinyl) alcohol as the stabilising agent.

Electron micrographs captured using scanning electron microscopy (SEM) reveal that PLGA nanoparticles synthesised using poly(vinyl) alcohol (PVA) as the emulsifier are predominantly cuboid, sheet-like polymer formations which vary substantially in size and form aggregates. (A-C) Representative micrographs of three separate batches of nanoparticles produced using PVA, indicating that PVA is not a suitable emulsifier for the PLGA nanoparticle production using the current protocol. Various magnifications were used and are provided along with scale bars on each individual micrograph.

All nanoparticles were thereafter synthesized using Vitamin E-TPGS as the emulsifying agent. SEM characterisation of the nanoparticles synthesized using a 100 mg/ml initial concentration

of PLGA polymer dissolved in dichloromethane (DCM) indicated that the nanoparticles were primarily spherical in shape, apart from a few nanoparticles which did not form completely, or were damaged as part of the characterisation process. Indeed, there were very clearly no sheet-like or cuboidal formations in any of the subsequent batches produced in this way. However, there remained a substantial size distribution within the raw, un-purified sample (Figure 20. A), with nanoparticle sizes ranging between less than 1 μm to greater than 10 μm . Consequently, we sought to centrifuge the samples at various high speeds in an attempt to remove the larger particles. Therefore, the sample was centrifuged at 8000 xg (Figure 20. B), 10 000 xg (Figure 20. C) and at 12 000 xg (Figure 20. D). Whilst this did remove most of the larger nanoparticles, there was a substantial loss in the number of nanoparticles overall and thus would not be an effective means in which to remove the larger particles for too much of the sample would need to be discarded. Thus, we attempted to synthesize the nanoparticles using a 50 mg/ml initial dilution of PLGA, however keeping the amount of PLGA polymer consistent with the 100 mg/ml batches. This resulted in a clear and considerable reduction in the size distribution, with most of the nanoparticles forming within a size range of 200 nm – 3 μm in the raw, un-purified sample (Figure 20. E). This sample was then centrifuged at 8000 xg (Figure 20. F), 10 000 xg (Figure 20. G) and at 12 000 xg (Figure 20. H), respectively. However, again this resulted in a substantial loss of sample when compared to the original raw sample and would not be an efficient way in which to narrow down the size range to the desired 200 nm – 500 nm.

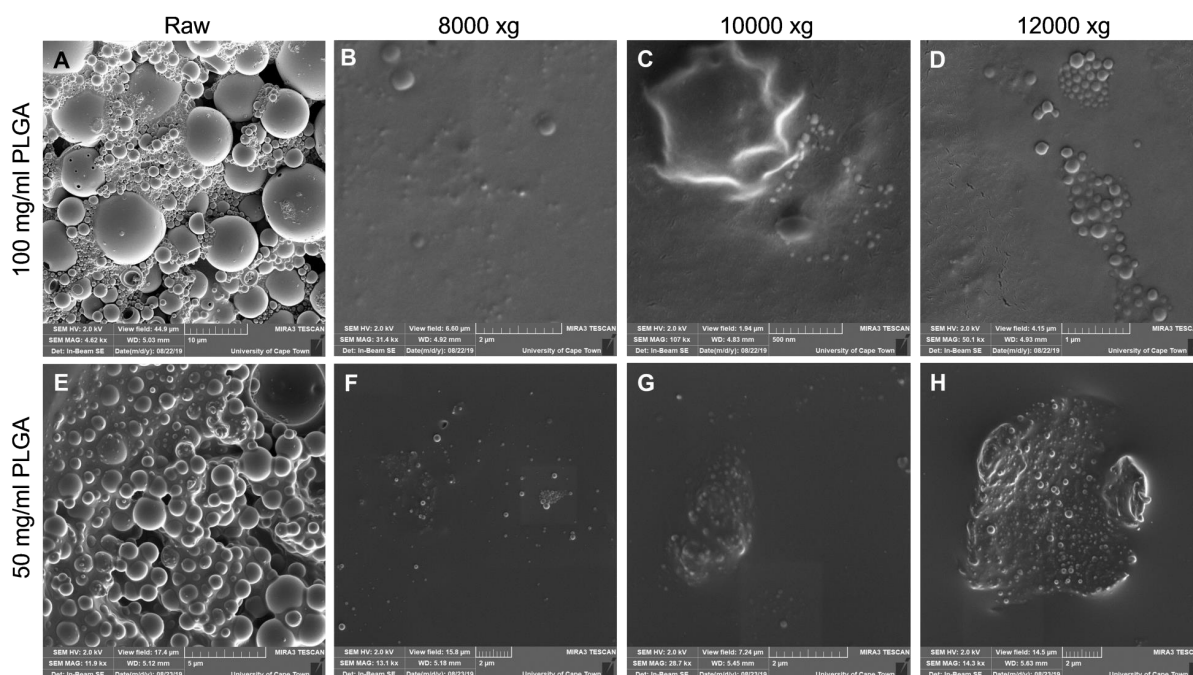


Figure 20: SEM characterisation of empty PLGA nanoparticles centrifuged at various speeds for optimisation of purification.

Representative electron micrographs captured using scanning electron microscopy (SEM) reveal that PLGA nanoparticles synthesised using a 100 mg/ml (A-D) and 50 mg/ml (E-H) initial dilution of PLGA exhibit a substantial loss of nanoparticles in the sample at higher centrifugation speeds. However, it is observed that the nanoparticles synthesised using the 50 mg/ml initial dilution produces nanoparticles that are considerably more uniform in size. (A & E) Raw, un-purified sample. (B & F) Raw sample centrifuged at 8 000 xg (C & G) Raw sample centrifuged at 10 000 xg (D & H) Raw sample centrifuged at 12 000 xg. Various magnifications were used and are provided along with scale bars on each individual micrograph.

Nevertheless, the sample produced using the 50 mg/ml initial dilution of PLGA polymer in DCM indicated a more efficient means of producing nanoparticles closer to the desired size range. Therefore, this dilution was used for all subsequent syntheses. Furthermore, SEM imaging confirmed that the nanoparticles were indeed hollow nanocapsules as desired, and not spheres of solid polymer (Figure 21. C) However, since the higher centrifugation speeds removed too much of the sample, we therefore attempted a short centrifugation with a low speed of 500 xg and indeed observed a sample that had a much lower size distribution, with nanoparticle sizes ranging between 200 nm and 500 nm (Figure 21. A, ii). Furthermore, we sought to try filtering the sample using a 0.45 μm filter, however learned that there was considerable loss of sample, and the remaining sample contained many particles outside of the desired size range (Figure 21. A, iii). This method was therefore not used in subsequent

experiments. Since the short centrifugation at 500 xg proved most efficient in isolating the nanoparticles with the desired characteristics, this method was used in all subsequent nanoparticle batch purification steps. In addition to this, dynamic light scattering (DLS) was thereafter used to assess the average hydrodynamic volume/size, as well as the polydispersity index (PDI) of the nanoparticles (Figure 21. B). The raw, un-purified sample exhibited a size of 324.87 nm, with a PDI of 0.71, while the sample that was centrifuged at 500 xg, but which was not yet further purified to remove the Vitamin E-TPGS, showed a size of 200.27 nm with a PDI of 0.78. This indicates that the mean nanoparticle size in both aforementioned samples did fall within the desired size range but that the batches remained polydisperse. Nevertheless, the purified sample, which was centrifuged at 500 xg, then further washed to remove the Vitamin E-TPGS had a mean size of 502.8 nm with a PDI of 0.38. Thus, indicating the sample fell within the desired size range and contained very little polydispersity. This procedure was therefore followed for all subsequent experimentation.

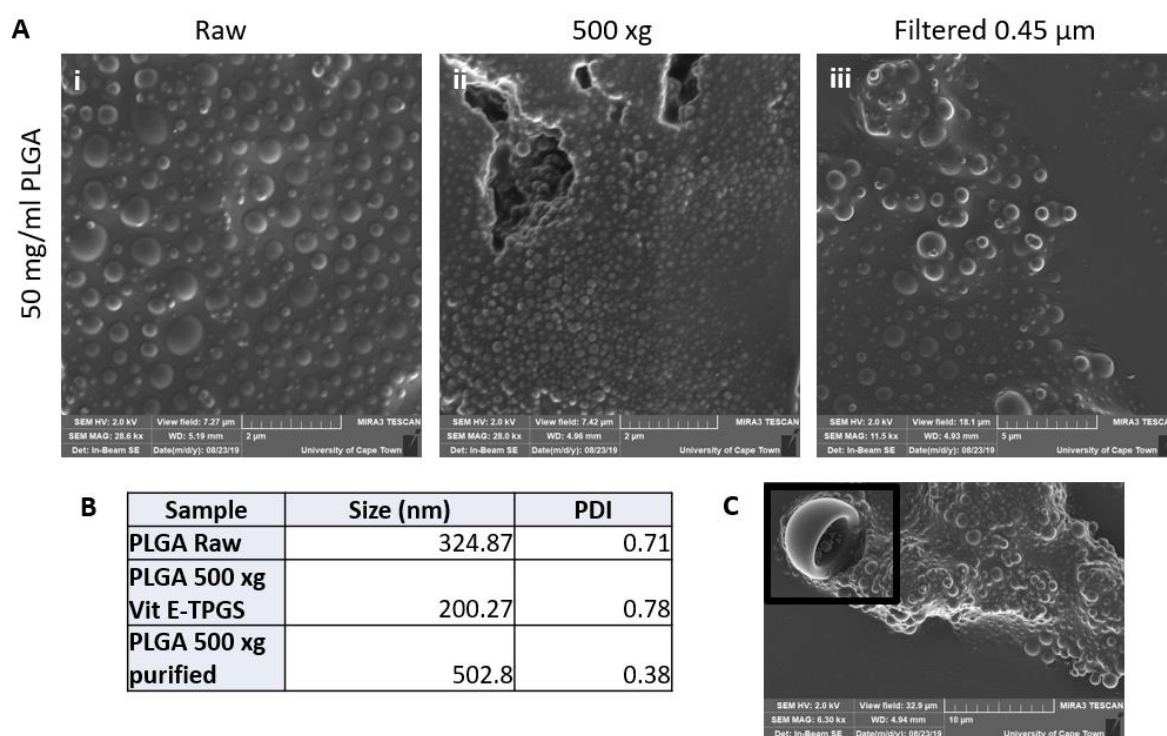


Figure 21: Characterisation of optimised empty PLGA nanoparticles.

(A) Representative electron micrographs captured using scanning electron microscopy (SEM) reveal that PLGA nanoparticles synthesised using a 50 mg/ml initial dilution of PLGA exhibit the desired characteristics after further purification using centrifugation at 500 xg. (i) The raw empty PLGA nanoparticles are reasonably uniform in size, however, still contain larger unwanted particles and require purification. (ii) The raw empty PLGA nanoparticles were centrifuged at 500 xg to remove the larger particles and it is observed that only nanoparticles in the desired size range (smaller than ± 500

nm) remain. (iii) The raw nanoparticles were filtered through a 0.45 μm filter and it can be seen that a large loss of sample occurred, with many of the remaining nanoparticles existing outside of the desired size range. (B) Dynamic light scattering results are tabulated, showing that the PLGA nanoparticles which are centrifuged at 500 xg and further purified to remove Vitamin E-TPGS have a hydrodynamic volume of 502.8 nm, with a polydispersity index (PDI) of 0.38, which confirms the correct size, as well as uniformity of size dispersion in the sample. (C) The nanoparticles produced are hollow inside (indicated by a black box), illustrating the formation of desired nanocapsules for drug encapsulation, rather than solid spheres of polymer. Various magnifications were used and are provided along with scale bars on each individual micrograph.

3.2.2. Empty PLGA nanoparticles have no cytotoxic effect in non-tumourigenic cells

Since the batch production of the nanoparticles had been optimised. These empty nanoparticles were thereafter assessed for cytotoxicity on the non-tumourigenic HEK293, as well as on the tumourigenic A549 cells.

Both cell lines were treated with 1%, 5% and 10% (v/v) of the empty nanoparticles and assessed via MTT cell viability assay in order to determine the minimum concentration at which the nanoparticles are cytotoxic (Figure 22). The non-tumourigenic HEK293 cells displayed no significant cell death after a 72-hour treatment with 1-10% of the empty PLGA nanoparticles when compared to the untreated cells (Figure 22. A). Thus, indicating that even at considerably high concentrations, the viability of the HEK293 cells was not affected by the nanoparticles, therefore, suggesting the nanoparticles do not have an effect on non-tumourigenic cells. The tumourigenic A549 cells exhibited no significant decrease in cell death after a 48-hour treatment with 1% empty nanoparticles when compared to the untreated cells. However, these cells displayed a significant 18.13% and 25.27% decrease in cell viability after a 48-hour treatment with 5% and 10% empty nanoparticles, respectively, when compared to the untreated cells (Figure 22. B). The A549 cells further showed a significant decrease in cell viability after a 72-hour treatment with the empty nanoparticles, indicating 90.81%, 85.81% and 83.12% viable cells after being treated with 1%, 5% and 10% nanoparticles, respectively, when compared to the untreated cells (Figure 22. B). This therefore indicates that although the nanoparticles themselves do not appear to have an effect on non-tumourigenic cells, they do significantly affect the viability of tumourigenic cells, such as the A549 lung cancer cells.

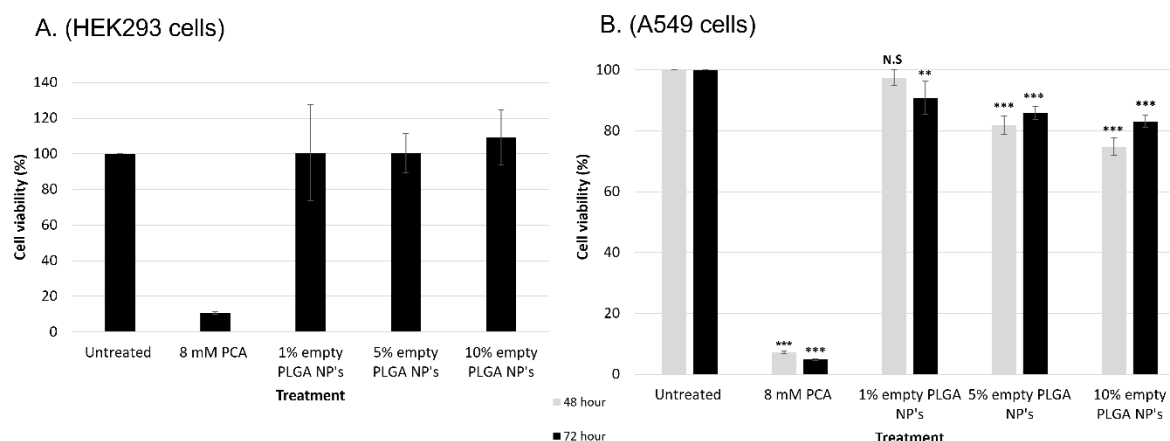


Figure 22: Assessing the cytotoxicity of empty PLGA nanoparticles on non-tumourigenic HEK293 and tumourigenic A549 cells.

Cellular viability was determined post-treatment with 1, 5 and 10% empty PLGA nanoparticles by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay in (A) HEK293 and (B) A549 cells. (A) Cell viability was assessed 72 hours post-treatment. One-way ANOVA analysis revealed no significant (N.S. $p=0.6996$) cytotoxicity on the non-tumourigenic HEK293 cells after treatment with 1-10% empty PLGA nanoparticles. (B) Cell viability was assessed 48- and 72 hours post-treatment. One-way ANOVA analysis indicates a significant impact of the treatments after both 48 ($***p=8E-27$) and 72 ($***p=1.17E-34$) hours in the tumourigenic A549 cells, which conveys an increase in cell death as the concentration of empty PLGA nanoparticles increases. Wherein, a significant 18.13% and 25.27% decrease in cell viability was observed after a 48-hour treatment with 5% and 10% empty nanoparticles and there were 90.81%, 85.81% and 83.12% viable cells after 72-hour treatment with 1%, 5% and 10% nanoparticles. The untreated samples were set to 100% and 8 mM PCA was used as the positive control. All values indicate the mean $\pm$ STD. Error bars represent standard deviation. $n=3$ biological repeats. N.S = nonsignificant, $* \leq p, 0.05$, $** \leq p, 0.01$, $*** \leq p, 0.001$; Student's t -test.

3.2.3. PLGA nanoparticles have no significant effect on LRP protein levels

Since the empty nanoparticles influenced the cell viability of the A549 cells, and not the HEK293 cells, western blotting was thereafter performed to determine whether the empty nanoparticles exhibited any effect on LRP/LR levels after a 72-hour treatment with 1%, 5% and 10% of the empty nanoparticles (Figure 23). The HEK293 cells showed no significant effect on LRP levels when treated with 1-10% empty nanoparticles compared to the untreated samples (Figure 23. A). Furthermore, even though the empty nanoparticle treatment had a significant effect on the cell viability of the A549 cells, no significant effect was observed on LRP levels in the A549 cells treated with 1-10% of the empty nanoparticles, when compared to the untreated samples (Figure 23. B). Altogether, this therefore indicates that the

nanoparticles have no effect on the cell viability or LRP protein levels in non-tumourigenic HEK293 cells, however even low concentrations of the nanoparticles affect cell viability in the tumourigenic A549 cells.

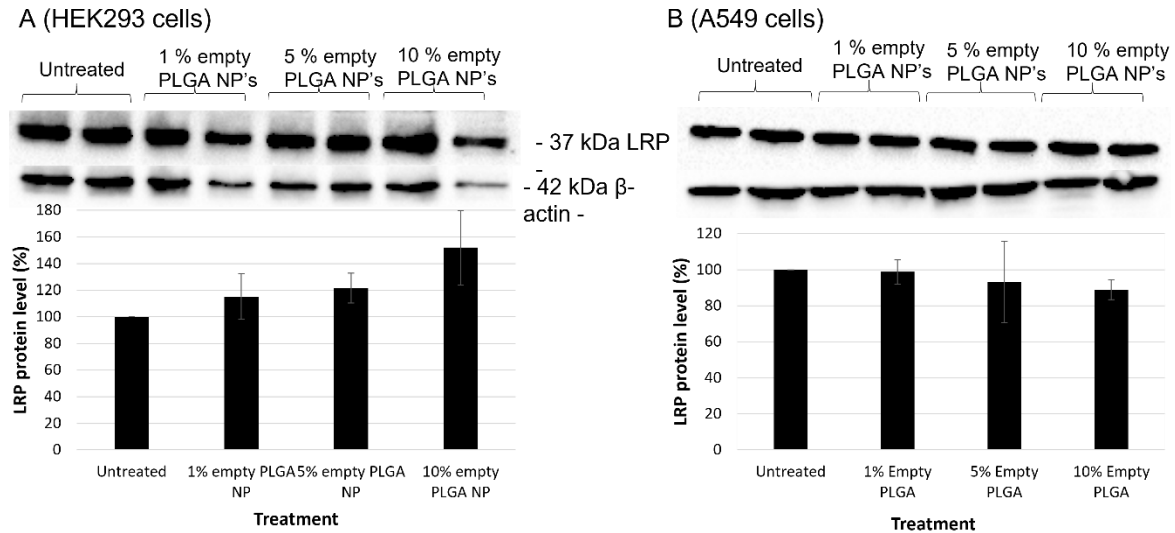


Figure 23: Treatment of non-tumourigenic HEK293 and tumourigenic A549 cells with empty PLGA nanoparticles does not affect total LRP/LR levels.

Western blotting with subsequent densitometric analysis indicates no significant effect on LRP levels was observed after a 72-hour treatment of both the HEK293 (A) and the A549 (B) cells with 1, 5 and 10% empty PLGA nanoparticles. Untreated is set to 100%, with all samples expressed as a percentage of the untreated samples. β -actin was used as the loading control. One-way ANOVA showed no significant impact of the treatment on LRP levels in the HEK293 (N.S. $p = 0.0837$) or A549 (N.S. $p = 0.7813$) cells. This data is representative of the mean $\pm$ SD, $n = 3$ biological repeats. N.S = nonsignificant, $* \leq p, 0.05$, $** \leq p, 0.01$, $*** \leq p, 0.001$; Student's t -test.

CHAPTER FOUR - DISCUSSION

4.1. Determining the effect of siRNA-mediated downregulation of LRP/LR on multiple cancer hallmarks in an *in vitro* lung cancer (A549 cells) model

Due to the many roles it plays in the cell, LRP/LR has become an increasingly popular target in the search for cancer therapeutics due to its characteristic overexpression in many cancer types. In particular, the overexpression of the receptor has been shown to aid in numerous tumourigenic processes, including apoptotic evasion and cell viability maintenance, enhancement of angiogenic processes, as well as enhancing the adhesive and invasive potential of cancer cells [124]. Furthermore, LRP/LR does not only serve as a receptor on the cell surface, but it also plays a role in intracellular processes through its nuclear and cytosolic localisation. These additional roles include regulation of the cell cycle, ribosomal anchorage to microtubules and regulating telomerase activity [123, 128, 129, 131], as well as protein synthesis and pre-rRNA processing [214]. Therefore, the overexpression of LRP/LR in cancer cells allows these cells to exploit these functions to aid in the tumourigenic process. However, various studies have observed that targeting LRP/LR for downregulation with LRP-specific siRNA successfully inhibits various tumourigenic processes, as mentioned above, in several different cancer cell types including breast (MDA-MB231 and MCF-7) [147], cervical (HeLa) [134], neuroblastoma (IMR-32), pancreatic (AsPC-1)[215], oesophageal (WHC01) [147], melanoma (A375 and A375M) [166] and colorectal (SW-480 and DLD-1)[167]. Moreover, siRNA-mediated downregulation has been performed in A549 lung cancer cells and has shown a decrease in cell viability caused by apoptosis [134]. The current study therefore served to confirm and extend these results and look further into the effects of downregulating LRP/LR on other cancer hallmarks in the A549 cells, using a different LRP-targeting siRNA.

To optimise the downregulation of LRP levels in the A549 lung cancer cells, transfection with LRP-specific siRNA (RPSA) as well as with a universal negative control siRNA (RLUC) was performed and the cells incubated for 72 hours. The downregulation was successful in that the RPSA siRNA transfected cells exhibited a significant 40% decrease in LRP levels, however the cells transfected with RLUC siRNA also displayed a significant 10% decrease in LRP levels (Figure 11. A). A significant downregulation of LRP protein levels by at least 60% in the RPSA siRNA transfected cells was desired, as this is what was previously observed using Dharmacon siRNA-LAMR1, where a downstream effect on cell viability was observed [134]. Thus, the siRNA

amounts were increased. Since there is not meant to be an effect observed in the negative control siRNA transfected cells, as a change in LRP levels implies off-target effects, a different negative control siRNA (a non-targeting control – NTC siRNA) was assessed, as well as an additional mock transfection control, included to account for any effects possibly caused by the transfection reagent. There were no apparent effects on LRP levels in either the NTC siRNA or mock transfected cells, when compared to the non-transfected cells. However, LRP levels in the RPSA siRNA transfected cells indicated a significant downregulation of 73%, as was desired (Figure 11. B). Thus, confirming the successful downregulation of LRP/LR in the A549 cells. These conditions were therefore carried through for all downstream experimentation.

4.1.1. Downregulation of LRP/LR decreases cell viability by inducing apoptosis

A vital hallmark of cancer includes the resistance of cancer cells to cell death processes, which includes apoptotic evasion and is a crucial way in which cancer cells continue to survive under conditions where normal cells would undergo apoptosis [7]. This is therefore a common focus as part of the search for cancer therapeutics. The overexpression of LRP/LR on cancer cells significantly contributes towards cell viability and inhibition of apoptotic pathways in cancer cells [124]. Thus, studies involving the downregulation of LRP/LR have placed emphasis on the subsequent effect on cell viability.

Cell viability was subsequently assessed via MTT and Trypan blue exclusion assays (Figure 12). A significant decrease in cell viability was observed in the A549 cells transfected with RPSA siRNA after both 48- and 72 hours. In particular, when assessed via MTT assay, the cells transfected with RPSA siRNA for 72 hours showed a significant 70.45% decrease in cell viability (Figure 12. A). Additionally, when assessed via the Trypan blue exclusion assay, the cells transfected in the same way showed that only 33.33% of the cells remained viable (Figure 12. B), thereby confirming the results as observed for the MTT assay. The NTC siRNA and mock transfected negative controls exhibited no significant decrease in cell viability when compared to the untreated cells, as anticipated by a lack of effect seen in the corresponding LRP levels. Furthermore, the cells treated with 8 mM of the known apoptotic inducer, PCA, exhibited a significant increase in cell death as expected of this positive control. Therefore, confirming that indeed, the downregulation of LRP/LR significantly reduces cell viability. Interestingly, however, the previous study showing a 60% decrease in LRP levels after siRNA-LAMR1 mediated downregulation corresponded with only a 13% decrease in cell viability [134]. Whereas, the current study showed a 73% downregulation of LRP levels that corresponded with a $\pm 70\%$ decrease in cell viability, which coincides more with what has been observed in other studies that have used the

esiRNA-RPSA in other cancer cell types [166, 167]. The corresponding decrease in cell viability observed in this study after siRNA-mediated LRP downregulation parallels that which has been observed in numerous other studies assessing this effect in the various abovementioned cancer cell types.

The cellular morphology was subsequently assessed via light microscopy to gain insight into the mode of cell death taking place. The A549 cells were therefore transfected as mentioned above, for 48- and 72 hours (Figure 13). The control NTC siRNA transfected cells displayed normal cellular morphology with no indication of cell death occurring, mirroring that of the healthy untreated A549 cells (Figure 13. A. i-ii & iv-v). However, apparent cell shrinkage and membrane blebbing, characteristic of apoptosis [208, 209], was observed after LRP/LR downregulation, therefore indicating apoptosis was occurring in these RPSA siRNA transfected A549 cells (Figure 13. A & B iii & vi). The APOPercentage™ assay did indeed show that the mode of cell death induced was apoptosis, as indicated by the light microscopy images of the stained cells (Figure 14. A), as well as the spectrophotometry readings after lysis (Figure 14. B iii & vi). This therefore confirms that downregulating LRP/LR in the A549 lung cancer cells induces apoptosis, resulting in a significant reduction in cell viability.

A critical role of LRP/LR, is the role it is known to play in apoptotic processes. Various studies have confirmed that siRNA-mediated downregulation of LRP/LR in cancer cells overexpressing LRP/LR, results in apoptotic induction through different pathways, depending on the cancer cell type. In particular, apoptosis was induced in breast, oesophageal [216], cervical and lung [134] cancer cells after LRP downregulation [216]. Additionally, other studies showed that knockdown of LRP/LR caused activation of caspase-8 and -9 in pancreatic and neuroblastoma cancer cells [215], in early and late stage colorectal cancer cells [167], as well in early and late-stage malignant melanoma cells [166]. The current study showing the induction of apoptosis in the A549 cells, therefore follows this trend observed in the increase in apoptosis after downregulation of LRP/LR in these other cancer cells. It has been described that the interaction between LRP/LR and laminin-1 facilitates an interaction between LRP/LR and FAK, which subsequently activates pro-survival pathways including the PI3k/Akt and MEK/ERK1/2 pathways. This then causes a subsequent upregulation of the anti-apoptotic Bcl-2 protein downstream of the intrinsic and extrinsic apoptotic pathways, thereby inhibiting apoptosis [164]. Furthermore, LRP/LR is known to bind to Midkine, a heparin-binding growth factor, resulting in the translocation of this complex to the nucleus. This interaction aids in

chromosomal stability and therefore, maintenance of cell viability. Midkine is known for its role in enhancing cell migration, proliferation and survival [217]. Additionally, the upregulation of Midkine in several cancer types has been associated with inhibition of the effector caspase-3, therefore impeding both intrinsic and extrinsic pathways, as well as inhibition of pro-survival factors [218]. Therefore, the interaction between LRP/LR and Midkine aids in cell viability maintenance. Thus, we suggest that the downregulation of LRP/LR is possibly inducing apoptosis by the impediment of the interactions between LRP/LR and FAK, as well as Midkine. It would therefore be interesting to look further into these processes in subsequent studies, to confirm this.

Furthermore, this effect is certainly time-dependent as observed by the small, nonetheless significant, effects seen in cell viability (11% decrease) (Figure 12) and apoptotic morphology (Figure 13), as well as apoptotic induction (1.65-fold) (Figure 14) 48 hours post-transfection compared to those at 72 hours as described above. This exponential increase in the effect observed at 72 hours versus 48 hours is indicative that once LRP levels have decreased below a certain level, the cells are no longer able to compensate for this, and the effect becomes detrimental to the entire cell population. Which is an outcome that shows potential for targeting entire tumours. The 72-hour transfection was therefore the time point chosen for assessment of the effect of LRP downregulation on telomerase activity as well as for proteome analysis.

4.1.2. LRP/LR downregulation decreases migration potential

Metastasis is the primary cause of poor prognoses and cancer lethality, whereby 90% of deaths caused by solid tumours are attributed to metastatic propagation. More specifically, the 5-year survival rate of metastatic lung cancer in 2021 was a dismal 7% (www.cancer.org). Understanding the multi-step process of migration, adhesion and invasion for each individual cancer type is a mammoth task [132], but one which is absolutely necessary in the search for therapeutic intervention. Therefore, a fundamental factor for consideration when developing novel treatment strategies for cancer is certainly determining the effect of the treatment on cell migration, adhesion and invasion [219]. Furthermore, LRP/LR has been implicated in the metastatic process through its interaction with laminin-1, whereby the overexpression of LRP/LR on cancer cells increases this interaction, causing enhanced migration and invasion of the tumorigenic cells [137, 138]. A crucial tool when investigating the therapeutic potential of novel anti-cancer treatments, is the quantification of *in vitro* migration processes in cancer cell lines

by means of time-lapse microscopy. Which can further provide a basic understanding of the metastatic processes of that particular cancer type *in vivo*. Thus, a scratch motility assay was performed after RPSA siRNA transfection to determine whether LRP downregulation would affect the migration potential of the A549 lung cancer cells.

The scratch assay was performed 24-hours post-transfection, with subsequent imaging 48 hours post-transfection since previous assays, as described above, had shown the downstream effects of LRP downregulation were visible, but minimally at 48 hours post-transfection. However, as the cells are only $\pm 30\%$ viable at 72 hours post-transfection, we were not able to accurately observe the effect on cell migration at this time-point (data not shown). Therefore, 24 hours post-scratch but 48 hours post-transfection, there was a significant decrease of 32.12% observed in migration potential in the A549 cells after LRP downregulation (Figure 15). Furthermore, since the anti-proliferative agent, Mitomycin C [220], was included during the scratch assay, it can confidently be concluded that the wound closure observed was due to cell migration, rather than cell proliferation. The core part of the enhanced interaction between laminin-1 and LRP in cancer cells overexpressing LRP, is that it causes the release of type IV collagenase, which degrades the basal lamina, thereby allowing the cells to migrate through the blood stream to distant sites [137, 138]. Previous studies have shown that downregulating LRP/LR decreases the adhesive and invasive potential of tumourigenic fibrosarcoma (HT1080) cells overexpressing LRP/LR [153]. Additionally, a study where mice were inoculated with lung cancer cells treated to display lower levels of LRP, showed prolonged survival rates [221]. Altogether, there is strong evidence showing the role of downregulating LRP/LR to reduce metastatic processes. Therefore, we suggest that the downregulation of LRP/LR reduces the migration potential of the cells, as the cell surface interactions, and subsequent signalling cascades facilitated by LRP/LR are inhibited.

4.1.3. Downregulation of LRP/LR inhibits telomerase activity

Not only is the upregulation of telomerase activity another way in which cancer cells supersede inhibitory signals, but high telomerase activity levels are also associated with cancer recurrence and chemotherapeutic resistance [77]. This elevated telomerase activity further permits the cells to proliferate in the presence of genomic instability, rather than undergoing senescence or cell death [57]. Accordingly, telomerase activity has been detected in 75% of NSCLC [222]. Importantly, we have previously demonstrated that LRP levels are directly associated with telomerase activity, in that LRP overexpression significantly increases TERT levels and

telomerase activity [128, 131], and downregulating LRP/LR suppresses telomerase activity [123]. Therefore, this study included assessing whether the downregulation of LRP/LR affects telomerase activity in the A549 lung cancer cells.

Therefore, the modified TRAP assay was performed via qPCR to detect telomerase activity levels, after the A549 cells were transfected with RPSA siRNA for 72 hours. Interestingly, the downregulation of LRP/LR caused a consequent, highly significant decrease in telomerase activity, whereby the cells transfected with RPSA siRNA displayed a 99.19% decrease in telomerase activity (Figure 16). Since the corresponding LRP levels in the NTC siRNA transfected samples reflected those of the untreated cells, the non-significant change in telomerase activity observed was expected. Thus, the downregulation of LRP/LR causes an almost complete impediment of telomerase activity in the A549 lung cancer cells and as such, hampers the cell's ability for replicative immortality, therefore targeting an additional cancer hallmark.

It is well-known that cancer cells are able to avoid the effects of senescence and apoptosis through the upregulation of telomerase activity. This thereafter permits the cells to maintain unstable telomere lengths [65], which stabilises the mutated genome and enables replicative immortality, whereafter macroscopic tumours are formed [7]. The downregulation or inhibition of these higher levels of telomerase activity has thus become a popular target for potential cancer therapeutics. Moreover, since telomerase activity is undetectable in most human somatic cells [60], targeting the upregulated telomerase levels in cancer cells or tumours should have few off-target effects. A previous study has shown that targeting hTERT causes a decrease in telomerase activity in lung cancer cells, which caused the cells to enter senescence via a p53-dependent mechanism [223]. Another study showed that by targeting TERT levels directly with siRNA in A549 lung cancer cells, telomerase activity was reduced. They further showed *in vivo*, that the downregulation of TERT via siRNA, reduced the size of lung xenograft tumours due to apoptotic induction [222]. Additionally, this study performed by Xie et al, demonstrated a complete inhibition of telomerase activity after 72-hour transfection that was causally associated with a 95% reduction in TERT levels at the same time point, which corresponds to the knockout of telomerase activity observed in the current study at 72 hours post-transfection. Interestingly, there was also a marked increase in apoptosis observed after telomerase inhibition [222]. Perhaps more pertinently to this study, it has very recently been demonstrated that siRNA-mediated downregulation of LRP in colorectal cancer cells, not only decreased

telomerase activity, but also decreased TERT and pTERT levels. Here, pTERT is the activated form of TERT, which specifically functions as the subunit in telomerase activity [165]. Therefore, although TERT levels were not directly assessed in this current study, we suggest that the inhibition of telomerase activity observed is due to the consequent downregulation of TERT levels after LRP downregulation. Furthermore, we propose that not only does this inhibition of telomerase activity reduce cell proliferation, but it also contributes to the increase in apoptosis observed in the present study, as described above. In particular, a study has shown that telomerase repression, either through inactivation of TERT or through shRNA inhibition of telomerase, increases cell cycle arrest and apoptosis in both *in vitro* and *in vivo* cancer models [74]. In addition, Xue et al, [74] observed that this decrease in telomerase caused an increase in expression of PML, resulting in its translocation to the nucleus, with subsequent PML-dependent recruitment of p53 into the nucleus and co-localisation between the two resulting in activation of p21 (to inhibit cell cycle progression) [74]. Thus, the inhibition of telomerase resulted in activation of the PML-dependent p53 pathway to induce cell cycle arrest and apoptosis. Interestingly, the downregulation of LRP/LR in the present study not only inhibited telomerase, but also caused the upregulation of PML (5.2.4). Therefore, we suggest that the mechanism by which the observed decrease in cell viability and induction of apoptosis is occurring in the present study, is because the downregulation of LRP subsequently downregulates hTERT and inhibits telomerase, which consequently activates PML-dependent p53 signalling. We did not observe a change in p53 levels during proteomic analysis, however, the nuclear translocation and activation of p53 does not necessarily mean the levels of p53 are affected.

4.1.4. LRP/LR downregulation causes differential expression of numerous proteins

Proteomics includes the application of technologies for identification and quantification of the overall protein content in cells and biological fluids. It is a well-known and reliable technique for studying large quantities of proteins via mass spectrometry in a single-shot analysis. We performed a 72-hour transfection of the A549 cells with RPSA-siRNA, whereafter the proteome of those cells was analysed using SWATH-MS. This was followed by protein set enrichment analysis with STRING database and the Reactome pathway browser tool in order to get an overview of the downstream effect on key pathways, which would provide insight into the mechanism of LRP downregulation. STRINGdb (string-db.org) is a biological database of known and predicted protein-protein interactions from numerous sources,

including experimental data, public text collections and computational prediction methods. Whereas the Reactome (reactome.org) Pathway Browser tool provides a means of viewing and interacting with pathways relating to a specific dataset, based on information within the Reactome database. The SWATH-MS analysis revealed 74 dysregulated proteins in total, between the control and RPSA siRNA transfected cells. Within the dataset of dysregulated proteins, it was revealed that 22 proteins were observed to be downregulated, whilst 52 proteins were found to be upregulated after LRP downregulation (Figure 17). For the full list of details regarding the observed dysregulated proteins and their function, refer to Table 8 and 9 in Appendix B. All identified dysregulated proteins from the SWATH-MS analysis were initially analysed using STRINGdb. However, though a clear interaction was observed between many of the dysregulated proteins, there were several proteins which currently do not have known existing interactions (seen as unconnected nodes) with the other query proteins (Figure 17). This makes it particularly challenging to deduce pathway commonalities, and thus it is difficult to group the proteins according to common functions and infer a relationship.

Upregulated proteins in re-establishing immune response

Various upregulated proteins identified after downregulation of LRP/LR appear to play a role in the MHC I antigen presentation pathway (Figure 24). PSMB8 and PSMB9 are proteasomal subunits involved in generating class I binding peptides through antigen processing and were observed to be upregulated. TAP1 (TAP) plays a role in transporting antigens from the cytoplasm to the ER for association to the MHCI molecules, while TAPBP (Tapasin) is part of the complex responsible for loading the peptide onto the MHC1 molecules [224] these proteins were also increased after downregulation of LRP. Although there was no evidence in STRINGdb for protein interactions between NAPA (seen as an unconnected node- Figure 18) and the other query proteins, Reactome showed a role of NAPA in vesicular transport between the ER and Golgi apparatus. Therefore, we suggest NAPA is involved in this pathway by possibly transporting the peptide-loaded MHC1 molecules from the ER to the Golgi. Furthermore, both HLA-A and HLA-B were found to be significantly upregulated and these proteins are the core subunits of the MHC1 molecules [225].

The MHC I antigen presentation pathway is the mechanism which allows CD8⁺ T cells to identify cells producing foreign proteins, such as from mutant genes in cancers [226]. Therefore, cancer cells that have defects in various places within this pathway, including making, MHC I-loading or transporting, become less visible to the immune system, thus impairing control of the tumours by CD8⁺ T cells [226]. Indeed, various cancer types lose

expression of MHC I molecules through a variety of different ways including, but not limited to, mutation or deletion of genes involved, transcriptional regulation, posttranslational modification and extrinsic stimuli from the tumour microenvironment. In this context, the loss of this pathway is a crucial way in which cancers are able to evade immune control. Furthermore, the loss of this pathway is not essential for cell viability or growth and is therefore not detrimental to the cancer cells. This is of clinical significance, in that the loss of this pathway is associated with poorer prognosis and worse clinical outcomes [227]; reviewed in [104]. Therefore, the observed upregulation of the abovementioned proteins that are involved in this pathway suggests how downregulating LRP/LR possibly plays a role in activating the MHC I antigen presentation pathway for the recognition of the cancer cells by CD8⁺ T cells.

Furthermore, we speculate that the mechanism of the loss of this pathway in the A549 cells, is likely due to transcriptional repression or post-translational modification, rather than mutation or tumour microenvironment signalling. The reasoning for this is three-fold, the first is that the observed upregulation of the proteins involved after LRP downregulation suggests a functional pathway, since these proteins are generally activated sequentially. The second reason is that as these cells were grown *in vitro*, we cannot consider the tumour microenvironment as playing a role. The third and perhaps most pertinent reason suggests the overexpression of LRP/LR is repressing this pathway because when LRP/LR is downregulated, the resulting upregulation of proteins involved in this pathway indicate transcription of the proteins involved. Ultimately, we propose a novel function of LRP/LR in cancer immune evasion, whereby the downregulation of LRP/LR activates the MHC I antigen presentation pathway for detection of the cancer cells by CD8⁺ T cells. Thus, targeting LRP/LR for downregulation is possibly re-establishing immune control over the cells. It would be interesting indeed, to be able to assess this *in vivo*, where the tumour microenvironment and immune system are able to act on the tumour cells.

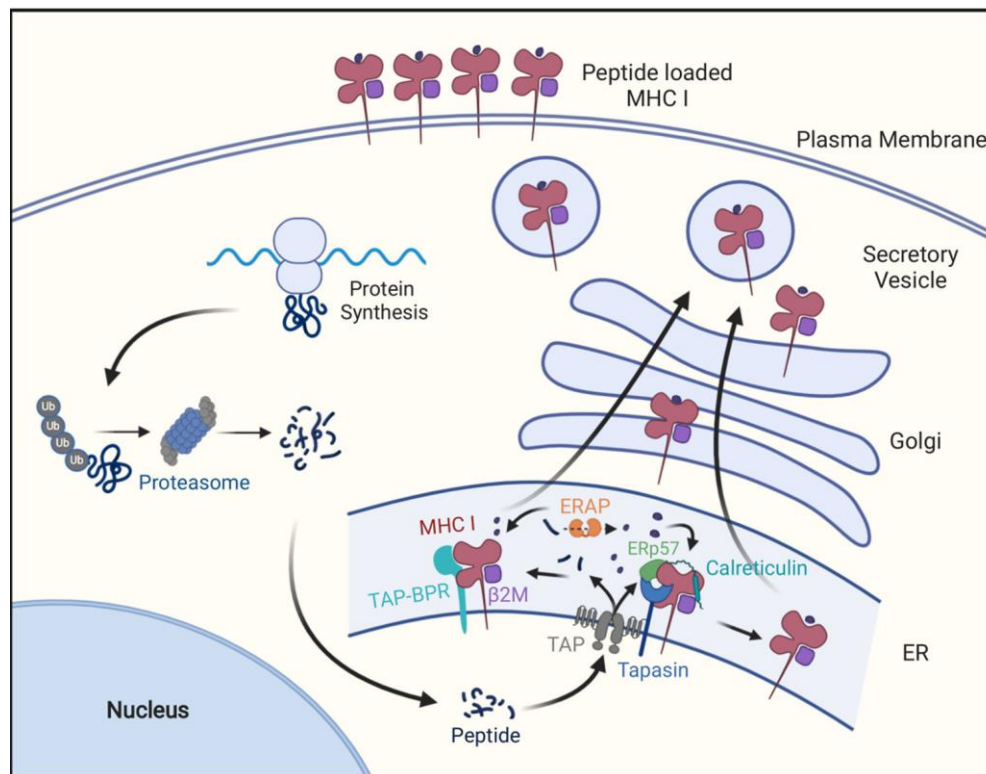


Figure 24: The MHC class I antigen presentation pathway

Proteins from the cell's expressed genes are processed into oligopeptides through the ubiquitin-proteasome pathway. These are then transported from the cytoplasm into the endoplasmic reticulum (ER) by TAP. Once in the ER, a peptide-loading complex including Tapasin bind the peptides to the MHC I molecules. Once bound, the MHC I molecules are transported to the cell surface for antigen presentation to CD8+ T cells. The CD8 + T cells are then able to identify the cells as pathological, particularly those synthesising abnormal proteins, such as cancer cells. (Adopted from [104].

Dysregulated proteins in inhibiting cell proliferation and inducing apoptosis

Moreover, most of the upregulated proteins identified after downregulation of LRP/LR have been shown to play important roles in inhibiting tumourigenesis. ISG15 showed a 6.71-fold increase and is a ubiquitin-like protein involved in stabilising and affecting protein activity similarly to ubiquitination, in a process known as ISGylation. A few studies have shown an anti-cancer effect of ISG15 [228-230] and indeed, it has been suggested to be a type of lung cancer tumour suppressor gene [231, 232] and has been found to be dysregulated in many different cancer types. Specifically, ISG15 has been associated with lung cancer growth suppression by its function in inhibiting cyclin D1 [232]. ISG20 was observed to be upregulated and is an RNase with 3'-5' exonuclease activity, much like RNase L (elaborated on below), which has mostly been studied in its role against pathogens, however has been indicated as a PML-nuclear bodies (PML-NB)-associated protein [233]. The promyelocytic leukaemia

protein (PML) was also found to be upregulated after LRP/LR downregulation. PML is a key component of PML-NBs and appears to play the role of a tumour suppressor by contributing to apoptotic induction, inhibiting angiogenesis as well as cell cycle progression [234, 235]. Furthermore, PML is also considered to be a tumour suppressor gene as it has been found to have potent growth suppression abilities when it is overexpressed in cancer cells [236]. PML is ubiquitously expressed at low levels in almost all cells and is involved in modulating cell death programs through both p53-dependent and p53-independent mechanisms via its role in modulating apoptosis at the transcriptional level. PML is additionally involved in checkpoint response to DNA damage, whereby it has a function in inducing G1-arrest and/or DNA repair (reviewed in [237]). In addition, OAS3, OAS1 and its paralog, OASL were found to be upregulated. These proteins belong to the OAS family and are able to synthesize 2'-5'oligoadenylates (2-5A) upon binding to dsRNA, the 2-5A then leads to activation of RNase L. RNase L can then promote DNA cleavage, nuclear condensation and apoptosis. Accordingly, studies have shown that restoring RNase L in lung cancer cells induced apoptosis [238]. Therefore, OAS proteins are considered to be tumour suppressors, indeed another study showed that activation and overexpression of OAS1 inhibits cancer cell proliferation and migration [239] through its role in activating RNase L [238]. Furthermore, DDX58 exhibited a 5.28-fold increase in expression and has been seen to play a role alongside OASL, whereby downregulation of these proteins is observed to enhance cancer cell motility and invasion [240]. In addition, EIF2AK2 was upregulated and is often also referred to as PKR, which is a double-stranded RNA-activated protein kinase. EIF2AK2 is activated by various cellular stresses including nutritional depletion and hypoxia [241], however, activation of this protein has additionally been reported to suppress tumour growth [242]. Additionally, analysis of NSCLC patient primary tissues showed not only a reduction in PKR, but also in OAS, further portraying their roles as lung cancer tumour suppressors [243]. H2A.z is upregulated in NSCLC which amplifies cell growth and survival, as its key role is in destabilising nucleosome core particles to allow the chromatin to be accessed for transcription of cell cycle genes, therefore enhancing tumour cell growth [244]. We observed a downregulation of -1.96-fold in H2A.z after LRP downregulation, which suggests a reduction in expression of cell cycle proteins that again correlates to the reduction in cell viability that was observed in the A549 cells.

Furthermore, the STAT1 transcription factor was shown to be upregulated after downregulation of LRP/LR. Although there was an observed increase in STAT1 expression, we do not have clarity on whether this is in its activated (pSTAT1) form. However, activated

STAT1 is known to translocate to the nucleus and induce expression of TAP1 and indeed we observed an upregulation in TAP1 expression, as described above. Increased STAT1 expression has been fundamentally correlated to tumour suppression (Koromilas and Sexl 2013). The loss of STAT1 has been observed in lung cancer tumours. Furthermore, it has been shown that cancer patients exhibiting high STAT1 expression tend to have better clinical outcomes compared to those with low STAT1 expression. STAT1 has been quite extensively studied for its tumour suppressor role, which it is able to carry out by inducing gene expression of proteins involved in cell growth arrest, apoptosis and angiogenic inhibition (reviewed in [245]). STAT1 is involved in cell cycle arrest and apoptosis, whereby it directly regulates the expression of pro-survival genes such as Bcl2 and various pro-apoptotic caspases, as well as through interactions with proteins such as p53. Moreover, multiple cell cycle regulatory genes such as p21 and cyclin D1 are transcriptionally regulated by STAT1 to negatively regulate the cell cycle [246]. STAT1 is further considered a negative regulator of angiogenesis, by downregulating pro-angiogenic factors such as FGF [247]. Interestingly, STAT1 has additionally been shown to be involved in promoting the activation of the MHC I antigen presentation pathway for processing and presenting tumour antigen molecules. It is therefore highly likely that, in the present study, the downregulation of LRP enhanced STAT1 expression, which activated transcription of the observed upregulated proteins in the MHC I pathway described above [248]. Furthermore, SAMHD1 was also found to be upregulated, with a fold increase of 3.36 observed. This protein is a dNTP hydrolase, involved in reducing dNTP concentration and is therefore involved in inhibiting cell proliferation, and tumourigenesis [249]. Indeed, overexpression of SAMHD1 has been found to reduce proliferation of A549 lung cancer cells [250].

Therefore, based on the significant upregulation of these proteins discussed above, as well as their functions, there is a clear role in immune presentation, cell cycle modulation and apoptosis, which corresponds with the reduction in cell viability and apoptotic induction as observed in the present study. Furthermore, we suggest that as the MTT assay can also be considered a cell proliferation assay, the reduction in cell viability shown in the current study is likely a combination of cell cycle arrest, as well as apoptosis. We also speculate that, since the cells are mostly apoptotic after a 72-hour transfection with RPSA siRNA, the immune and proteasomal degradation systems are being activated to clear the apoptotic cells and any resulting debris to regulate the cell death process and avoid tumour-promoting inflammation

[251]. This is further suggested by the lack of dysregulated proteins identified in pro-inflammatory pathways, indicating no apparent inflammatory response.

Dysregulated proteins involved in improving therapeutic response

Moreover, several upregulated proteins identified include IFIT1, IFIT2, IFIT3 and IFI35. The interferon-induced protein with tetratricopeptide repeats (IFIT) gene family form part of the interferon-stimulating genes (ISGs). Recent studies into the role of the IFIT family of proteins have shown an anti-oncogenic function, aside from their role in host immunity and viral response (Pidugu, Pidugu et al. 2019). IFIT proteins have been shown to be involved in a variety of processes in the cell including cell migration and proliferation, dsRNA signalling and virus induced translation, initiation and replication [252]. It has further been shown that the IFIT proteins are activated by STAT1 [252], which we have already seen was upregulated after LRP downregulation. Therefore, it appears that the downregulation of LRP induces activation of the STAT1 transcription factor, which is then able to activate/induce transcription of several of the proteins that have been observed to be upregulated. Furthermore, increased IFIT1 and IFIT3 expression has been correlated to improved therapeutic response to chemotherapeutics and immunostimulating agents [253, 254]. It has been further shown that IFIT1 and IFIT3 form an interdependent complex to carry out their functions [255]. However, IFIT2 appears to have a more relevant function in tumour suppression, whereby low IFIT2 expression has been correlated to enhanced migration and increased levels of metastasis [256]. Moreover, increased IFIT2 expression in the tumour tissues of patients was associated with improved patient survival [256]. IFI35 has been shown to be involved in regulation the radiosensitivity of cancer cells and is therefore considered to be a radiotherapy-associated gene [257]. IFI35 was additionally found to be associated with cellular immune responses, including in the proteasome accessory complex in lung adenocarcinoma [258].

Furthermore, TYMP and NT5C3A were observed to be upregulated and these two proteins are important enzymes forming part of the pyrimidine salvage pathway [259]. This is a crucial pathway involved in converting 5' deoxy-5-fluorocytidine (5'DFCR) to 5-fluorouracil, a key chemotherapeutic agent for treating solid tumours [260]. Therefore, the downregulation of LRP has also led to the upregulation of proteins known to be involved in sensitising cancer cells to current chemotherapeutics and radiotherapy. It is therefore possible, that targeting LRP/LR for downregulation could be used as a means of enhancing therapeutic response to these existing treatment strategies to perhaps improve the clinical outcome for patients.

Dysregulated proteins with no direct role in pathways identified after LRP downregulation

LGALS3BP, PC, LCN2 and ZC3H11A exhibited fold increases of 2.91, 2.3, 2.04 and -3.72 respectively, upon LRP downregulation. However, these 4 proteins appear to have functions which do not coincide with the experimental results observed in this study, as well as with the other dysregulated proteins observed to play roles in tumour suppression. Furthermore, as can be seen (Figure 18), PC, LCN2 and ZC3H11A were seen as unconnected nodes, suggesting that there is no current evidence within STRINGdb to recommend interactions with the other proteins that were observed to be dysregulated after LRP downregulation.

LGALS3BP has been shown to be elevated in NSCLC tumour tissues and was involved in poor clinical outcomes of those patients [261]. Controversially, LGALS3BP was seen to exhibit anti-tumour activity in colorectal carcinoma, through its suppression of Wnt signals by the ISGylation of β -catenin [262]. Under normal conditions, activated β -catenin is involved in cell proliferation, however, when it undergoes proteasomal degradation, cell proliferation is inhibited. Since this agrees more with the results observed in this study, we suggest that the upregulation of the ISGylation associated proteins (ISG15 and ISG20 described above) is perhaps playing a role in ISGylation-dependent ubiquitination of β -catenin by overexpression of LGALS3BP and its role in suppressing this pathway. However, there is currently limited information on the role of LGALS3BP in lung cancer. Furthermore, LCN2 is suggested to be associated with radioresistance in lung cancer cells, as the radiosensitivity of these cells were enhanced when LCN2 was downregulated [263]. PC is known for its role in catalysing the conversion of pyruvate to oxaloacetate in the tricarboxylic acid (TCA) cycle. Specifically, NSCLC uses PC to support the cancer cell growth and indeed, downregulating PC expression restricted tumour growth [264]. However, since we conclusively observed both a reduction in cell viability and induction of apoptosis, it is possible that the cells are attempting to compensate for this by increasing PC production and enhancing the TCA pathway for biosynthesis.

Furthermore, ZC3H11A is involved in nuclear to cytoplasmic transport of mRNA [265], however, has been predominantly studied in its function in assisting viral replication [266]. Therefore, little is known of its involvement in a cancer setting. However, since this protein was seen to be downregulated, it is attractive to speculate that as part of the growth arrest and apoptotic pathways, the resultant reduction in mRNA transport assists in inhibiting translation of the proteins involved in cell cycle progression and apoptotic evasion. Indeed, this is entirely

speculative, as we were unable to obtain evidence for protein interactions between these 3 proteins (LCN2, PC and ZC3H11A) and the rest of the data set using STRINGdb and Reactome. Therefore, we are unable to confirm the role of these proteins at this time, however, it would prove interesting to delve further into the relationship between LRP downregulation and the upregulation of these 4 proteins to either confirm or deny the suggested function.

4.2. Optimisation and assessment of PLGA nanoparticles as a delivery system

Polymers have become progressively more popular for use as a drug delivery system for various applications from targeted tumour therapy [267] to immune system modulation (such as vaccination) [268]. The encapsulation and/or conjugation of drugs to polymers have shown to be more efficacious than their counterparts that are freely delivered. Specifically, encapsulation allows for a longer half-life, with improved effectiveness and reduced off-target effects [183]. In particular, PLGA demonstrates several ideal characteristics of a nanoparticle delivery system, including that the particles are degraded into the biocompatible components, glycolic and lactic acid, and they provide sustained release of their contents [269]. Indeed, it has been demonstrated that PLGA encapsulated small molecules, nucleic acids and proteins display improved activity in an assortment of applications in disease [270]. In addition, PLGA demonstrates the ability for straightforward customization of charge, size and surface modification, therefore allowing for precision and targeted therapy [271]. Moreover, as PLGA has previously been used in humans in FDA approved biodegradable clinical sutures [185] as well as drug carriers [272], there is considerable potential for translation of the PLGA nanocapsules to a clinical setting. Furthermore, since there have been numerous *in vitro* studies conveying the potential of using siRNA to target and downregulate LRP/LR as a cancer therapeutic, the translation of this research to an *in vivo* setting requires the use of an effective delivery system. Therefore, the second part of this study aimed to optimise the synthesis of PLGA nanocapsules, and to investigate the effect of the empty/unloaded nanoparticles on cytotoxicity as well as LRP levels in non-tumourigenic HEK293 and tumourigenic A549 cells.

Since siRNA can be quite costly, we first optimised the synthesis of the nanoparticles to achieve the required morphology and size in order to reduce the cost of the study as well as time. Therefore, PLGA nanoparticle production was optimised on empty, unloaded (no siRNA) nanoparticles, to optimise for smooth, uniformly sized, spherical nanocapsules (hollow)

predominantly in the 200-500 nm size range. The surface morphology and particle size range are important, as these factors play a role in drug release, as well as the foreign body response *in vivo*. Previous studies have shown that nanoparticles smaller than 1 μm in the circulatory system are not tagged and removed by the mononuclear phagocyte system, whilst microparticles (5-10 μm) are too large to be phagocytosed and therefore, result in a foreign body response when the particles were administered orally [186]. Furthermore, nanoparticles in the 250-500 nm size range allows for the administration of the particles, without risking the blockage of blood vessels. Accordingly, this size range allows for the particles to be delivered orally, rectally, transdermally, ocularly, nasally, subcutaneously, intraperitoneally, intramuscularly, as well as directly into the systemic circulatory system [187-189]. Moreover, since the aim was to ultimately encapsulate siRNA, barriers to drug release needed to be taken into consideration during the optimisation process. In this context, the particle size also plays a role whereby, the larger the particle, the longer it takes for the drug to be released from the particle, because of a longer diffusion path length. Specifically, there is an exponential relationship between the diffusion of the drug and the radius of the nanoparticle [190]. Thus, the optimal size range decided on for this study was 200-500 nm. Furthermore, the spherical nature of the particles allows them to be endocytosed more readily by cells [191]

The double-emulsification solvent evaporation protocol used for the synthesis of the nanocapsules is described by McCall and Sirianni [195]. This protocol was used as it provides various ways and means in which to optimise the nanoparticle synthesis based on the desired characteristics for the study. The protocol was followed closely, with small modifications only, including testing two different emulsifying agents, as well as different solvent: polymer ratios, based on recommendations within the protocol [195]. Initially, PVA was used as the emulsifier, however, exhibited primarily cuboid/sheet-like polymer formations which greatly varied in size and formed very large aggregates. Further, we could not confirm whether the nanoparticles were hollow (Figure 19). However, when using Vitamin E-TPGS as the emulsifier/stabilising agent as recommended in the protocol, it was observed that only spherical nanoparticles were synthesized (Figure 20). Indeed, it has been shown that using vitamin E-TPGS as the emulsifier allows for higher drug encapsulation efficiency, as well as increased cellular uptake of the nanoparticles, with a consequent increase in therapeutic effect, when compared to PVA [273]. Therefore, all nanoparticles were thereafter synthesized using Vitamin E-TPGS as the emulsifying agent. Not only were we able to confirm reduced batch to batch variability, but another factor to take into consideration is the safety profile to avoid unwanted toxicity and

adverse effects. Notably, Vitamin E-TPGS appears to have a selective response against normal and cancer cells [274, 275] and like PLGA, is FDA approved. It is of particular importance to achieve the correct safety profile, as it makes a substantial difference to the patient and clinical outcome of the intended treatment, since it allows for an increased maximum tolerated dose of the active agent, whilst avoiding tolerability issues caused by toxic components [276].

In addition, two different solvent: polymer ratios were used, 100 mg/ml and 50 mg/ml initial dilutions of PLGA in DCM. SEM characterisation of the nanoparticles synthesized using these two dilutions indicated that the nanoparticles were primarily spherical in shape (Figure 20). However, there remained a substantial size distribution within the raw, un-purified samples, with nanoparticle sizes ranging between less than 1 μm to greater than 10 μm in the 100 mg/ml sample and 200 nm – 3 μm in the 50 mg/ml sample (Figure 20. A&E). Consequently, we sought to centrifuge the sample at various high speeds in attempt to remove the larger particles and whilst this did remove most of the larger nanoparticles, there was a substantial loss in the number of nanoparticles overall (Figure 20. B-D & F-H) and thus would not be an effective means in which to remove the larger particles for too much of the sample would need to be discarded. Nevertheless, the sample produced using the 50 mg/ml initial dilution indicated a more efficient means of producing nanoparticles closer to the desired size range. Therefore, this dilution was used for all subsequent syntheses. Furthermore, it was confirmed that the nanoparticles were indeed hollow nanocapsules (Figure 21. C) as desired, and not spheres of solid polymer. It has been observed that although solid polymeric PLGA nanocapsules have a high encapsulation efficiency, these nanoparticles trap the siRNA resulting in a very low drug release efficiency, therefore causing a very slow release time with reduced therapeutic outcome [192]. Accordingly, Raicher et al, 2015 showed that hollow nanocapsules with the siRNA encapsulated in an aqueous core exhibited a much-improved release profile [193], therefore warranting the decision made in the current study to use hollow nanocapsules. Since the higher centrifugation speeds caused large loss of sample, an additional suggestion in the protocol recommended a fast, low-speed centrifugation for removal of large particles. We therefore attempted a short centrifugation with a low speed of 500 xg and observed a much lower size distribution, with nanoparticle sizes ranging between 200 nm and 500 nm (Figure 21. A. ii). Consequently, DLS analysis of the samples confirmed a mean hydrodynamic size of 502.8 nm with a PDI of 0.38 (Figure 21. B), indicating the sample fell within the desired size range and contained very little polydispersity after purification. Furthermore, the protocol recommended filtering the sample, thus this was performed using a 0.45 μm filter, however, it was not

efficacious as the larger nanoparticles appeared to block the filter, resulting in considerable loss of sample (Figure 21. A. iii). This method was therefore not used in subsequent experiments.

The short centrifugation at 500 xg was used in all subsequent nanoparticle batch purifications and was followed by multiple long, high speed centrifugations (17 000 xg) to collect the smaller particles in the desired size range and remove the large amounts of Vitamin E-TPGS. Based on the relationship between diffusion and nanoparticle radius [190], higher centrifugation speeds than this were not used for nanoparticle collection so as to also ensure the removal of the ultra-small nanoparticles (smaller than 100 nm), since these smaller nanoparticles have a high burst release, which can cause cytotoxicity.

Effect of nanoparticles on cytotoxicity and LRP levels

With the nanocapsule production optimised for safety, size and morphology, the empty/unloaded nanoparticles were subsequently assessed for cytotoxicity on non-tumourigenic HEK293, as well as on tumourigenic A549 cells. Therefore, both cell lines were treated with 1%, 5% and 10% (v/v) of the empty nanoparticles and assessed via the MTT cell viability assay (Figure 22). The non-tumourigenic HEK293 cells displayed no significant cell death after a 72-hour treatment with varying percentages of the empty PLGA nanoparticles (Figure 22. A). Thus, indicating that even at considerably high concentrations, the viability of the HEK293 cells was not affected by the nanoparticles. However, the tumourigenic A549 cells did exhibit a significant decrease in cell viability ranging between 9-25% after both 48 and 72 hours (Figure 22. B). This therefore indicates that although there is no apparent effect of the nanoparticles on non-tumourigenic cells, they do significantly affect the viability of tumourigenic cells.

Vitamin E-TPGS is a water-soluble analogue of naturally occurring vitamin E, which is notably effective at being selectively toxic to cancer cells. It has been shown that vitamin E-TPGS can both inhibit cancer cell growth and induce apoptosis [277]. When cancer cells are exposed to vitamin E analogues, such as TPGS, there is a consequent rapid accumulation of ROS, resulting in destabilization of the mitochondria and thus activation of mitochondrial-mediated apoptosis [278]. Interestingly though, this is selective towards cancer cells, as they generally produce lower levels of anti-oxidant enzymes (such as superoxide dismutase) when compared to normal cells [279]. Furthermore, since vitamin E-TPGS is used as the emulsifier when the PLGA

nanoparticles are synthesized, the core of the nanocapsules therefore contains vitamin E-TPGS. Thus, the observed effect of the empty nanoparticle treatment on the cell viability of the A549 cells is suggested to be due to the subsequent degradation of the nanocapsules consequently releasing the internal vitamin E-TPGS. However, ROS levels will need to be assessed to make a definitive conclusion. Moreover, the cell viability of the cells was observed to decrease as the percentage of the treatment increased, thereby indicating a concentration-dependent effect on the cells, which is congruent with the increasing number of nanoparticles containing vitamin E-TPGS. Moreover, as the HEK293 cells are non-tumourigenic, the resultant release of the vitamin E-TPGS did not affect cell viability.

Since the nanoparticles were synthesised with the intention of being used as a delivery system for LRP-targeting siRNA, it was pertinent to determine whether the empty/unloaded PLGA nanocapsules would influence LRP levels. When treated in the same way as previously described, the HEK293 and A549 cells exhibited no significant difference in LRP levels. Therefore, although there was an observed effect on the cell viability of the A549 cells, the nanoparticles do not appear to have significant off-target effects on LRP levels at treatments of 10% and below. Thus, only percentages below 10% v/v (volume nanoparticles/blood volume) of the nanoparticles will be used for *in vivo* treatments.

Although we have yet to encapsulate LRP-specific siRNA using the now optimised protocol for PLGA nanocapsule synthesis and test the therapeutic potential, if we do encounter a problem with low knockdown efficiency, there are various means to improve this. This includes creating hybrid nanoparticles, as well as numerous different types of surface modification. Liposomes have shown to be efficient in this regard and have both low toxicity and low immunogenicity, but are robust structurally [280]. Therefore, creating hybrid nanoparticles, which take advantage of the positive aspects of both PLGA and liposomes, would create an optimal siRNA delivery system that has a high encapsulation efficiency, a sustained siRNA release profile and high serum stability [281, 282]. In this way, the siRNA is encapsulated in the polymeric PLGA core and coated with a lipid shell, which enables the nanoparticle hybrid to be taken up by the cell. Indeed, this principle has been used to efficiently knockdown gene expression in prostate cancer, with minimal cytotoxicity [283]. Furthermore, surface modifications such as chitosan, PEGylation or attachment of ‘self’ peptides and cancer specific moieties will allow for a more active targeting approach, as well as avoiding phagocytosis.

Rather than relying on the enhanced permeability and retention effect (passive targeting) and risking sequestration [284].

Altogether, this therefore suggests that the optimised protocol for PLGA nanoparticle synthesis allows for hollow nanocapsule production in the 200-500 nm size range, with uniform batch-to-batch variability. Furthermore, that these nanocapsules selectively exhibit cytotoxicity on cancer cells and display no significant off-target effects on LRP levels. Ultimately, these nanocapsules show great potential for use as a delivery system for LRP-targeting siRNA, which will be the focus of future studies.

CHAPTER FIVE – CONCLUSION

The global occurrence and mortality rates of cancer are growing at an exponential rate. In particular, lung cancer is the most frequently occurring cancer and the foremost cause of cancer-related deaths. The fundamental cause of the extremely complex, multi-step process of tumourigenesis are the hallmarks of cancer and their enabling characteristics. Since there is co-dependence and complementarity amongst these hallmarks, prospective treatments need to consider targeting them on multiple levels, rather than independently. The 37 kDa laminin receptor precursor/67 kDa laminin receptor (LRP/LR) is overexpressed in many different cancer types, and as a result is involved in promoting the tumourigenic process by its involvement in enhancing cell migration, adhesion and invasion, maintaining cell viability, inducing angiogenesis and evading apoptosis, as well as increasing telomerase activity. The current study revealed that siRNA-mediated downregulation of LRP/LR in A549 lung cancer cells significantly inhibits the characteristics of multiple cancer hallmarks. Indeed, we observed a significant reduction in cell viability and attributed this to the induction of apoptosis, as well as a reduction in migration potential and telomerase activity. Therefore, targeting four of the known hallmarks. Furthermore, proteomic analysis performed after LRP downregulation revealed upregulation of several proteins in the MHC I antigen presentation pathway, indicating the possible re-establishment of immune control over the cells. Not only does this suggest the inhibition of another key cancer hallmark, but also indicates a novel function of LRP/LR in its oncogenic form, as contributing to cancer cell evasion of immune destruction. Thus, offering a novel facet to the potential use of downregulating LRP/LR for the treatment of lung cancer. In addition, proteomic analysis revealed upregulation of numerous proteins with tumour suppressor functions that are specifically involved in cell cycle modulation and apoptosis, further confirming the induction of apoptosis, as well as suggesting the induction of cell growth repressors with consequent cell cycle arrest. However, translation of this research to an *in vivo* setting requires an effective delivery system for the siRNA. Therefore, this study also included the assessment of whether PLGA nanoparticles would provide an effective, minimally cytotoxic delivery system and revealed that optimised PLGA nanocapsules were selectively cytotoxic to the A549 cancer cells and exhibited no off-target effects on LRP levels.

In conclusion, this study has revealed siRNA-mediated downregulation of LRP/LR inhibits several key cancer hallmarks in lung cancer cells including resistance to cell death, evasion of growth suppressors, activation of metastasis, enabling replicative immortality and evasion of

immune destruction. Therefore, this strategy of targeting LRP/LR has a 5-fold function in impeding key cancer hallmarks in the lung cancer cells, which therefore has the potential for improving the prognosis of those suffering from lung cancer. Furthermore, PLGA nanocapsules will provide an effective delivery system for the LRP-targeting siRNA to be tested *in vivo*. Altogether, we suggest targeting LRP/LR for downregulation provides a possibly powerful therapeutic for inhibiting multiple cancer hallmarks.

5.1. Future studies

To next step in the assessment of the effect of the PLGA nanocapsules on toxicity is a pilot *in vivo* study for optimisation of tumour formation using lung (A549 cells) xenografts in nude mice, which will then be treated with the empty PLGA nanoparticles and LRP-targeting siRNA-loaded nanocapsules. Ethics clearance has been obtained for this study. (Ethics certificate: 2019/10/61/D). Furthermore, since we observed promising results for the use of PLGA nanocapsules as a delivery system for LRP-targeting siRNA, subsequent studies will involve the encapsulation of the RPSA siRNA in the PLGA nanocapsules. However, once the siRNA is encapsulated, we will perform further *in vitro* testing to investigate whether the effects of the siRNA-loaded nanocapsules compare with those observed in the present and previous studies using ‘naked’ siRNA. Furthermore, we will assess the release profile and loading efficiency *in vitro* before the siRNA-loaded PLGA nanoparticles will be tested *in vivo*.

The first part of this study involving the assessment of the effect of LRP downregulation in the A549 cells has certainly provided promising results for the use of LRP-targeting siRNA as a potential therapeutic. Therefore, subsequent studies will delve further into the results observed in the current study to better understand the mechanism behind these observed effects. Since we observed a decrease in cell viability, as well as an upregulation of several proteins involved in growth arrest, it will be interesting to perform cell cycle analysis to both confirm this, as well as to investigate the specific mechanism involved. In addition, as we observed a reduction in migration potential, subsequent studies can include specifically assessing the adhesive and invasive potential of the A549 cells after LRP downregulation, to provide a better substantiation of the inhibition of these metastatic processes. Additionally, ensuing studies could include assessing TERT levels after LRP downregulation. This is because, not only is TERT considered to be the limiting factor in telomerase activity, but TERT itself plays non-canonical functions in preventing apoptosis and rescuing cells from ROS-mediated cytotoxicity. Therefore, a decrease in TERT levels after LRP downregulation would provide

further evidence to the knockout of telomerase and induction of apoptosis observed in this study. It is also of interest to perform analysis on the effect on mitochondrial function and energy metabolism since this is another cancer hallmark that could possibly be affected.

In terms of the proteomic analysis aspect of this study, firstly, it would prove interesting to attempt to define the role of the four proteins not identified in Reactome. Secondly, subsequent studies will include confirming the levels of the identified dysregulated proteins through other methods, such as western blotting and mRNA analysis. Since there is a likelihood that the reduction in cell viability observed was due to a combination of cell cycle arrest and apoptosis, and that there were no dysregulated proteins identified in pro-inflammatory pathways, it would therefore also be interesting to perform cell cycle analysis and perform specific assessment of pro-inflammatory markers after downregulation of LRP in these A549 cells. Lastly, as there is an earlier response to LRP downregulation, subsequent analysis will include earlier proteomics analysis, at 48 hours post-transfection, as well as phosphoproteomics analysis. This will give insight into the earlier response genes activated, as well as a better understanding of the signalling pathways involved.

Supplementary results

Table 2: All downregulated proteins identified via SWATH-MS after downregulation of LRP/LR.

Fold change	Protein Identifier (UniProt ID)	Protein Name	p-value	Function
^a -3.7197	ZC3H11A (O75152)	Zinc finger CCCH domain-containing protein 11A	0.012118	Involved in nuclear mRNA export
-1.9631	H2AFZ (P0C0S5)	Histone H2A.Z	0.000631	Variant histone H2A which replaces conventional H2A in a subset of nucleosomes
-1.4441	HPGD (P15428)	15-hydroxyprostaglandin dehydrogenase [NAD (+)]	7.59E-09	-Prostaglandin inactivation -Catalyses the NAD-dependent dehydrogenation of lipoxin A4 to form 15-oxo-lipoxin A4 -Inhibits in vivo proliferation of colon cancer cells
-1.3834	LAMP1 (P11279)	Lysosome-associated membrane glycoprotein 1	1.24E-06	-Presents carbohydrate ligands to selectins. -Implicated in tumour cell metastasis
-1.3683	ACO1 (P21399)	Cytoplasmic aconitate hydratase	6.62E-10	-Binds a 4Fe-4S cluster and functions as aconitase when cellular iron levels are high -Functions as mRNA binding protein that regulates uptake, sequestration and utilization of iron when cellular iron levels are low -Binds to iron-responsive elements (IRES) in target mRNA species when iron levels are low
-1.3569	LAMTOR1 (Q6IAA8)	Ragulator complex protein LAMTOR1	0.000213	Involved in amino acid sensing and activation of mTORC1, a signalling complex promoting cell growth in response to growth factors, energy levels, and amino acids
-1.3474	CTSD (P07339)	Cathepsin D	1.10E-08	-Acid protease active in intracellular protein breakdown -Involved in the pathogenesis of several diseases such as breast cancer and possibly Alzheimer disease
-1.3157	HRSP12 (P52758)	2-iminobutanoate/2-iminopropanoate deaminase	0.003533	Catalyzes the hydrolytic deamination of enamine/imine intermediates that form during normal metabolism
-1.2833	GBE1 (Q04446)	1,4- α -glucan-branching enzyme	4.02E-07	Required for normal glycogen accumulation
-1.2697	GCLC (P48506)	Glutamate-cysteine ligase catalytic subunit	5.41E-08	
-1.2633	GBA (P04062)	Glucosylceramidase	0.000575	-Plays a central role in the degradation of complex lipids and the turnover of cellular membranes -Plays a role in cholesterol metabolism

-1.2419	CNTN1 (Q12860)	Contactin-1	4.02E-07	-Mediate cell surface interactions during nervous system development
-1.2083	TIMM9 (Q9Y5J7)	Mitochondrial import inner membrane translocase subunit	0.000288	Mitochondrial intermembrane chaperone that participates in the import and insertion of multi-pass transmembrane proteins into the mitochondrial inner membrane
-1.1543	PCYOX1 (Q9UHG3)	Prenylcysteine oxidase 1	0.000215	-Involved in the degradation of prenylated proteins.
-1.1333	DDX27 (Q96GQ7)	Probable ATP-dependent RNA helicase	4.62E-06	-Component of the nucleolar ribosomal RNA (rRNA) processing machinery that regulates 3' end formation of ribosomal 47S rRNA
-1.0995	UBE3C (Q15386)	Ubiquitin-protein ligase E3C	1.09E-05	E3 ubiquitin-protein ligase that accepts ubiquitin from the E2 ubiquitin-conjugating enzyme UBE2D1 in the form of a thioester and then directly transfers the ubiquitin to targeted substrates
-1.0725	HNRNPUL1 (Q9BUJ2)	Heterogeneous nuclear ribonucleoprotein U-like protein 1	2.07E-06	-Acts as a basic transcriptional regulator. Represses basic transcription driven by several virus and cellular promoters. When associated with BRD7, activates transcription of glucocorticoid-responsive promoter in the absence of ligand- stimulation. -Plays a role in mRNA processing and transport
-1.0429	PARP14 (Q460N5)	Poly [ADP-ribose] polymerase 14	0.001432	-By mono-ADP-ribosylating STAT1 at 'Glu-657' and 'Glu-705' and thus decreasing STAT1 phosphorylation -Negatively regulates pro-inflammatory cytokines production in macrophages in response to IFNG stimulation.
-1.0416	PRKAR2A (P13861)	cAMP-dependent protein kinase type II-alpha regulatory subunit	0.000303	-involved in cAMP signalling in cells -Type II regulatory chains mediate membrane association by binding to anchoring proteins, including the MAP2 kinase
-1.0155	RAD23B (P54727)	UV excision repair protein RAD23 homolog B	2.64E-06	Multiubiquitin chain receptor involved in modulation of proteasomal degradation
-1.0023	DBI (P07108)	Acyl-CoA-binding protein	1.93E-06	Binds medium- and long-chain acyl-CoA esters with very high affinity and may function as an intracellular carrier of acyl-CoA esters

^a proteins in **bold** are those with a fold change of 2 and above.

Table 3: All upregulated proteins identified via SWATH-MS after downregulation of LRP/LR.

Fold change	STRING Identifier (UniProt ID)	Protein Name	p-value	Function
1.0380	NDUFB10 (O96000)	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10	4.20E-09	Accessory subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I)
1.0593	NUB1 (Q9Y5A7)	NEDD8 ultimate buster 1	3.25E-05	Specific down-regulator of the NEDD8 conjugation system
1.1022	KPNA2 (P52292)	Importin subunit alpha-1	6.89E-06	Functions in nuclear protein import as an adapter protein for nuclear receptor KPNB1
1.1092	NCOR2 (Q9Y618)	Nuclear receptor corepressor 2	0.00343	Mediates the transcriptional repression activity of some nuclear receptors by promoting chromatin condensation
1.1113	RRBP1 (Q9P2E9)	Ribosome-binding protein 1	1.19E-06	Acts as a ribosome receptor and mediates interaction between the ribosome and the endoplasmic reticulum membrane
1.1190	CYCS (P99999)	Cytochrome c	3.15E-09	Electron carrier protein
1.1424	SQRDL (Q9Y6N5)	Sulfide:quinone oxidoreductase, mitochondrial	1.47E-08	Catalyses the oxidation of hydrogen sulphide with the help of a quinone, such as ubiquinone, giving rise to thiosulfate and ultimately to sulfane (molecular sulphur) atoms.
1.1589	RNF213 (Q63HN8)	E3 ubiquitin-protein ligase	1.05E-05	Involved in angiogenesis -Involved in the non-canonical Wnt signalling pathway in vascular development
1.1594	MIA2 (Q96PC5)	Melanoma inhibitory activity 2	0.000265	
1.1757	TST (Q16762)	Thiosulfate sulfotransferase	0.000148	Formation of iron-sulfur complexes, cyanide detoxification or modification of sulfur-containing enzymes
1.1872	ZC3HAV1 (Q7Z2W4)	Zinc finger CCCH-type antiviral protein 1	2.50E-07	Antiviral protein which inhibits the replication of viruses by recruiting the cellular RNA degradation machineries to degrade the viral mRNAs
1.2062	MRPS36 (P82909)	Mitochondrial ribosomal protein S36	0.014676	
1.3110	EPHA2 (P29317)	Ephrin type-A receptor 2	0.007786	Receptor tyrosine kinase which binds promiscuously membrane-bound ephrin-A family ligands residing on adjacent cells
1.3332	LIMA1 (Q9UHB6)	LIM domain and actin-binding protein 1	7.65E-06	Binds to actin monomers and filaments. --- Increases the number and size of actin stress fibres and inhibits membrane ruffling - Inhibits actin filament depolymerization. Bundles actin filaments, delays filament nucleation and reduces formation of branched filaments
1.3612	PSME2 (Q9UL46)	Proteasome activator complex subunit 2	2.43E-07	Implicated in immunoproteasome assembly and required for efficient antigen processing
1.5087	PSME1 (Q06323)	Proteasome activator complex subunit 1	1.82E-11	Implicated in immunoproteasome assembly and required for efficient antigen processing.
1.5478	SPATS2L (Q9NUQ6)	Spermatogenesis associated serine rich 2 like protein	5.12E-09	Belongs to the SPATS2 family
1.5699	EHD4 (Q9H223)	EH domain-containing protein 4	4.80E-07	ATP- and membrane-binding protein that probably controls membrane reorganization/tubulation upon ATP hydrolysis

1.6533	MRPL15 (Q9P015)	Mitochondrial ribosomal protein L15	2.06E-06	is a member of MRPL, which provides energy in the form of ATP for cell growth
1.6601	STK4 (Q13043)	Serine/threonine-protein kinase 4	9.50E-05	-Stress-activated, pro-apoptotic kinase which, following caspase-cleavage, enters the nucleus and induces chromatin condensation followed by internucleosomal DNA fragmentation - Key component of the Hippo signalling pathway which plays a pivotal role in organ size control and tumour suppression by restricting proliferation and promoting apoptosis
1.7610	TGM2 (P21980)	Protein-glutamine gamma- glutamyltransferase 2	0.000436	Catalyses the cross-linking of proteins and the conjugation of polyamines to proteins
1.7854	CNP (P09543)	2',3'-cyclic-nucleotide 3'- phosphodiesterase	2.42E-07	May participate in RNA metabolism in the myelinating cell
1.8448	OGFR (Q9NZT2)	Opioid growth factor receptor	1.35E-09	Seems to be involved in growth regulation
^a 1.9839	C15orf48 (Q9C002)	Normal mucosa of esophagus-specific gene 1 protein	1.34E-07	Belongs to the complex I NDUF4A subunit family
2.0217	APOL2 (Q9BQE5)	Apolipoprotein L2	1.19E-06	May affect the movement of lipids in the cytoplasm or allow the binding of lipids to organelles
2.0361	LCN2 (P80188)	Neutrophil gelatinase- associated lipocalin	0.015405	Iron-trafficking protein involved in multiple processes such as apoptosis, innate immunity and renal development
2.0493	NAPA (P54920)	Alpha-soluble NSF attachment protein	2.43E-08	Required for vesicular transport between the endoplasmic reticulum and the Golgi apparatus
2.2511	PNPT1 (Q8TCS8)	Polyribonucleotide nucleotidyltransferase 1, mitochondrial	3.02E-08	RNA-binding protein implicated in numerous RNA metabolic processes
2.3045	PC (P11498)	Pyruvate carboxylase, mitochondrial	0.002444	Catalyses in a tissue specific manner, the initial reactions of glucose (liver, kidney) and lipid (adipose tissue, liver, brain) synthesis from pyruvate
2.3345	EIF2AK2 (P19525)	Interferon-induced, double- stranded RNA-activated protein kinase	4.52E-08	Plays a key role in the innate immune response to viral infection and is also involved in the regulation of signal transduction, apoptosis, cell proliferation and differentiation
2.3584	PSMB8 (P28062)	Proteasome subunit beta type-8	1.28E-05	Involved in antigen processing to generate class I binding peptides
2.6578	PSMB9 (P28065)	Proteasome subunit beta type-9	5.99E-10	Involved in antigen processing to generate class I binding peptides
2.7058	PML (P29590)	Protein PML	0.000747	Functions via its association with PML-nuclear bodies (PML-NBs) in a wide range of important cellular processes, including tumour suppression, transcriptional regulation, apoptosis, senescence, DNA damage response, and viral defence mechanisms
2.7725	TAP1 (Q03518)	Antigen peptide transporter 1	4.03E-08	Involved in the transport of antigens from the cytoplasm to the endoplasmic reticulum for association with MHC class I molecules
2.9067	LGALS3BP (Q08380)	Galectin-3-binding protein	4.03E-07	- Promotes integrin-mediated cell adhesion - May stimulate host defence against viruses and tumour cells

2.9820	IFIT2 (P09913)	Interferon-induced protein with tetratricopeptide repeats 2	0.008082	- inhibits expression of viral messenger RNAs lacking 2'-O-methylation of the 5' cap - provide a molecular signature to distinguish between self and non-self mRNAs by the host during viral infection
3.0249	TYMP (P19971)	Thymidine phosphorylase	4.54E-10	- May have a role in maintaining the integrity of the blood vessels - Has growth promoting activity on endothelial cells, angiogenic activity in vivo and chemotactic activity on endothelial cells in vitro
3.0590	ISG20 (Q96AZ6)	Interferon-stimulated gene 20 kDa protein	3.51E-06	Interferon-induced antiviral exoribonuclease that acts on single-stranded RNA and also has minor activity towards single- stranded DNA
3.1678	NT5C3A (Q9H0P0)	Cytosolic 5'-nucleotidase 3A	1.31E-10	Nucleosidase which shows specific activity towards cytidine monophosphate (CMP) and 7-methylguanosine monophosphate (m(7)GMP)
3.1814	LAP3 (P28838)	Cytosol aminopeptidase	2.52E-08	- Presumably involved in the processing and regular turnover of intracellular proteins - Catalyses the removal of unsubstituted N-terminal amino acids from various peptides
3.1882	TAPBP (O15533)	Tapasin	2.37E-10	Involved in the association of MHC class I with transporter associated with antigen processing (TAP) and in the assembly of MHC class I with peptide (peptide loading)
3.3612	SAMHD1 (Q9Y3Z3)	Deoxynucleoside triphosphate triphosphohydrolase	6.29E-08	Host restriction nuclease involved in defence response to virus
3.4444	IFI35 (P80217)	Interferon-induced 35 kDa protein	2.65E-10	Not yet known
3.4587	OAS3 (Q9Y6K5)	2'-5'-oligoadenylate synthase 3	5.64E-06	-Interferon-induced, dsRNA-activated antiviral enzyme which plays a critical role in cellular innate antiviral response - may also play a role in other cellular processes such as apoptosis, cell growth, differentiation and gene regulation
3.5885	STAT1 (P42224)	Signal transducer and activator of transcription 1	1.88E-09	Signal transducer and transcription activator that mediates cellular responses to interferon-alpha, interferon-gamma, EGF, PDGF and IL6 -mediates the expression of a variety of genes, which is thought to be important for cell viability in response to different cell stimuli and pathogens
3.9890	HLA-A (P04439)	HLA class I histocompatibility antigen, A-3 alpha chain	2.02E-09	Involved in the presentation of foreign antigens to the immune system
4.9251	HLA-B (P01889)	HLA class I histocompatibility antigen, B-7 alpha chain	4.93E-07	Involved in the presentation of foreign antigens to the immune system
5.2837	DDX58 (O95786)	Probable ATP-dependent RNA helicase	7.02E-09	Innate immune receptor which acts as a cytoplasmic sensor of viral nucleic acids and plays a major role in sensing viral infection and in the activation of a cascade of antiviral responses including the induction of type I interferons and proinflammatory cytokines

5.6279	OAS1 (P00973)	2'-5'-oligoadenylate synthase 1	1.80E-10	Interferon-induced, dsRNA-activated antiviral enzyme which plays a critical role in cellular innate antiviral response - may also play a role in other cellular processes such as apoptosis, cell growth, differentiation and gene regulation
5.6556	IFIT3 (O14879)	Interferon-induced protein with tetratricopeptide repeats 3	1.52E-12	FN-induced antiviral protein which acts as an inhibitor of cellular as well as viral processes, cell migration, proliferation, signalling, and viral replication.
6.5920	OASL (Q15646)	2'-5'-oligoadenylate synthase-like protein	5.85E-08	Does not have 2'-5'-OAS activity, but can bind double- stranded RNA - antiviral activity -paralog of OAS1
6.7055	ISG15 (P05161)	Ubiquitin-like protein ISG15	8.41E-11	Ubiquitin-like protein which plays a key role in the innate immune response to viral infection either via its conjugation to a target protein (ISGylation) or via its action as a free or unconjugated protein
7.2337	IFIT1 (P09914)	Interferon-induced protein with tetratricopeptide repeats 1	5.21E-12	Interferon-induced antiviral RNA-binding protein that specifically binds single-stranded RNA bearing a 5'-triphosphate group (PPP-RNA), thereby acting as a sensor of viral single- stranded RNAs and inhibiting expression of viral messenger RNAs

^a proteins in **bold** are those with a fold change of 2 and above.

Enlarged figures

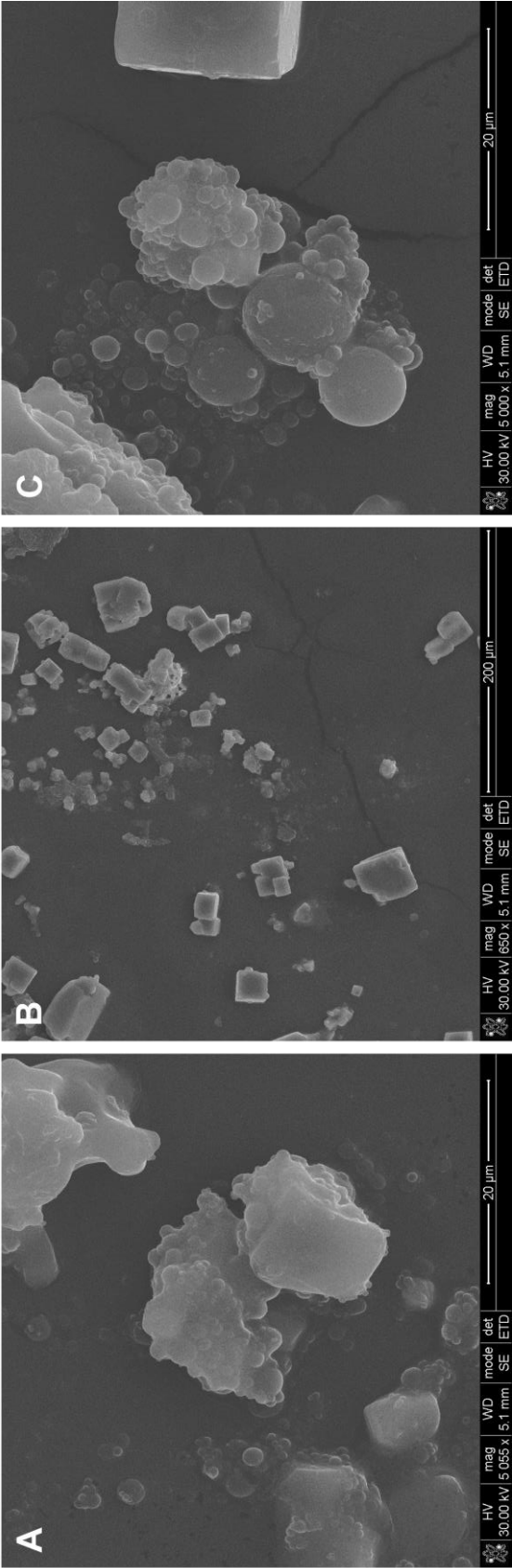


Figure 19 (Enlarged): SEM characterisation of empty PLGA nanoparticles synthesized using poly(vinyl) alcohol as the stabilising agent.

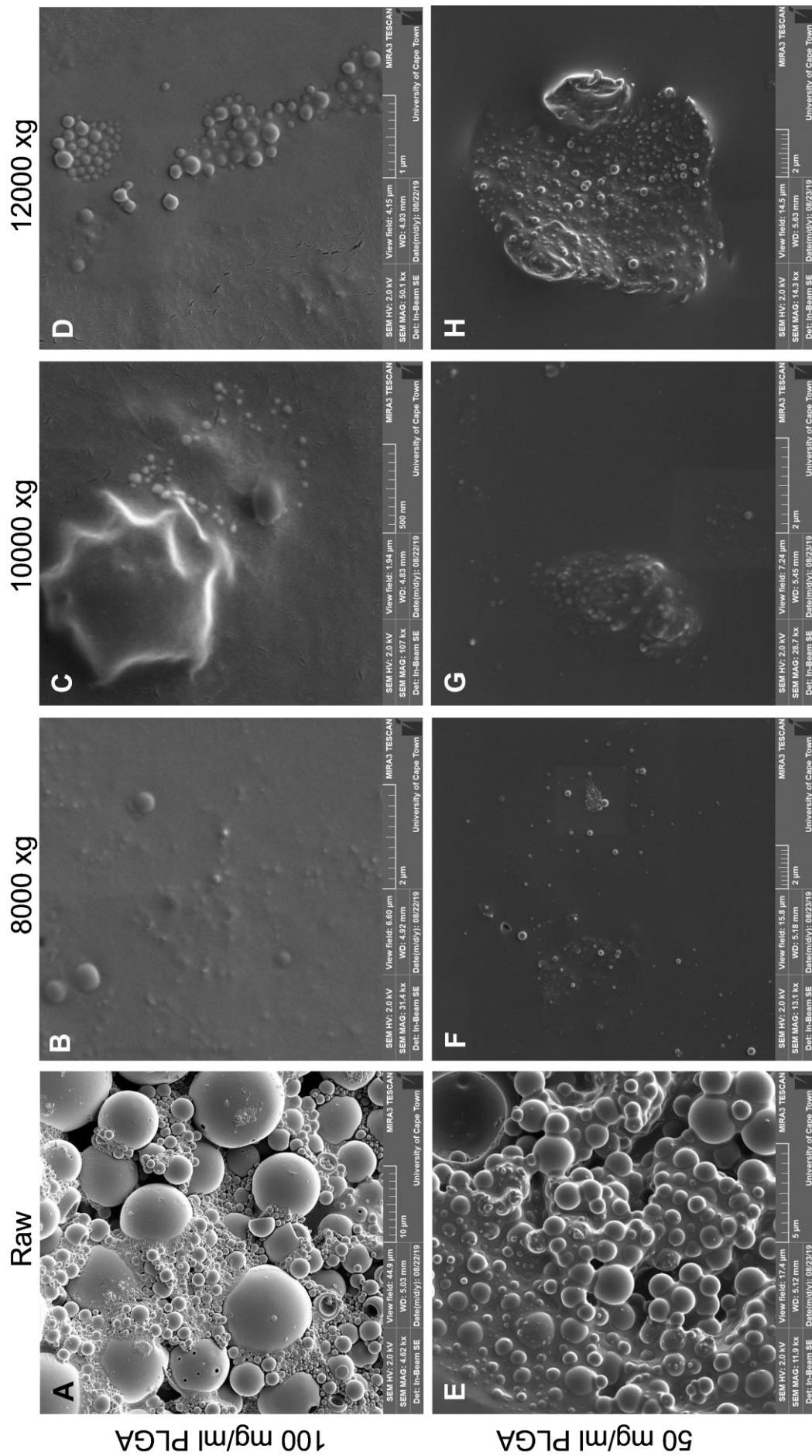


Figure 20 (Enlarged): SEM characterisation of empty PLGA nanoparticles centrifuged at various speeds for optimisation of purification.

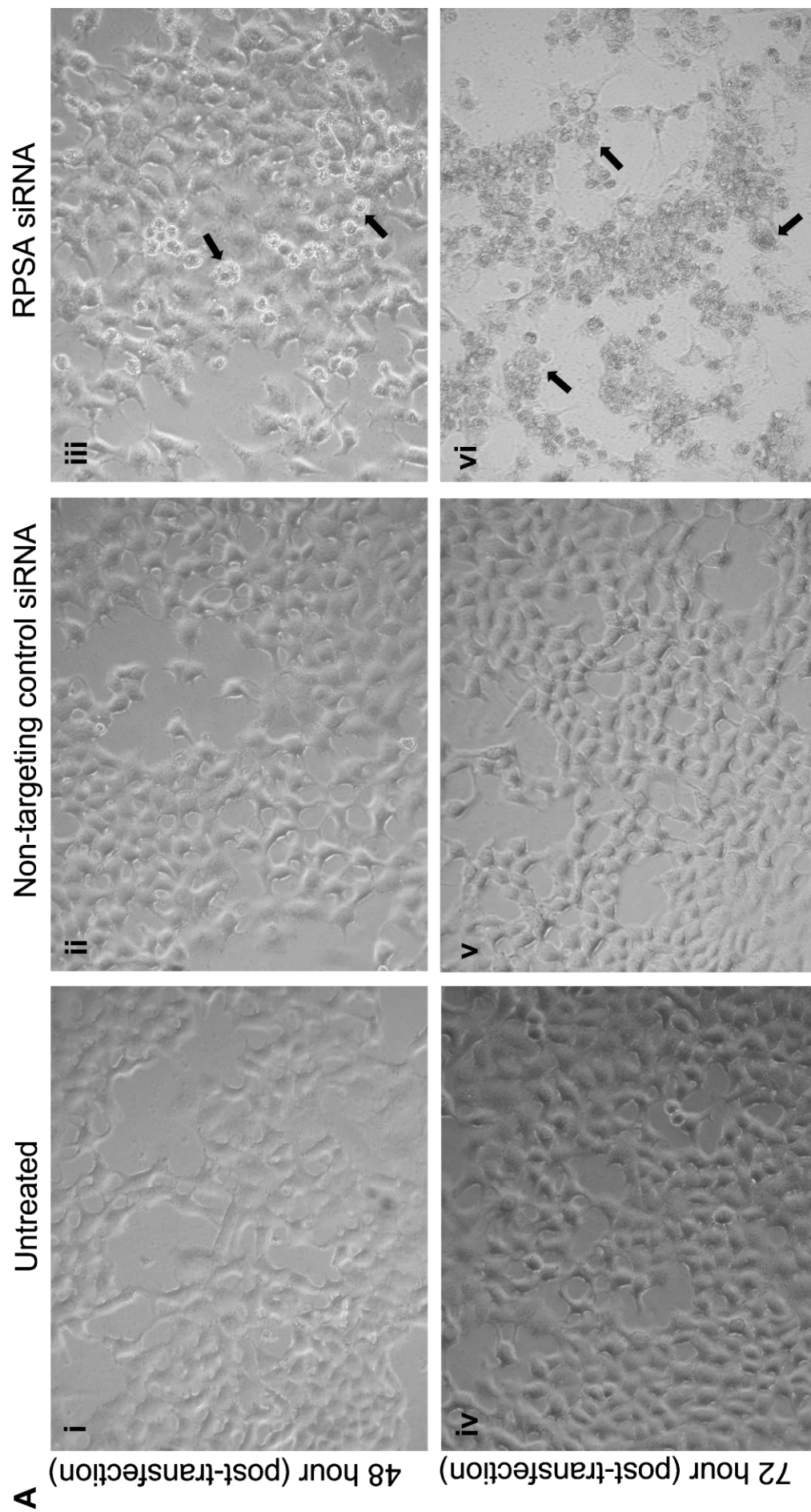


Figure 13 (enlarged): Downregulation of LRP/LR induces an apoptotic morphology in A549 cells

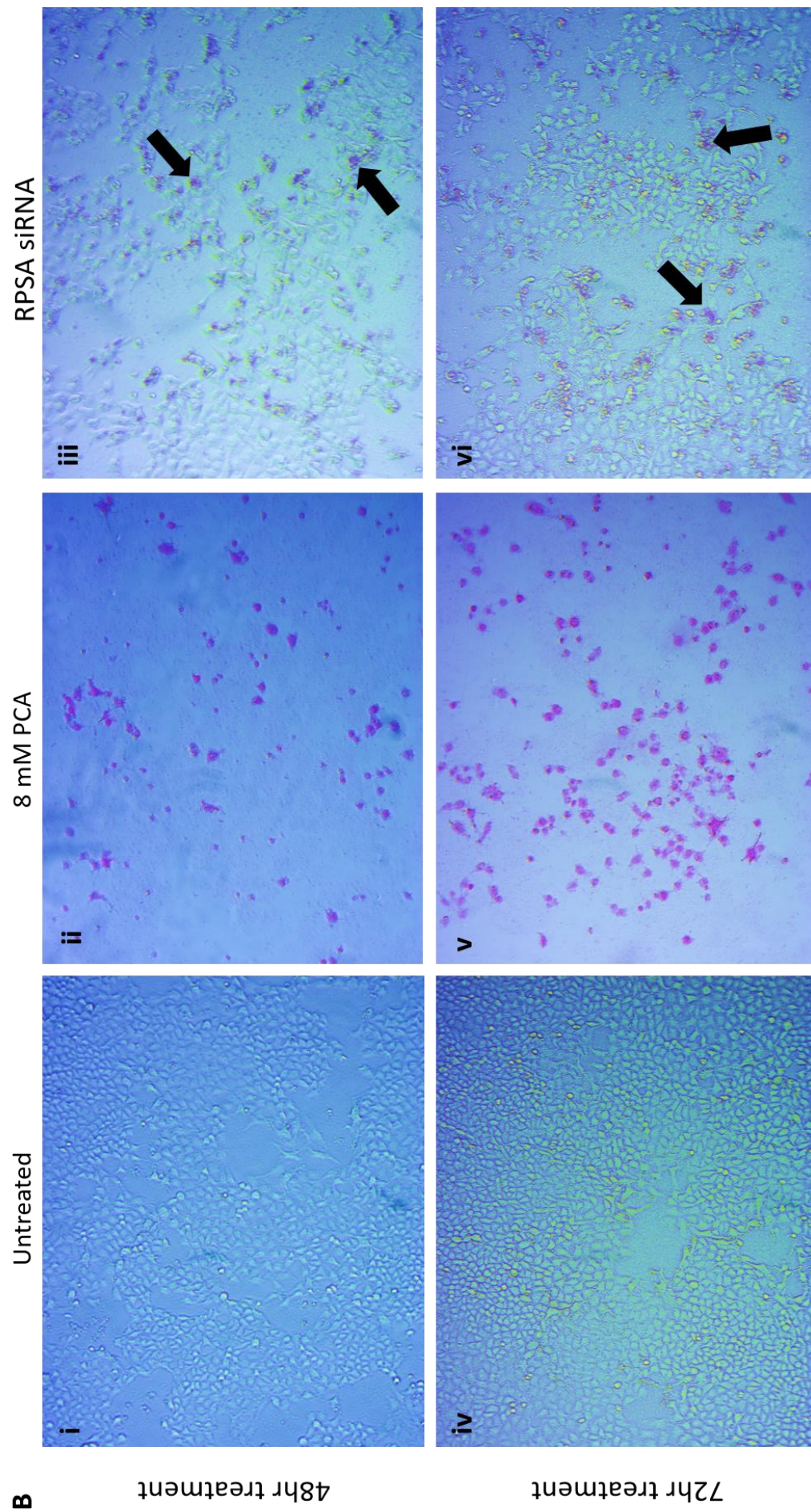


Figure 14. A (Enlarged): siRNA-mediated downregulation of LRP/LR significantly increase apoptosis in A549 lung cancer cells.

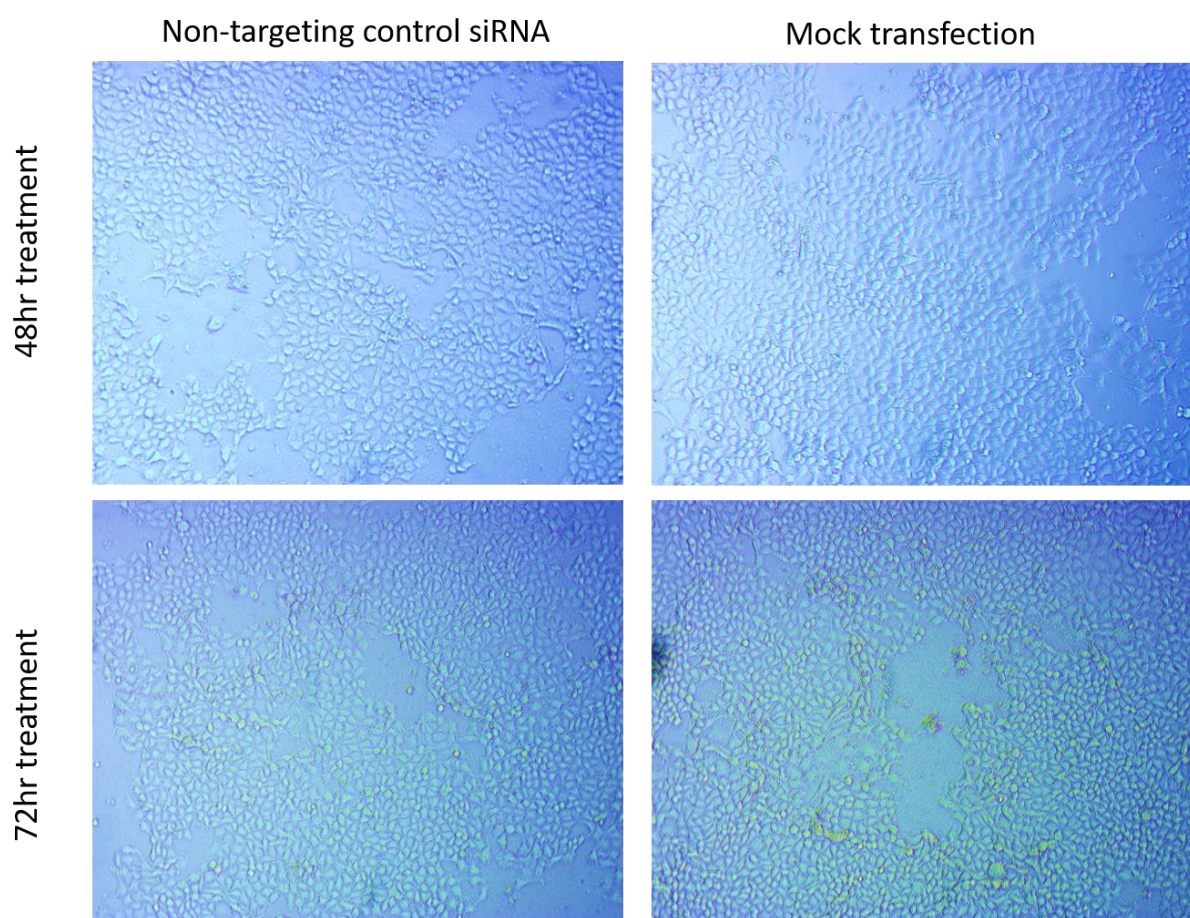


Figure 14. B (Enlarged): Control transfected A549 cells show no apparent apoptosis (APOPercentage™ assay).

There are no apparent cells-stained pink with the APOPercentage dye in the control transfected A549 cells after both 48- and 72-hour transfections.

A

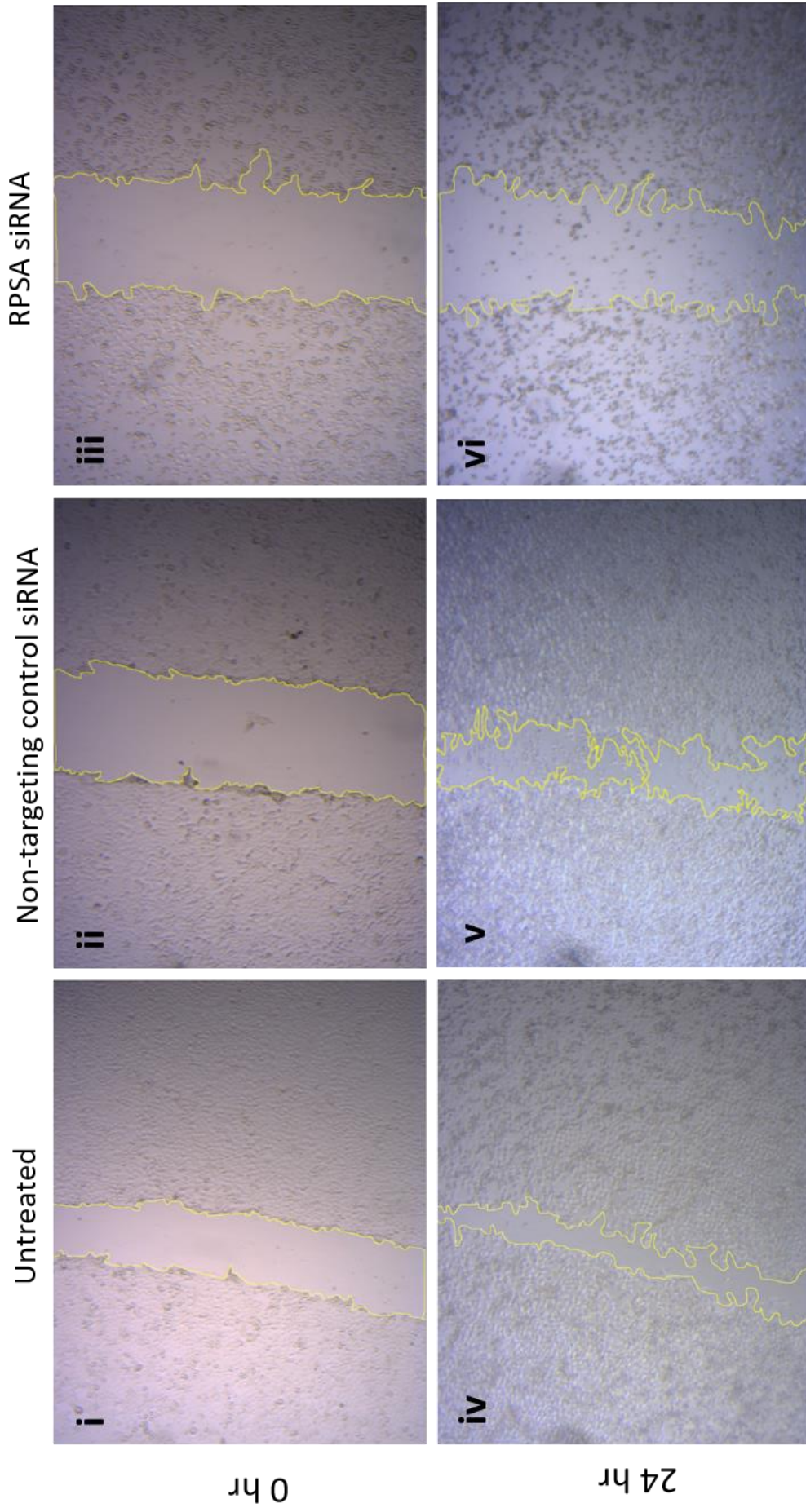


Figure 15 (enlarged): Downregulation of LRP/LR significantly decreases A549 cellular migration

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Appendix A – Supplementary information

Recipes

Table 4: Radioimmunoprecipitation assay (RIPA) lysis buffer

Reagent	1X	2X
NaCl (58.44 g.mol)	150 mM (0.876 g)	300 mM (1.7532 g)
Triton X-100	1 % (1 ml)	2 % (2 ml)
Sodium deoxycholate	0.5 % (0.5 g)	1 % (1 g)
SDS	0.1 % (1 ml of 10 %)	0.2 % (2 ml of 10 %)
Tris-HCl (pH 8.0)	50 mM (10 ml of 500 mM)	100 mM (20 ml of 500 mM)
EDTA (pH 8.0)	5 mM (1 ml of 500 mM)	10 mM (2 ml of 500 mM)

Made up to 100 ml with dH₂O and stored at 4 °C.

500 mM / 0.5M EDTA (pH 8.0)

18.2 g Na₂EDTA

80 ml dH₂O

Make up to 100 ml → pH 8.0 with highly concentrated NaOH (+-20 pellets in 10 ml dH₂O)

- While pH, warm slightly and be aware of overshooting the volume
- Only goes into solution at pH 8.0!

Stored at 4 °C

10 % (w/v) SDS

10 g SDS

Make up to 100 ml dH₂O

Warm gently to dissolve and store at RT.

30% acrylamide/ 0.8% bis-acrylamide

0.8g/100ml bis-acrylamide 4g/500 ml

30g/100ml acrylamide 150g/500ml

Made up to volume with dH₂O and stored at 4 °C

Resolving/ Separation gel buffer (pH 8.8)**2X Tris-HCl/SDS (pH 8.8)**

45.5g/500ml Tris

1g/500ml SDS

Make up to 500ml dH₂O and stored at 4 °C**Stacking gel buffer (pH 6.8)****4x Tris-HCl/SDS (pH6.8)**

30.2g/500ml Tris

2g/500ml SDS

Make up to 500ml dH₂O and stored at 4 °C**10 % (w/v) APS**

0.01 g/ml in

Make up to 100 µl dH₂O

Stored at 4 °C

Table 5: SDS-PAGE gel (30% acrylamide, 2X resolving and 4X stacking).

Reagent/ Number of gels	12 % Separating		15% Separating		5 % Stacking	
	1	2			1	2
dH ₂ O (ml)	1.6	3.2	-		1.83	3.66
30% /0.8% Acrylamide/bis (ml)	3.2	6.4	4	8	0.4	0.8
2X Resolving gel buffer (ml)	4	8	4	8	-	-
4X Stacking gel buffer (ml)	-	-	-	-	0.75	1.5
10% APS (µl)	27	54	27	54	45	90
TEMED(µl)	5	10	5	10	4.5	9
Total vol (ml)	8.8	17.6	8	16	3	6

Table 6: Electrophoresis tank (Running) Buffer (pH 8.3)

Reagent	1X	10X
Tris	3 g	30 g
Glycine	14.4 g	144 g
SDS	1 g	10 g

Made up to 1L with dH₂O and stored at 4 °C.

Table 7: Transfer Buffer

Reagent	10X
Tris	30 g
Glycine	144 g

Made up to 1L with dH₂O and stored at 4 °C.

Reagent	1X
10X Transfer Buffer	100 ml
Methanol	200 ml (20 %)
Cold dH ₂ O	700 ml

Stored at 4 °C.

Blocking buffer**3% (w/v) BSA**

3 g Bovine serum albumin

Made up to 100 ml with 1X PBST

Store at 4 °C

OR 5% blocking buffer

5 g non-fat milk powder

Table 8: Phosphate Buffered Saline (PBS) (pH 7.2/7.4)

Reagent	1X	10X	20X
NaCl	8 g	80 g	160 g
KCl	0.2 g	2 g	4 g
Sodium dihydrogen phosphate dodecahydrate (Na ₂ HPO ₄ ·12H ₂ O)	1.44 g	14.4 g	28.8 g
Monopotassium phosphate (KH ₂ PO ₄)	0.24 g	2.4 g	4.8 g

Made up to 1L with dH₂O and stored at room temperature.

To make PBS-Tween: 1X PBS and 1% (final) Tween20.

Table 9: Western blot stripping buffer (pH 2.2)

Reagent	1L	500 ml	250 ml	100 ml
Glycine	15 g	7.5 g	3.75 g	1.5 g
SDS	1 g	0.5 g	0.25 g	0.1 g
Tween20	10 ml	5 ml	2.5 ml	1 ml

Dissolve in 80 % final volume dH₂O, pH 2.2 then make up to final volume.

Stored at 4 °C for up to 3 days.

Table 10: TRAP qPCR Mastermix

Reagent	Amount per well (µl)
SYBR green/Luna® universal qPCR Mastermix	6.5
10 µM Substrate Primer (TS)	0.5
10 µM Reverse Primer (ACX)	0.5
25 mM EGTA	0.5
DNase/ RNase free dH ₂ O	2.5
Sample/control	2
Total volume/well (half reaction)	12.5

Reagents and Materials

- **Associate chemical enterprise (ACE), Southdale, RSA:** Methanol; Paraformaldehyde; Acetic acid; Hydrochloric acid; Sodium hydroxide (NaOH)
- **Bioline, London, UK:** Sensi-Fast™ SYBR green master mix
- **Biorad Hercules, CA:** Clarity™ Western ECL Substrate; Laemmli sample buffer; Cell counting slides; Triton X-100
- **Biowest/Gibco, Nuaille - France:** Penicillin/streptomycin; PBS; Opti-MEM
- **Capricorn Scientific, Hessen, Germany:** FCS
- **Cleaver Scientific Ltd, Warwickshire, UK:** Agarose
- **Corning Inc. Corning, NY, USA:** 15 ml and 50 ml centrifuge tube; 1.8 ml cryogenic tube, 24-well, 6-well plates as well as 25 cm³ and 75 cm³ cell culture flasks
- **Eppendorf AG, Hamburg, Germany:** 2 ml microfuge tubes
- **Fermentas Life Sciences, Glen Burnie, Burlington, Canada:** Loading buffer
- **GE Life sciences /Hyclone, Massachusetts, USA:** Media (DMEM)
- **Labocare, Secunderabad, India:** Microscopy slides and coverslips
- **Lonza Walkersville, MD:** Trypsin/EDTA; Pen/Strep; Gentamycin
- **Macherey-Nagel, Düren, Germany:** RNase A
- **Merck Millipore, Massachusetts, USA:** Tween 20; DMSO; CHAPs lysis buffer; Ethylenediaminetetraacetic acid (EDTA); Glycine; Isopropanol; KCl; Potassium dihydrogen phosphate (KH₂PO₄); Sodium chloride (NaCl); Sodium dodecyl sulfate (SDS); Tris(hydroxymethyl) aminomethane (Tris)
- **New England Biolabs Inc. Massachusetts, USA:** Luna® Universal qPCR Mastermix
- **Pall corporation, NY, USA:** PVDF membrane
- **Sigma-Aldrich, Missouri, USA:** Non-essential amino acids; BCA reagents; APS; acrylamide; TEMED; fluoromount mounting fluid; SDS; Triton-X; Agarose gel stain; β-mercaptoethanol; Bisacrylamide; Ammonium persulphate; Formaldehyde; Dapi
- **Thermo Fisher Scientific, Massachusetts, USA:** TurboFect™ *in vivo* Transfection Reagent; GeneRuler 1 kb DNA ladder; PageRuler™ Plus prestained protein ladder
- **VWR Life sciences, Pennsylvania, USA:** BSA; Ethanol

Equipment list

- Light microscope – Zeiss Primovert
- Centrifuge – Eppendorf 5417C
- CFX Maestro™ thermo cycler
- Gel casting and running apparatus, Chemidoc imaging machine (Biorad)
- Laminar flow – Labotec
- VICTOR® Nivo™ Multimode Microplate Reader (PerkinElmer)
- NanoDrop One (Thermo Fisher Scientific, Massachusetts, USA)
- pH meter – Eutech instruments
- Pipettes and micropipettes – Eppendorf research
- Trans-Blot® Turbo™ Transfer System – Biorad

Software

- Biorad CFX Maestro™ (version 1.0)
- Image Lab (version 5.1)
- Microsoft Excel 365 ProPlus (Microsoft Corporation)
- Zen v2.6 (Blue edition)