



**The effect of the siRNA-mediated
downregulation of the non-integrin
laminin receptor on cancer cell viability**

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A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science.
Johannesburg, 2013

Declaration

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

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26 day of February 2013 in Johannesburg

Abstract

Cancer is a hypernym used to describe a group of diseases characterised by the uninhibited growth and spread of abnormal cells in the body. An estimated 7.6 million annual deaths are attributed to the disease while 12.7 million new cases are reported every year. The severity of this disease demonstrates the urgent requirement of novel anti-cancer therapeutic agents. The non-integrin laminin receptor, here designated the 37 kDa/67 kDa laminin receptor (LRP/LR), is a multifunctional protein located on the surface, in the cytoplasm, in the perinuclear compartment and in the nucleus of cells. While this receptor is imperative for normal cellular functioning, it has also been implicated in many diseases – it serves as a cell surface receptor for numerous viruses, infectious prion proteins as well as certain respiratory tract pathogens. Additionally, LRP/LR has been found to have some involvement in zoonotic diseases and those involving neurodegeneration, such as Alzheimer's disease. Most importantly for this study, LRP/LR has been implicated in cancer progression, where it was found to be overexpressed on the surface of various cancer cell lines, this overexpression correlating to increased metastasis. The aim of this study was to investigate the effect of the siRNA-mediated downregulation of LRP expression on the viability of tumorigenic lung and cervical cancer cells (A549 and HeLa, respectively). The cell surface LRP/LR and total LRP levels were investigated using flow cytometry and western blotting, respectively, in A549 and HeLa cells, the results revealing high percentages of both cell lines expressing LRP/LR on their surface. Additionally, A549 and HeLa cells express similar total levels LRP. The transfectability of these cells was confirmed and siRNA-LAMR1 was shown to significantly downregulate LRP expression (80% and 60% in A549 and HeLa cells, respectively). MTT assays revealed that the significant 13% and 18% reduction in cellular viability in A549 and HeLa cells, respectively, was as a consequence of LRP downregulation. This reduction in cellular viability was found to be a consequence of induced apoptosis (identified by the visualisation of the loss in nuclear integrity, as well as the significantly increased activity of the apoptosis-associated protein caspase-3) and inhibited cellular proliferation in the aforementioned cells. These findings suggest that siRNA targeting LRP mRNA may act as a potential alternative therapeutic tool for the treatment of cancer.

“The fear of the Lord *is* the beginning of wisdom, and the knowledge of the Holy One *is* understanding”

– Proverbs 9:10

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List of Symbols

°C	Degree Celsius
cm	Centimetre
h	Hour
M	Molar
mA	Milliampere
min	Minute
mL	Millilitre
mm	Millimeter
mM	Millimolar
ng	Nanogram
nm	Nanometer
pg	Picogram
pmol	Picomole
rpm	Revolutions per minute
s	Second
V	Volts
α	Alpha
β	Beta
μg	Microgram
μL	Microlitre
μM	Micromolar
μmol	Micromole

List of Abbreviations

37LBP	37-kDa laminin-binding protein
67LR	67-kDa laminin receptor
A549	Adenocarcinoma human alveolar basal epithelial cells
aa	Amino acid
Ago	Argonaute
AIDS	Acquired immune deficiency syndrome
AMD	Age-related macular degeneration
APS	Ammonium persulfate
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
Bcl2	B-cell lymphoma 2
Bid	Bcl-2 Interacting Domain
bp	Base pair
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
C-terminus	Carboxyl-terminus
CaCl ₂	Calcium chloride
CAD	Caspase-Activated DNase
CAM	Cell-cell adhesion molecule
CO ₂	Carbon dioxide
DD	Death domain
DED	Death effector domain
dH ₂ O	Distilled water
DISC	Death inducing signalling complex
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPBS	Dulbecco's Phosphate-Buffered Saline
dsRNA	Double stranded RNA

<i>E. coli</i>	<i>Escherichia coli</i>
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FADD	Fas-associated protein with death domain
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FXR	Fragile X-related
GFP	Green fluorescent protein
GS	Growth signals
HBV	Hepatitis B virus
HeLa	Human cervix carcinoma cell line
Hep3B	Human hepatoma cell line
HepG2	Liver hepatocellular cell line
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HRP	Horse radish peroxidase
HT1080	Human fibrosarcoma cell line
ICAD	Inhibitor of Caspase-Activated DNase
IOD	Integrated optical density
IPTG	Isopropyl β -D-1-thiogalactopyranoside
KCl	Potassium chloride
kDa	Kilo Dalton
LB	Lysogeny broth
LBP-p40	Laminin-binding protein precursor p40
LRP	Laminin receptor precursor
LRP/LR	37-kDa/67-kDa laminin receptor precursor/laminin receptor
mRNA	messenger RNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
N-terminus	Amino-terminus
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NuMA	Nuclear mitotic apparatus protein
OD	Optical Density
PBS	Phosphate buffered saline

PCA	Protocatechuic acid
PCR	Polymerase chain reaction
pRb	Retinoblastoma protein
PTGS	Post-transcriptional gene silencing
PVDF	Polyvinylidene fluoride
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RPSA	Ribosomal protein SA
rRNA	Ribosomal ribonucleic acid
RSV	Respiratory syncytial virus
RT	Room temperature
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	Small hairpin RNA/short hairpin RNA
siRNA	Small interfering RNA
TBE	Tris/Borate/EDTA
tBid	Truncated Bid
TEMED	Tetramethylethylenediamine
TGS	Transcriptional gene silencing
TNFR	Tumour necrosis factor receptor
TRAIL	(TNF)-related apoptosis-inducing ligand
Tudor SN	Tudor-Staphylococcus nuclease
v/v	volume/volume
w/v	weight/volume
Xgal	5-bromo-4-chloro-indolyl- β -D-galactopyranoside

1. CHAPTER 1 - INTRODUCTION

1.1. Cancer

Cancer is a hypernym used to describe a group of diseases characterised by the uninhibited growth and spread of abnormal cells in the body ¹. If the growth of these cells is not restricted, death is often the result ². Cancer is an indiscriminatory disease, however, diagnosis is directly proportional to age with the majority of cases occurring in the middle aged and older adults ².

Cancers are currently the global leading cause of mortality, accounting for approximately 7.6 million deaths (13% of all deaths) in 2008 ³ while an estimated 12.7 million new cancer cases were reported in the same year ⁴. One in every eight worldwide deaths is attributed to cancer ¹ and in 2007 the disease caused more fatalities than AIDS, tuberculosis and malaria combined ¹. The most commonly diagnosed cancers globally are lung, breast and colorectal cancers while the most common cause of cancer related deaths are lung, stomach and liver cancers ⁴.

According to 2008 GLOBOCAN statistics, Southern Africa has the 7th highest cancer incidence rate, with breast cancer being the most common, followed by prostate, oesophageal and cervix uteri cancers (11%, 10%, 9% and 8% of all cases, respectively) (Figure 1.1). Among men (Figure 1.2a) in Southern Africa, prostate cancer is the most frequent, followed by lung cancer and oesophageal cancer, while in women (Figure 1.2b), breast cancer, cervix uteri cancer and oesophageal cancer are the most common ⁴.

These statistics demonstrate the urgent requirement for the search and development of novel anti-cancer therapeutic agents.

Cancer Incidence Worldwide

Breakdown of the estimated 12.7 million new cases, age standardised incidence rates and the most commonly diagnosed cancers by the different regions of the world, 2008.

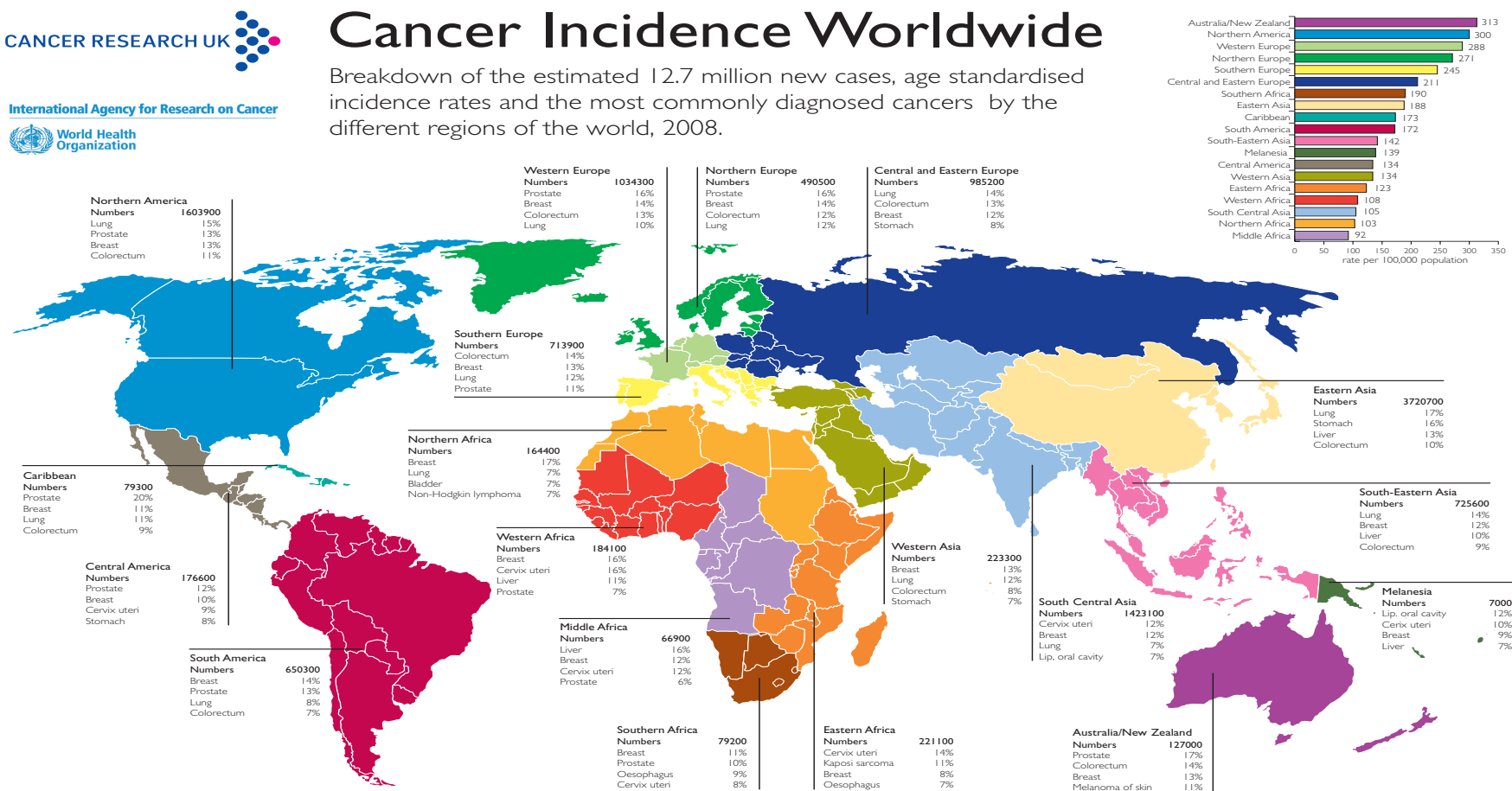
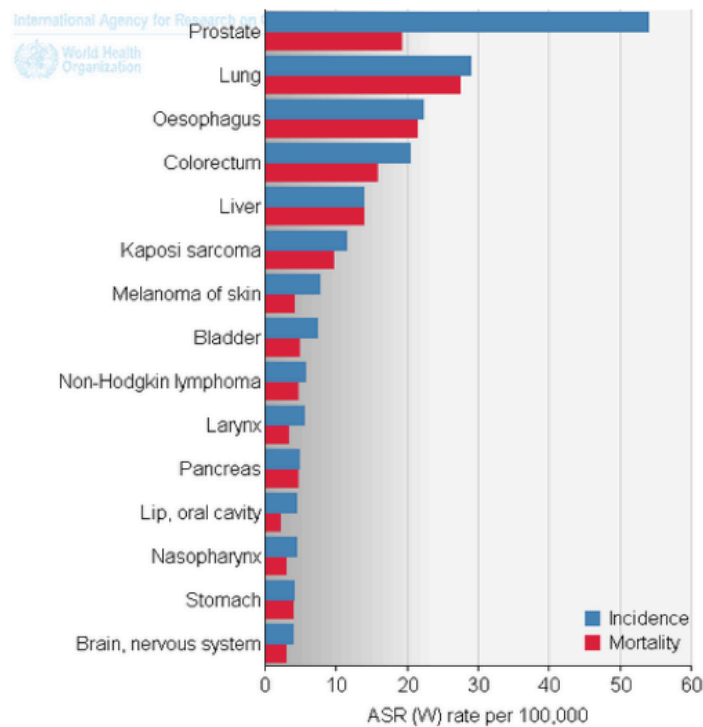


Figure 1.1 – Global incidences of cancer. A map indicating the cancers of highest incidence with respect to country (adopted from GLOBOCAN 2008).

a) Most frequent cancers: men



b) Most frequent cancers: women

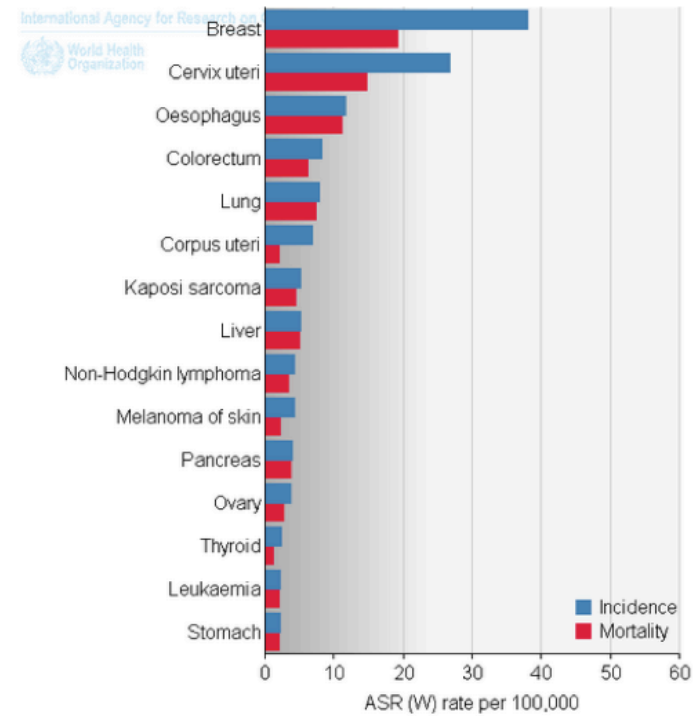


Figure 1.2 – Most frequently diagnosed gender specific cancer types in Southern Africa. Most frequent cancers among a) men and b) women in Southern Africa (adopted from GLOBOCAN 2008).

1.1.1. Cancer cause and prevention

Numerous causes of cancer have to date been identified, both preventable and non-preventable, all concerning malfunctioning genes and proteins involved in the control of cellular growth and division ². The disease can be initiated by internal (inherited mutations, hormones, immune conditions and mutations occurring from metabolic processes) and external (tobacco, infectious organisms, chemicals and radiation) factors ².

Successful prevention of this disease has been observed by decreasing an individual's exposure to certain external causative factors, such as cigarette smoking and excessive alcohol intake (30% of all cancer related deaths in the United States in 2011 were expected to be caused by tobacco use) ². Certain lifestyle choices are also of interest, as it is estimated that 33% of all cancer deaths in the United States in 2011 were expected to be related to obesity, poor nutrition and physical inactivity, therefore also preventable ². Additionally, approximately 2 million of the annually diagnosed cases of skin cancer could be prevented through adequate protection from the sun's rays as well as the avoidance of indoor tanning ².

Certain cancers are caused by infectious agents such as viruses e.g. hepatitis B virus (HBV) ^{5; 6}, human papillomavirus (HPV) ^{7; 8}, human immunodeficiency virus (HIV) ⁹ as well as bacteria such as *Helicobacter pylori* (*H. pylori*) ^{10; 11}. These cancers can be prevented by vaccination ¹², behavioural changes (for sexually transmitted diseases) or by antibiotics ².

1.1.2. Mechanisms of cancer progression

Cells must attain a number of features in order to make the transition from being normal to becoming cancerous. These features, which are necessary in controlled measures for normal cellular development, homeostasis and survival, become exploited by cancerous cells, allowing them to propagate themselves with little impediment. Some of these features include the ability of cancer cells to grow in the absence of growth signals, their unresponsiveness to antigrowth signals, infinite

replicative potential, maintained angiogenesis, invasion and metastasis, and the evasion of apoptosis¹³ (Figure 1.3).

1.1.2.1. Cancer cells self-sufficiency in growth signals

Mitogenic growth signals (GS) are required by normal cells for their progression from a quiescent to an actively proliferative state¹³. The growth signals are transduced into the cell via specific transmembrane receptors, which bind ligands on the extracellular cell surface and subsequently relay the proliferative message along a signal pathway into the cell. It is not possible for normal cells to propagate in the absence of these growth signals; however cells that have become tumorigenic possess this ability¹³. Tumour cells can also possess the ability to produce their own growth stimuli allowing them to proliferate without restriction¹³.

1.1.2.2. Cancer cells unresponsiveness to antigrowth signals

Antigrowth signals, received by transmembrane receptors and subsequently integrated to intracellular signalling pathways, are present in normal cells, and are responsible for the prevention of cellular propagation as well as the maintenance of cellular quiescence and tissue homeostasis¹³. In some cases, when forced out of the proliferative phase of the cell cycle by the action of antigrowth signals, cells maintain the ability to emerge from this state and proliferate in the presence of growth signals¹³. In contrast, antigrowth signals can also permanently prevent further cellular proliferation in certain cells e.g. cells which are fully differentiated¹³. Normal cells are responsive to these signals due to cell cycle regulation; specifically the mechanism that assist in the passage of the cell through G₁ (section 1.3 – the cell cycle). In normal cells, the decision to proliferate is governed by the retinoblastoma protein (pRb) – when hypophosphorylated, pRb blocks proliferation by requisitioning and altering E2F transcription factors, essential proteins involved in the transcription of genes which are required for the cells to progress through G₁¹³. It is essential for cancerous cells to become unresponsive to antigrowth signals if they are to flourish, therefore tumorigenic cells block the pRb pathway, liberating the E2F transcription factors, thus allowing for cellular proliferation¹³. This disruption renders the cells insensitive to these antigrowth factors, therefore promoting tumour formation¹³.

1.1.2.3. Cancer cells infinite replicative potential

In 1997, Hayflick demonstrated that normal cells have a limited replicative potential, showing that the proliferation of cells in culture is finite ¹⁴ and once these cellular populations have attained their set number of proliferative steps, they become senescent (their propagation is discontinued) ¹³. It was shown that senescence could be avoided by disrupting the tumour suppressor proteins pRb and p53, enabling the cells to continue their multiplication, a step known as immortalisation ¹⁵. The replicative potential of normal cells is governed by telomeres (the ends of chromosomal DNA which become 50-100 bp shorter with every replicative cycle due to the inability of DNA polymerases to fully replicate their 3' ends) ¹⁶. In almost all types of cancerous cells, telomere maintenance is observed ¹⁷, where the cells have an upregulated expression of telomerase (functions in the addition of hexonucleotide repeats onto the ends of telomeric DNA), thus allowing the telomeres to counteract their erosion ¹⁸. Evidence for tumour cells possessing an infinite replicative potential is gained from the observation that most cancer types propagated in culture appear to be immortalised ¹⁴.

1.1.2.4. Cancer cells maintain angiogenesis

Cellular functionality and survival is dependent on the adequate receipt of oxygen and nutrients, factors that are transported in the body by the vascular system. The importance of this process is further demonstrated by the observation that all cells and tissue are found to reside within 100 µm of a capillary blood vessels ¹³.

Angiogenesis, a term used to describe the production of new blood vessels in the body, holds high importance in early organ development, however, once tissue formation has been completed, this process is both transitory and tightly regulated ¹³. Since cancerous cells have increased cellular proliferation, they require higher concentrations of oxygen and nutrients; this is achieved through the initiation of tumour angiogenesis by the cells, a step necessary for their maintained growth ¹⁹.

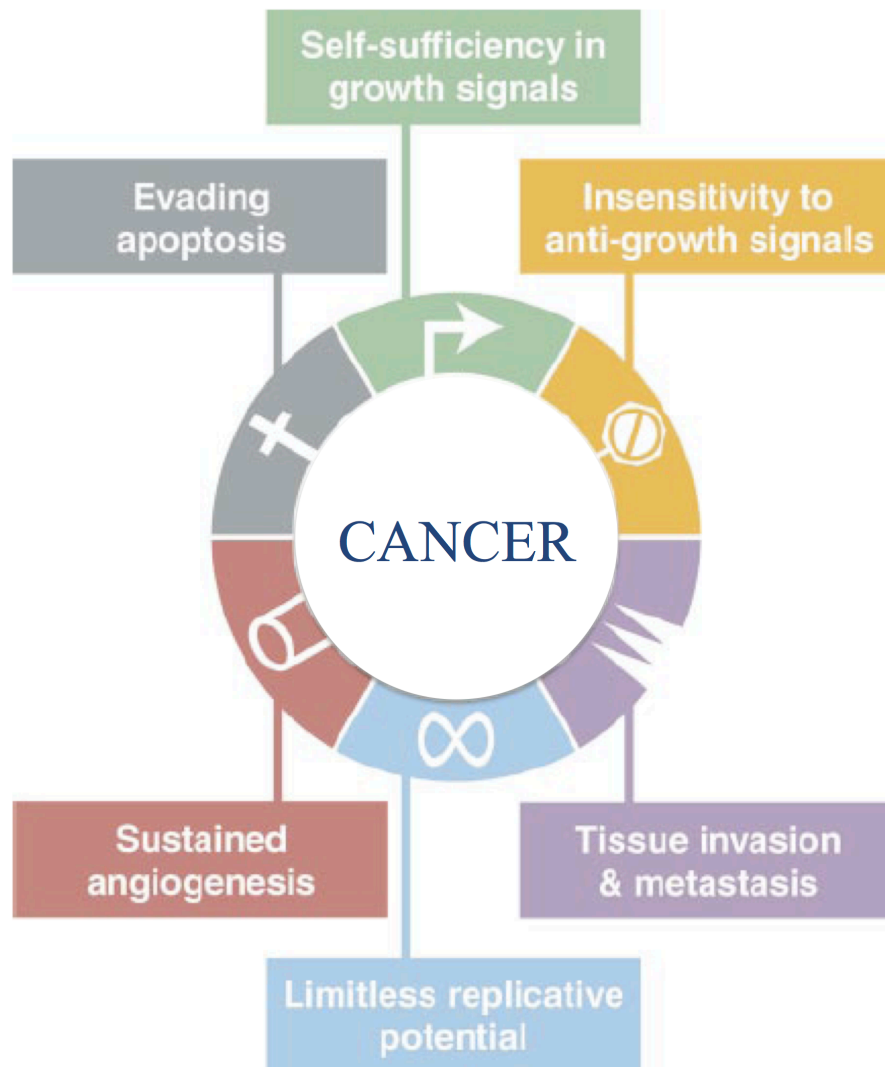


Figure 1.3 – The six main hallmarks of cancer. The six main mechanisms by which cells become cancerous (adopted from ¹³).

1.1.2.5. Cancer cells invasion and metastasis

In the developmental process of most human cancers, cells are able to secede from the primary tumour site and invade adjacent tissues where it is possible for them to establish new colonies¹³, a progression termed metastasis. This process allows cancer cells to inhabit new areas of the body where initially there is little spatial constraint and nutritional restriction¹³. Metastasis has been found to be the cause of 90% of all human cancer deaths²⁰, as it is often difficult to inhibit the spread of these cells.

The molecular mechanisms of invasion and metastasis have been investigated and a number of influential proteins have been identified, with several involved in the tethering of cells to their surrounding environment. These proteins become altered in tumours exhibiting invasive or metastatic tendencies¹³. Some such proteins include the cell-cell adhesion molecules (CAMs)¹³, extracellular proteases^{21; 22} and the cadherin²³ and integrin^{24; 25} families.

1.1.2.6. Cancer cells evasion of apoptosis

Tumour cells expand by increased proliferation and maintain this by a decreased level of cell death. Apoptosis is a form of programmed cell death that is essential for homeostasis, the elimination of cells during development and the eradication of cells possessing harmful mutations²⁶. The acquired resistance of cancerous cells to this process was first observed in 1972 by Kerr *et al.* – subsequent to hormone elimination from hormone-dependent tumours, substantial cell death was observed²⁷. Apoptosis is a tightly regulated process, with a number of triggers and pathways; a detailed description of this form of cell death will be outlined in the section to follow. It is increasingly being observed that the evasion of apoptosis is a hallmark noticed in most if not all cancer types¹³, therefore targeting this process could be a possible therapeutic for the treatment of this disease. However, it must be noted that the evasion of apoptosis can be effectuated through the mutation or dysregulation of a number of proteins and/or genes, e.g. *bcl-2*^{28; 29}, *p53*³⁰, *myc*³¹, adding to the complexity of targeting this process.

1.2. Apoptosis

Apoptosis or programmed cell death is a physiological form of cell death which involves a cell actively committing suicide³². Apoptosis is a fundamental element for many cellular processes including normal cell turnover, which is important for homeostasis, proper development and the functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death²⁶. Tissue damage occurs in cases where cells undergo too much apoptosis and this event is observed in medical conditions such as strokes and neurodegenerative disorders such as Alzheimer's, Huntington's and Parkinson's diseases²⁶. In cases where cells evade apoptosis, medical conditions such as cancers may evolve²⁶. Due to the consequences of the activation of this process, it is both stringently controlled and well regulated in the cell³³, with a large number of regulatory and effector proteins involved in the initiation and execution of the apoptotic pathways.

1.2.1. Apoptotic pathways

The process of apoptosis involves a number of proteins all implicated in intricate and cross-linked pathways. These proteins function in activation cascades, which increase the regulation of this form of cell death. The process of apoptosis takes place in the cell via two major pathways namely the death receptor (extrinsic) pathway and the mitochondrial-dependent (intrinsic) pathway (Figure 1.4). These two apoptosis-inducing pathways can be triggered by a number of signals arising either extra- or intracellularly³⁴.

1.2.1.1. Death receptor (extrinsic) pathway

The death receptor pathway (Figure 1.4) is mediated by the activation of cell surface receptors known as death receptors. Death receptors belong to the tumour necrosis factor receptor (TNFR) gene super family, including TNFR-1, Fas/CD95, and the TRAIL receptors DR-4 and DR-5³⁵. These receptors span the membrane, and once an appropriate ligand has bound to them, transmit the apoptotic signals from the extracellular space into the cytoplasm of the cell²⁶. The binding of the ligand to the

cell surface receptor/death receptor results in the exposure of a conserved amino acid sequence, known as the death domain (DD), on the receptor to the cytosolic side of the plasma membrane³⁶. This results in the recruitment of adaptor molecules such as the Fas-associated protein with death domain (FADD) via DD-DD interactions. Furthermore, FADD also consists of a death effector domain (DED) which is involved in the recruitment and binding of procaspase-8 via DED-DED interactions³⁶. The binding of the FADD to the receptor death domains forms the death inducing signalling complex (DISC), and when concentrated at the DISC, procaspase-8 possess the ability to undergo autocatalytic cleavage and activation, resulting in the production of active caspase-8²⁶. Active caspase-8 is then able to cleave inactive procaspase-3 forming active caspase-3, which then cleaves specific substrates, ultimately resulting in the death of the cell.

Where the apoptotic signals generated by the activation of the death receptor is not sufficient to activate caspase-8 which in turn will activate caspase-3 via the death receptor pathway, the activated caspase-8 cleaves Bid (a member of the Bcl-2 family of proteins) to truncated Bid (tBid). tBid then activates the mitochondrial-dependent (intrinsic) apoptotic pathway as described below³³.

1.2.1.2. Mitochondrial-dependent (intrinsic) pathway

The mitochondrial-dependent apoptotic pathway (Figure 1.4) can be initiated by apoptotic signals from the death receptor pathway (described above) as well as death signals that originate inside the cell³⁷. The death signals originating inside the cell include damage to the DNA, oxidative stress, lack of nutrients, as well as chemotherapeutic drugs²⁶. The aforementioned intracellular death signals cause a shift in the ratio of pro-apoptotic and anti-apoptotic proteins, which subsequently leads to an increase in the permeability of the outer mitochondrial membrane³⁸. The increase of the membrane permeability allows for the release of many pro-apoptotic proteins such as cytochrome c from the mitochondria to the cytosol³⁹. Once in the cytosol, cytochrome c binds to a monomeric molecule called Apaf-1, which then undergoes an ATP-dependent conformational change and oligomerisation, leading to recruitment of procaspase-9 and the formation of the apoptosome. In the apoptosome,

procaspase-9 is cleaved to active caspase-9, which in turn cleaves procaspase-3 to active caspase-3. Active caspase-3 is involved in the initiation of downstream events, e.g. DNA degradation, which ultimately results in the death of the cell ²⁶.

The degradation of DNA is accomplished through the action of the Caspase-Activated DNase (CAD), however, this endonuclease is found complexed to an inhibitor, Inhibitor of Caspase-Activated DNase (ICAD) and is therefore inactive ²⁶. In apoptotic cells, active caspase-3 cleaves ICAD, releasing CAD, which then degrades DNA, causing cell death ²⁶.

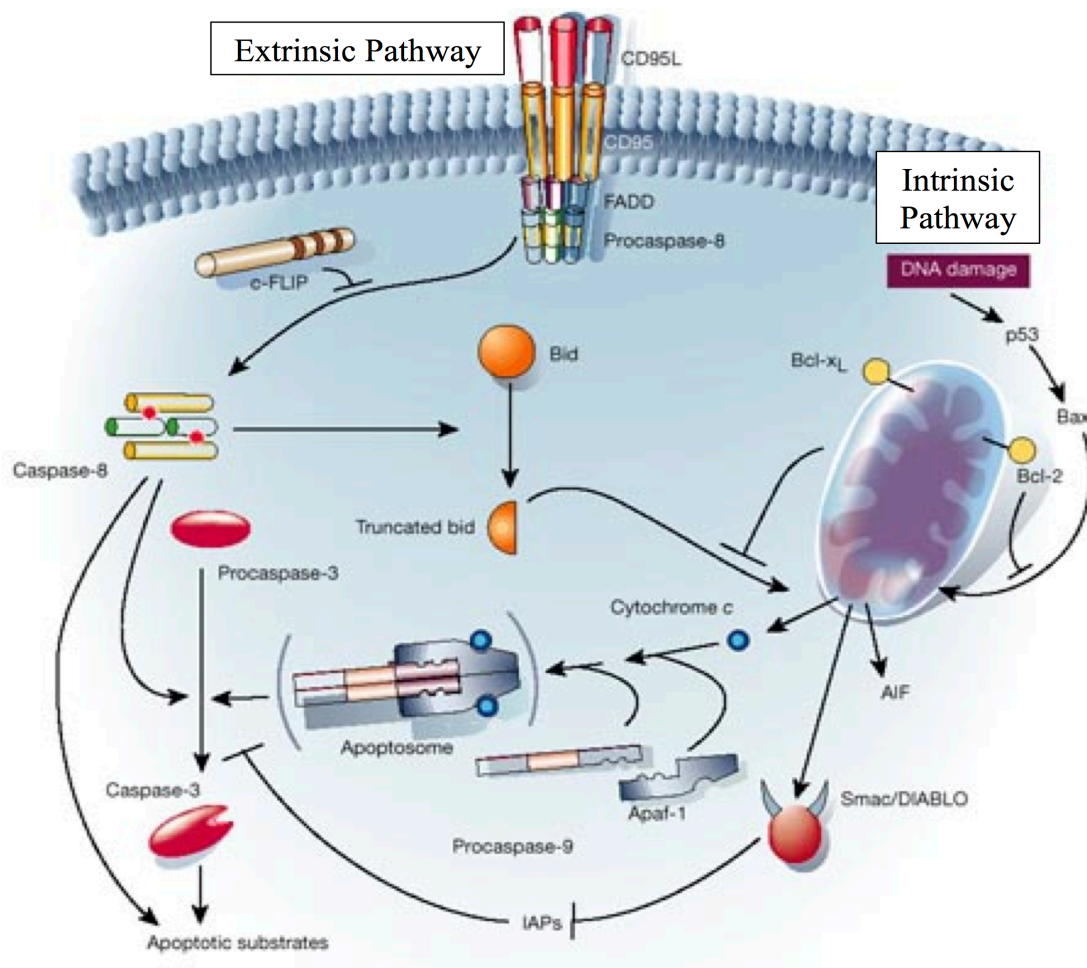


Figure 1.4 – The two main apoptotic pathways in mammalian cells. The death receptor (extrinsic) pathway receives a signal from the exterior of the cell e.g. binding of a ligand to the CD95L receptor, which activates the apoptotic pathway. The binding of a ligand to this death receptor recruits adaptor molecules and procaspase-8. Procaspase-8 is cleaved to form active caspase-8, which in turn cleaves procaspase-3 activating it to caspase-3. The mitochondrial-dependent apoptotic (intrinsic) pathway is activated by intracellular signals e.g. damaged DNA, stress, lack of nutrients, chemotherapeutic drugs etc. These signals cause a change in the permeability of the mitochondrial membrane causing the release of cytochrome c from the mitochondrion into the cytosol. In the cytosol, cytochrome c interacts with Apaf-1 to form the apoptosome. The apoptosome is able to activate procaspase-9 to caspase-9, which in turn activates caspase-3. The intrinsic and extrinsic apoptotic pathways both converge at the activation of caspase-3 which then mediates the downstream pathways responsible for the biochemical and morphological changes observed in apoptotic cells (adopted from ³⁶).

1.2.2. Cellular alterations resulting from apoptosis

Once cellular initiation of programmed cell death pathways has occurred, morphological and biochemical changes in the cell become apparent.

1.2.2.1. Morphological features of apoptosis

At the onset of apoptosis, cellular and nuclear shrinkage is observed. Additionally, the chromatin undergoes condensation, forming sharply defined masses on the periphery of the nuclear membrane ⁴⁰. The nucleus then becomes increasingly condensed in a process known as karyorrhexis and the cell detaches both from the surrounding tissue and the outer membrane, becoming convoluted and forming extensions ⁴⁰. These extensions, called membrane blebs, then detach and form separate membranes surrounding cellular material, and are termed apoptotic bodies ⁴⁰. The apoptotic bodies become phagocytosed into the surrounding cells and are ultimately degraded (Figure 1.5).

The abovementioned process occurs exclusive of inflammation, which would be resultant from the release of intracellular contents into surrounding tissues ⁴⁰.

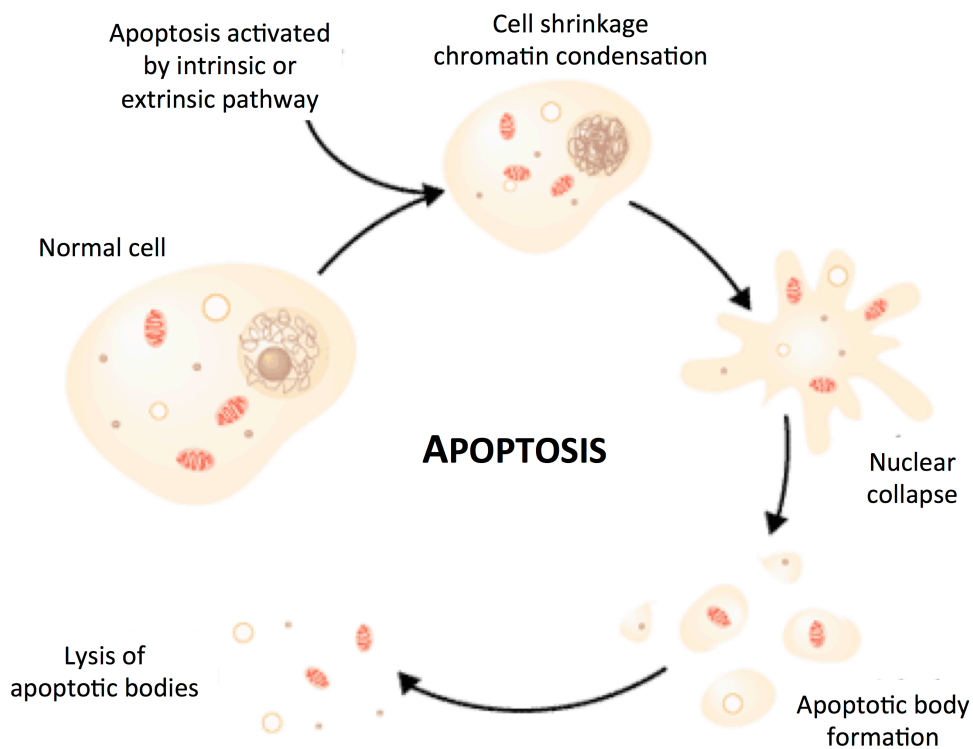


Figure 1.5 – The morphological features associated with apoptosis in the cell.

Once apoptosis has been initiated, the cell shrinks and the chromatin becomes condensed. The nucleus then constricts and collapses, following which the outer membrane of the cell loses its rigidity, becoming convoluted and forming extensions. The extensions, known as apoptotic bodies, surround cellular material and detach from the cell (adopted from <http://cancerbattlefield.com/research/apoptosis/>).

1.2.2.2. Biochemical features of apoptosis

In addition to the abovementioned cellular morphological changes associated with apoptosis, certain biochemical alterations are also observed in apoptotic cells.

The degradation of DNA is an imperative biochemical alteration occurring when cells undergo apoptosis. This process is mediated by the activity of endogenous DNases (e.g. Caspase-Activated DNase), which restrict double-stranded DNA internucleosomally, resulting in 180-200 bp fragments⁴¹ which contain both blunt ends⁴² as well as single base 3' overhangs⁴³.

1.2.3. Caspases and apoptosis

Cysteine-dependent aspartate-specific proteases, commonly referred to as caspases, are a family of proteins that have little association with nonspecific degradative processes, rather, they are involved in specific signalling cascades⁴⁴. They are enzymatically active and show specificity toward protein substrates containing aspartate, using their cysteine side chain to catalyse the cleavage of the peptide bond^{45; 46}.

The physiological functions of the caspases have predominantly been determined by genetic mouse knockout studies which indicate that these proteins have numerous functions in the cell, including involvement in the regulation of cellular proliferation⁴⁷, differentiation⁴⁷, migration⁴⁷ and the bodies innate immunity⁴⁸. While caspases are associated with the aforementioned processes, they are fundamental in the regulation of apoptosis.

Caspases are identified and grouped according to function; those associated with innate immunity (group I) and those involved in the regulation of apoptosis (group II)⁴⁸. Group II/apoptosis associated caspases are further divided into two groups, namely initiator/apical (caspase-2, -8, -9 and -10) and effector/executioner (caspase-3, -6 and -7) caspases⁴⁸. Initiator caspases are produced as ineffective zymogens and require the formation of multicomponent complexes, which trigger their dimerisation, to become active⁴⁹. Effector caspases are also produced as ineffective zymogens, and

become functional through activated initiator caspase-mediated proteolytic processing

⁴⁸.

In multicellular organisms, it is imperative for an appropriate balance between cellular proliferation and death to be maintained ⁴⁷. Because of the fundamental role of apoptosis in the elimination of damaged cells, aberrant expression of the caspases (either in excess or inhibited) holds implications in a number of pathological conditions, ranging from autoimmune diseases, sepsis, immunodeficiency, neurodegenerative disorders and cancer ⁴⁷.

The evasion of apoptosis is a well known hallmark of cancer ¹³ (discussed in section 1.1.2.6), and an overwhelming amount of experimental evidence has suggested that active group II caspases play a fundamental role in bringing about the morphological and biochemical alterations associated with this form of cell death (discussed in sections 1.2.2.1 and 1.2.2.2, respectively) ⁴⁰. The process is not fully elucidated, however, it is known that the effector caspase-mediated cleavage of a number of target proteins (Table 1.1) involved in the maintenance of cellular structure ⁵⁰ is partly responsible.

Since caspases are so essential in the execution of apoptosis, the study of their activation for the determination of the presence of apoptotic cells could therefore be advantageous for the establishment of the efficacy of potential cancer therapies.

Table 1.1 – Apoptotic morphology associated proteins cleaved by caspases

Protein	Function/Localisation
Actin	Microfilament forming protein with various localisations and functions, i.e. regulation of cell shape in the cortical cytoskeleton ^{51; 52; 53}
Spectrin / fodrin	Actin cross-linking protein in cortical cytoskeleton ^{54; 55; 56}
Beta-catenin	Intracellular attachment protein in cell-to-cell junction sites ⁵⁷
Gelsolin	Microfilament protein ⁵⁸
Gas2	Microfilament organising protein ⁵⁹
PAK2	Protein kinase involved in cytoskeleton regulation ⁶⁰
MEKK-1	Regulation of cell survival at the cell-matrix and cell-cell contacts ⁶¹
FAK	Regulation of cell adhesion at cell-matrix and cell-cell contacts ⁶²
Keratins 18 & 19	Intermediate filament protein in keratinocytes ⁶³
Rabaptin 5	Membrane protein that regulates intracellular vesicle traffic ⁶⁴
Lamin A and B	Intermediate filament that forms the nuclear lamina ^{65; 66}
NuMa	Mediator of nuclear chromatin-matrix protein interactions ⁶⁷

1.3. The cell cycle

The cell cycle is a term referring to the highly regulated process by which a cell grows ⁶⁸, undergoes duplication ⁶⁹ and accurately replicates, transferring its genetic information to daughter cells ⁶⁹. This progression is divided into 5 major phases (Figure 1.6), namely gap 0 (G_0), gap 1 (G_1), gap 2 (G_2), mitosis (M phase) and DNA synthesis (S phase), each tightly regulated through the action of a number of proteins ⁶⁹.

A cell is referred to as quiescent or senescent in G_0 when it is either temporarily or permanently excluded from the cell cycle, respectively. Cells temporarily in G_0 enter G_1 in response to extracellular signals such as growth factors and mitogens, and after entering this stage, proceed to S phase where DNA replication occurs in preparation for mitosis ⁶⁹. G_2 phase involves the rapid growth of the cell and protein synthesis in preparation for M phase, the final step in the cell cycle. M phase is an intricate and multi-step process involving the generation of mitotic spindles, sister chromatid segregation and cell division ⁶⁹, which ultimately results in the formation of two identical daughter cells.

The progress of the cell through the cell cycle must be stringently controlled to ensure that the initiation of each of the aforementioned phases occurs only once the preceding phase has adequately reached its completion, consequently, a number of cell cycle checkpoints exist ⁶⁹. This refers to the monitoring of critical cell cycle events e.g. DNA replication, and in response to possible errors in these processes, the progression of the cell through the cell cycle is halted while the error is fixed, if this error is irreparable, the death of the cell is induced ⁷⁰.

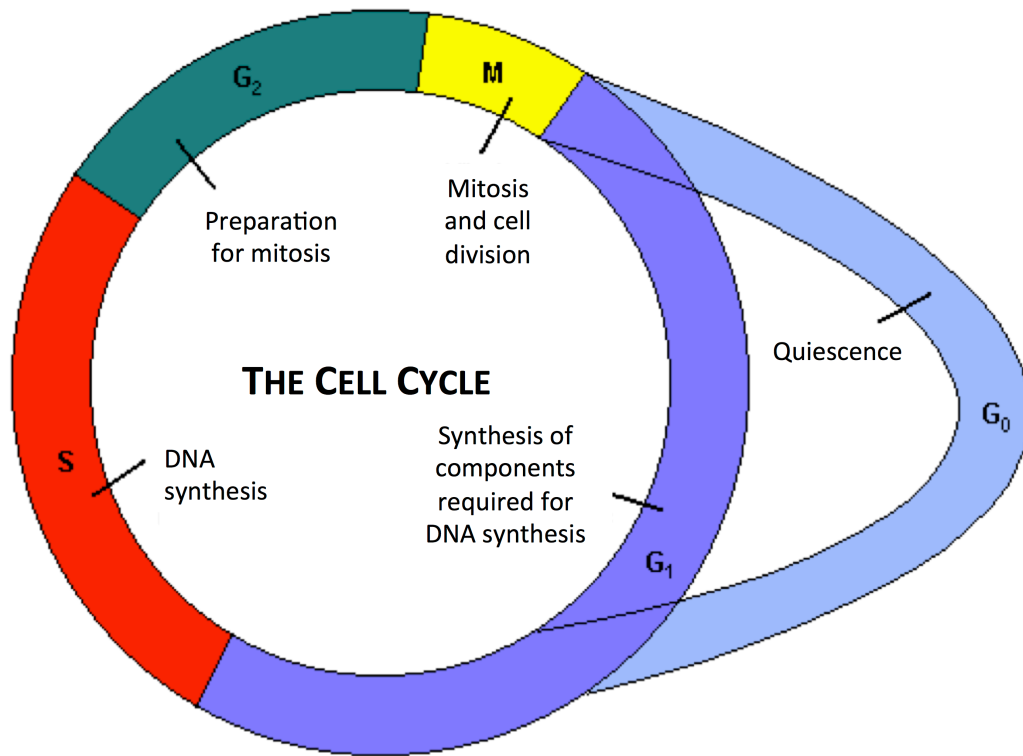


Figure 1.6 – The cell cycle. The cell cycle is divided into 5 phases; G₀ – where cells are temporarily or permanently excluded from the cell cycle, G₁ – cells enter this phase from G₀ in response to growth signals, S – the replication of DNA, G₂ – a phase of rapid growth and protein synthesis in preparation for M phase, M – the generation of mitotic spindles, sister chromatid segregation and cell division. (Image adopted from http://www.ch.ic.ac.uk/local/projects/s_liu/Html/Introduction.html).

In some cases, excessive expression of growth factors and mitogens, which propel the cell out of G₀ (as discussed above), leads to the unnecessary progression of the cell through the cell cycle, generating a mass of non-functional cells. Damaged or mutated cells allowed to bypass cell cycle checkpoints pose a serious problem, as these mutations often permit aberrant cellular proliferation and insensitivity to death signals⁷⁰. These enlightenments reveal the importance of the correct regulation of the cell cycle in disease progression, especially with respect to cancer.

1.4. The 37-kDa/67-kDa laminin receptor precursor/laminin receptor

Laminins belong to a large family of extracellular matrix proteins that are involved in a number of biologically significant processes, including cellular differentiation, migration, adhesion, growth and signalling⁷¹. The effects of these proteins are often mediated through their interaction with integrin and non-integrin laminin-binding proteins, which function as receptors in the linkage of laminin in the extracellular matrix to intracellular signalling cascades⁷¹.

A major laminin binding partner is the multifunctional protein, designated in this study, as the 37-kDa/67-kDa laminin receptor precursor/laminin receptor (LRP/LR). This receptor has been variously labelled as the 37/67-kDa high affinity laminin receptor protein⁷², the 37-kDa laminin-binding protein (37LBP)⁷³, the laminin receptor precursor (LRP)⁷², the 67-kDa laminin receptor (67LR)⁷⁴, laminin receptor (LAMR)⁷⁵, laminin-binding protein precursor p40 (LBP-p40)⁷² and the ribosomal protein SA (RPSA)⁷⁶.

1.4.1. Structure of LRP/LR

The gene encoding LRP/LR consists of seven exons, six of which correspond to the coding sequence⁷⁷, however, it contains no signal sequences directing the protein to its various cellular locations, e.g. the nucleus or the cell membrane^{78;79}. The crystal structure of this protein was solved at 2.15 Å resolution⁸⁰, and was found to consist of 295 amino acid residues, with a theoretical molecular mass of 32854 Da⁷⁷. In immunoblots of cellular extracts, however, LRP and LR appear at molecular weights

of 37 and 67 kDa, respectively, showing dissimilarity to the theoretical molecular weight ⁷⁷.

The 67 kDa laminin receptor may be formed from the 37 kDa laminin receptor precursor ^{81; 82}, with the exact mechanism of this conversion currently still elusive. However, some studies have suggested that the unglycosylated 37 kDa form becomes acylated at Ser2 through the action of fatty acids; and this acylation is a critical step in the conversion of the 37 kDa form to the 67 kDa form ^{83; 84}.

LRP/LR is divided into two main domains, namely the N-terminal (aa 1-209) and C-terminal (aa 210-295) domains ⁷⁷. It belongs to a family of type II membrane proteins, which span the plasma membrane once (between amino acids 86-101), with its N-terminus found on the cytoplasmic side of the cell membrane and its C-terminus exposed to the extracellular space ⁸⁵. It has also been found to possess binding sites for laminin (aa 161-180 and aa 205-229), however these sites are not exclusive to laminin binding; the 161-180 amino acid laminin binding sequence is also able to bind Heparin, while PrP binds to the 205-229 sequence in addition to laminin ^{86; 87} (Figure 1.7).

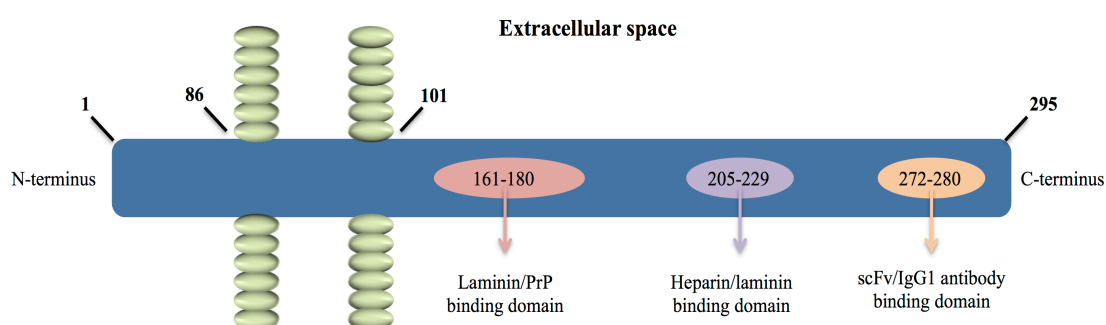


Figure 1.7 – Structure of LRP/LR. LRP/LR is a 295 aa transmembrane protein. It has a cytoplasmic N-terminus and an extracellular C-terminus. It contains binding sites for laminin, PrP and Heparin.

1.4.2. Cellular locations and physiological roles

LRP/LR has been discovered in numerous cellular locations; it is found localised in the cytoplasm^{8; 75; 88}, in the cell membrane on the cell surface⁸⁹, in the perinuclear compartment^{75; 90; 91} and in the nucleus^{90; 91}.

In the cytoplasm, LRP/LR (in the form LBP-p40) has been found to be involved in protein synthesis⁸⁸ and the maturation of the 40S ribosomal subunit⁸⁸. It also interacts with the 18S rRNA and the ribosomal protein S21, playing a role in the maturation and assembly of the ribosome^{76; 88; 92; 93; 94}. On the cell surface, it acts as a membrane receptor for ECM components such as laminin-1, carbohydrates and elastin⁹⁵ as well as certain growth factors e.g. Midkine^{96; 97; 98; 99}. This protein is reported to interact with prion proteins in the perinuclear compartment¹⁰⁰ and in the nucleus, it is associated with DNA and some histones⁹⁰, functioning as a constituent of the nuclear machinery^{94; 98; 101}.

In addition to the abovementioned physiological roles of LRP/LR, this receptor is also imperative in a variety of cellular processes required for normal functionality and homeostasis, namely cellular proliferation/growth⁸⁶, migration¹⁰², invasion¹⁰³, angiogenesis¹⁰⁴, extracellular remodelling¹⁰⁵ and differentiation¹⁰⁶, further demonstrating its importance.

1.4.3. Pathological implications of LRP/LR

While LRP/LR is imperatively functional in a number of physiological and homeostasis related processes, it has also been implicated in many diseases. LRP/LR serves as a cell surface receptor for numerous viruses (Dengue virus stereotypes 1, 2 and 3¹⁰⁷, Sindbis virus^{108; 109}, Venezuelan Equine Encephalitis Virus¹¹⁰ and Adeno-Associated Virus serotype 2, 3, 8 and 9¹¹¹), infectious prion proteins¹¹², as well as the respiratory tract pathogens *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae*, the causative agents of meningitis¹¹³. LRP/LR has also been found to have some involvement in zoonotic diseases⁸⁵ and those involving neurodegeneration, such as Alzheimer's disease⁸⁵. Additionally, this receptor is suggested to regulate microgliosis¹¹⁴ and function in the binding of inflammatory

macrophages to the basement membrane ¹¹⁵, both of which can contribute to pathological conditions. Furthermore, a number of studies have implicated this protein in cancer progression.

1.4.3.1. LRP/LR and its role in cancer and metastasis

The pathological implications of LRP/LR are further observed in cancer, where the overexpression of this protein is detected in several cancerous tissues (Table 1.2). An increased level of this protein on the cell surface has been shown to correlate with the aggressiveness of the cancer ⁷⁴ as well as malignancy ^{116; 117}.

Table 1.2 – Cancer types overexpressing LRP/LR

Human tumours overexpressing LRR/LR	Reference
Colon cancer	118
Cervical preneoplastic & neoplastic squamous epithelial lesions	119
Breast carcinoma	120; 121
Uterine adenocarcinoma	122
Colorectal carcinoma	123
Gastric adenocarcinoma	124
Prostate cancer	125
Laryngeal squamous carcinoma	126
Small cell lung cancer	127
Acute myeloid leukaemia	128
Ovarian carcinoma	116

A possible role of LRP/LR in cancer was first reported by Rao *et al.* in 1983 where the 67 kDa laminin receptor was purified from BL6 murine melanoma cells⁹⁶. It was suggested in this study that the laminin receptor may play a role in the interaction of metastasising tumour cells with the basement membrane⁹⁶. An imperative step in cancer metastasis is the erosion of the basal lamina by primary tumour cells, allowing them to enter and travel through the bloodstream, and form secondary tumours elsewhere in the body (Figure 1.8). LRP/LR plays an important role in this process, owing to its interaction with the cell adhesion molecule laminin-1⁸⁵. This interaction induces laminin-1 mediated basement membrane attachment in conjunction with the local degradation of the basal lamina, permitting cell movement⁸⁵. It has been demonstrated that blocking cell surface LRP/LR with anti-LRP/LR specific antibodies significantly decreases the invasive potential of cancerous cells^{85; 129; 130; 131; 132}, providing further evidence for the role of this receptor in cancer metastasis.

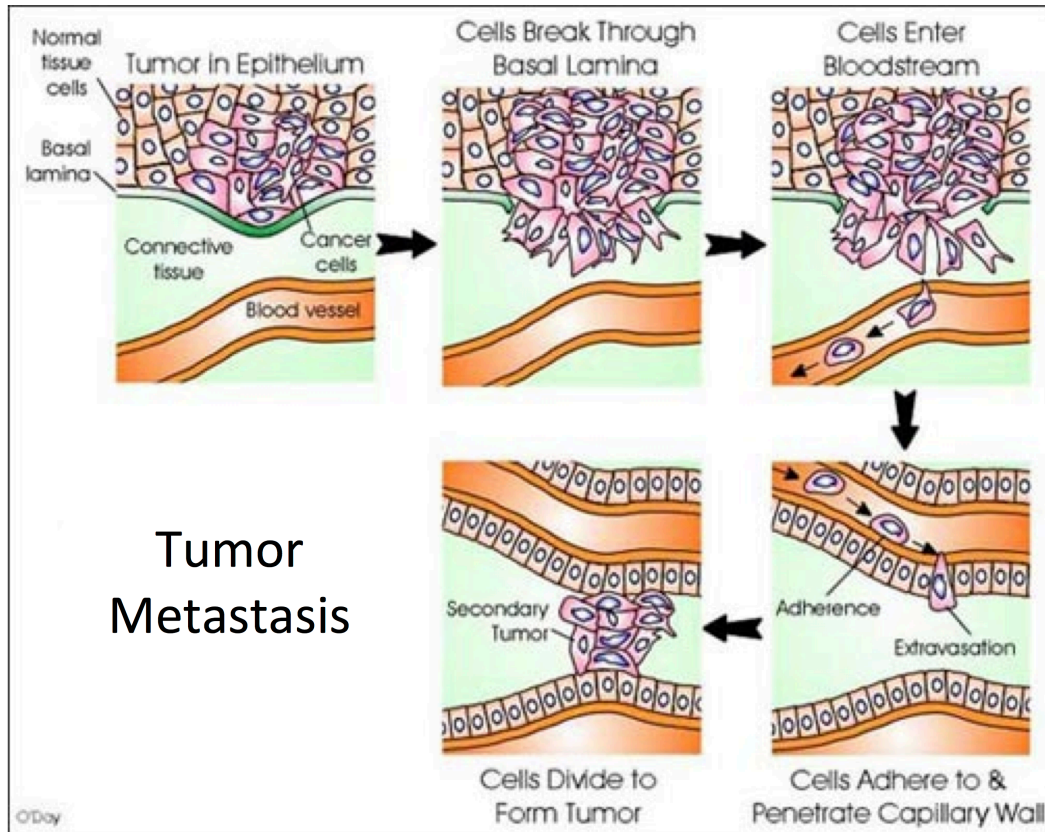


Figure 1.8 – The process of tumour metastasis. Tumour metastasis involves the erosion of cancerous cells through the basal lamina and their subsequent entrance into the blood stream. The cells travel through the blood stream, adhere to and penetrate the wall of the capillary and finally establish secondary tumours (adopted from <http://www.utm.utoronto.ca/~w3bio315/picts/lectures/lecture20/>).

1.4.3.2. LRP/LR and its role in cancer angiogenesis

The angiogenic process (the formation of blood vessels) is imperative for the growth of cancerous tumours, and LRP/LR is further implicated in this process through its interaction with the extracellular molecule laminin-1¹³³. An imperative step in the angiogenic process is the formation of tubular structures; this requires the action of certain cell surface receptors, which recognise extracellular matrix proteins and mediate cell adhesion¹³³, since LRP/LR is involved in mediating these processes, its role in angiogenesis is likely. It was observed that the interaction between the 67 kDa LR and the β -chain of laminin is implicated in the formation of tubes in the angiogenic process¹³⁴ as well as being functional in later aspects of this process¹³³. Additionally, a study undertaken in our lab discovered that blocking cell surface LRP/LR with the anti-LRP specific antibody, W3, completely destroyed angiogenic tube formation in HUVECs (Human umbilical vein endothelial cells)¹³⁵. These findings implicate LRP/LR in the angiogenic process.

1.4.3.3. LRP/LR and cellular viability

The human laminin receptor gene sequence has homologues in a number of organisms⁷⁵ and the protein sequence shares structural homology with Archaea⁸⁰. This conservation of LRP/LR gene and protein homology indicates that it is an essential molecule, involved in basic cellular functionality⁷⁵. Yeast homologues of LRP/LR have been found to be essential for cellular viability¹³⁶, where it functions in the processing of the 20S-18S rRNA as well as ribosome assembly¹³⁷. Additionally, LRP/LR has been found to be associated with the ribosome in mouse⁸⁸ and plant¹³⁸ models, indicating a role of the receptor in cellular viability in these organisms. In humans, the laminin receptor's importance in cellular viability is found in its localisation to the nucleus where it has been implicated in pathways involving cell signalling¹¹⁷. In addition to nuclear localisation, certain extraribosomal functions associated with the C-terminus of the human laminin receptor is also suggested to alternatively regulate cellular viability⁷⁵.

Since LRP/LR holds much importance in the maintenance of cellular viability, the dysregulation of this protein may cause aberrant cell survival, and therefore, targeting this protein could be beneficial for disease treatment.

1.4.3.4. LRP/LR and the evasion of apoptosis

LRP/LR has been implicated in the blockage of the apoptotic process ⁷². siRNA-mediated downregulation of LRP/LR stimulated the induction of apoptosis in Hep3B (transformed liver) cells, detected by Annexin V/propidium iodide staining as well as by double staining with Annexin V and an antibody directed against the 37/67 kDa laminin receptor ⁷². Furthermore, the reduction of LRP/LR expression in HeLa cells also resulted in apoptosis ¹³⁹. These results suggest that the 37/67 kDa LRP/LR plays a fundamental role in preventing the apoptotic form of cell death in these cells, and therefore could be a potential target for the treatment of cancers displaying increased expressions of this receptor.

1.4.3.5. LRP/LR and cellular proliferation

Recent evidence has implicated this protein in the progress of the cell through the cell cycle, where the reduction of LRP/LR expression in HT1080, HeLa and HepG2 cells resulted in G₁-phase cell cycle arrest through the alteration of the expression of cell cycle regulatory proteins ¹⁴⁰.

Taking into account that the progression of the cell through the cell cycle allows for cellular proliferation (an important hallmark of cancer), and that LRP/LR can be implicated in the dysregulation of this process, targeting this receptor could be advantageous for the treatment of this disease.

1.5. RNA silencing

RNA silencing refers to a regulatory mechanism which functions in either suppressing transcription (known as transcriptional gene silencing [TGS]), or activating sequence-specific RNA degradative processes (known as posttranscriptional gene silencing

[PTGS] or RNA interference [RNAi])¹⁴¹. This mechanism was initially reported in plants, however, following this discovery, PTGS/RNAi has been reported in almost all eukaryotic organisms including protozoa¹⁴², flies¹⁴³, nematodes¹⁴⁴, insects¹⁴⁵, parasites¹⁴⁶ as well as mouse¹⁴⁷ and human¹⁴⁸ cell lines. The main components in PTGS/RNAi are the double-stranded RNA (dsRNA) molecules which act as inducers and activators of these processes through their cleavage of inducer molecules, which cause the destruction of cellular or viral mRNA molecules¹⁴¹.

1.5.1. Components of gene silencing

Dicer and the RNA-Induced Signalling Complex (RISC) are enzyme complexes that play integral roles in PTGS/RNAi. Dicer is involved in PTGS/RNAi initiation, while RISC functions in targeting and degrading the mRNA¹⁴⁹.

1.5.1.1. Dicer

Dicer is a member of the RNase III family of proteins which are nucleases that demonstrate specificity for dsRNA¹⁵⁰. It contains four distinct domains: an amino terminal helicase domain, dual RNase III motifs, a dsRNA binding domain and a PAZ domain^{151; 152}. Dicer possesses the ability to digest dsRNA, forming uniform small sized RNA molecules called small interfering RNA (siRNA) of approximately 22 bp¹⁴¹. Complete digestion of the dsRNA using RNase III nucleases results in fragments ranging from 12-15 bp¹⁵³. This is due to the RNase III enzyme acting as a dimer, therefore digesting the dsRNA with the aid of two catalytic centres¹⁴¹. A model for the digestion of dsRNA to siRNA by Dicer suggests that this enzyme folds on the dsRNA substrate producing four sites for catalysis¹⁵⁴. This results in the two terminal sites, which have maximum homology with the RNase III catalytic sequence, remaining active while the other two sites which contain partial homology lose their functional significance¹⁵⁴. The dsRNA processed by Dicer is therefore double in size when compared to the fragments produced by the other RNase III enzymes¹⁵⁴.

1.5.1.2. RNA-Induced Silencing Complex

A study by Hammond *et al.*¹⁴³ demonstrated that targeting exogenous genes of *Drosophila* S2 cells in a sequence specific manner could be accomplished by the transfection of these cells with dsRNA. In this study, the inability of nuclease treated cellular extracts to degrade the associated mRNA, and the lack of this outcome with DNase I treatment established that RNA was an indispensable constituent of the nuclease activity¹⁴³. The sequence-specific nuclease activity observed in these cellular extracts responsible for the degradation of target mRNA was termed the RNA-induced silencing complex (RISC)¹⁴³. The RISC enzymatic complex consists of Argonaute (Ago), fragile X-related (FXR) and Tudor-Staphylococcus nuclease (Tudor SN) domain proteins¹⁴⁹.

1.5.2. Mechanism of RNA interference

Many aspects of this pathway remain elusive, however, various *in vivo* and *in vitro* experimental results have been combined to propose a mechanism for the PTGS/RNAi pathway¹⁴¹. This pathway has been simplified into three basic steps:

Step 1 is referred to as the initiation step and involves the binding of the RNA III nuclease Dicer to dsRNA, cleaving it into 21-25 nucleotide RNA fragments called siRNA¹⁴¹ (Figure 1.9a).

Step 2 is known as the effector step. The siRNAs are joined to a multinuclease complex known as RISC¹⁴¹ (Figure 1.9b). The organization of siRNA (double-stranded duplexes with two-nucleotide 3' overhangs and 5'-phosphate termini) is essential for the incorporation of these molecules into the RISC complexes^{155; 156} by the dsRNA-binding protein R2D2¹⁵⁷. RISC zymogens become enzymatically active upon the addition of ATP and the unwinding of the double stranded siRNA, resulting in only the most stable strand of siRNA remaining attached to RISC¹⁵⁴.

Step 3 involves the silencing of the gene¹⁴¹ (Figure 1.9c). The activated RISC is then able to cleave the target mRNA¹⁵⁸, identified by Watson-Crick base-pairing, with the single siRNA strand bound to the complex¹⁴³. The degradation of the target mRNA has been suggested to be endonucleolytic¹⁵⁹ and occurs at the central point of the region complementary to the siRNA, ten nucleotides upstream of the nucleotide paired with the 5' end of the guide siRNA¹⁶⁰. The cleavage process is not ATP-

dependent ¹⁶¹, however, several repetitions of mRNA cleavage requiring the release of cleaved products are more efficient in the presence of ATP ¹⁴³.

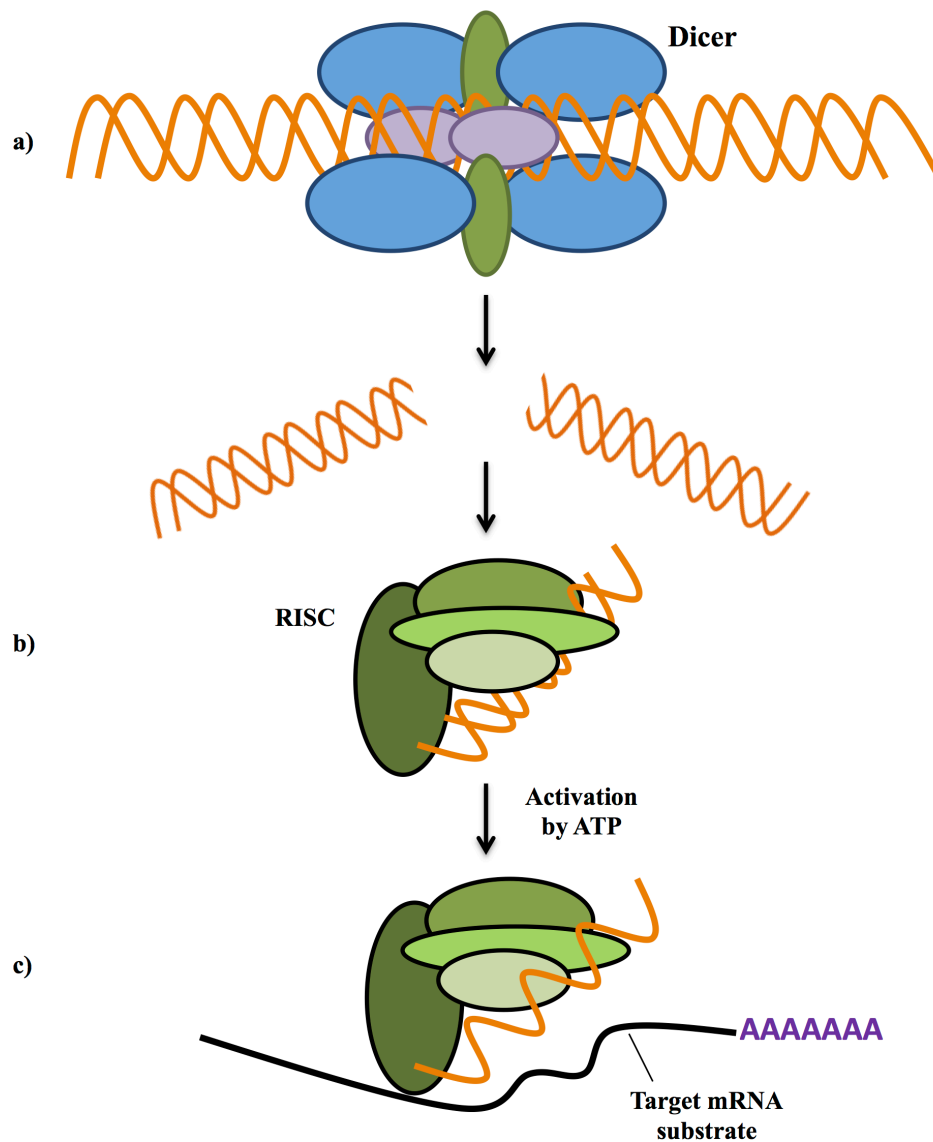


Figure 1.9 – The mechanism of RNA interference. a) The pathway is initiated by Dicer processing dsRNA into ~22 nucleotide small interfering RNAs (siRNA). b) The siRNAs are integrated into a multicomponent nuclease called RISC, activating it. c) RISC then uses the siRNA as a guide to the target mRNA for degradation ¹⁵⁴.

1.5.3. Regulation of RNA interference

RNA interference controls downstream gene expression but is itself also regulated. dsRNA acts as the substrate from which siRNA is produced by the catalytic action of Dicer¹⁶², however, the dsRNA also competes with an RNA editing process, catalysed by dsRNA-specific adenosine deaminases¹⁶³, which catalyse the conversion of adenosine to inosine¹⁶². This RNA editing reaction causes both the elimination of complementarities between the dsRNA and the target RNA, and the destabilisation of edited RNA, making them poor substrates for Dicer¹⁶⁴.

1.5.4. Applications of RNA interference

RNAi is of much interest as a research tool in determining gene function, and therefore can also be implemented as a therapeutic agent. Much research has been focussed on RNA interference as a therapeutic approach for the treatment of various diseases¹⁶⁵. It is possible that many if not all human diseases, including cancer, autoimmune diseases, dominant genetic disorders and viral infections, caused by the activity of one or more genes can be corrected by RNAi based therapeutics¹⁶² and RNA interference is therefore a very attractive and motivating field of research. While the introduction of synthetic siRNA into a system is reasonably simple and usually results in effective gene silencing, the results are ephemeral, however, DNA-based methods have the potential of being permanently established¹⁶². Furthermore, it must be stressed that modes of targeting the siRNA solely to the intended diseased/affected cells is essential for effective therapeutic functionality.

The treatment of age-related macular degeneration (AMD) was the first clinical application of RNAi^{166; 167}. RNAi based therapies are also currently being developed for the treatment of viral infections such as human immunodeficiency virus (HIV) hepatitis B and C viruses (HBV and HCV) and respiratory syncytial virus (RSV)¹⁶⁸. RNAi approaches for the treatment of neurodegenerative diseases and cancers have been performed¹⁶⁵.

1.6. Rationale, research question, aims and objectives

1.6.1. Rationale

LRP/LR has been shown to play a significant role in cancer metastasis ⁹⁶, angiogenesis ^{134; 135} and cellular proliferation ⁷⁵, all major hallmarks of cancer ¹³. Additionally, the downregulation of LRP/LR expression has been found to reduce the viability of cells ⁷⁵, a desirable phenomenon with respect to cancer. The role of this receptor in cancer progression cannot be denied, and therefore the mechanism resulting in the reduction of cellular viability in cancerous cells due to decreased expression of LRP/LR was a topic of great interest.

1.6.2. Research question

Will the shRNA/siRNA-mediated downregulation of LRP/LR reduce the viability of A549 (human lung carcinoma) and HeLa (human cervix carcinoma) cells, and if so, what are the possible mechanisms for this decrease.

1.6.3. Aim

The aim of this study was to determine the effects of the shRNA/siRNA-mediated downregulation of LRP/LR on the viability of A549 (human lung carcinoma) and HeLa (human cervix carcinoma) cells, and to define possible mechanisms for the observed changes.

1.6.4. Objectives

- Determination of cell surface levels of LRP/LR and total levels of LRP on the aforementioned cell lines using flow cytometry and western blotting, respectively.
- Successful downregulation of LRP expression in A549 and HeLa cells by transfection of cells with shRNA/siRNA (quantification by densitometric analysis of western blot signals).

- Determination of the effect of the shRNA/siRNA-mediated downregulation of LRP on cellular viability using MTT assays.
- Identification of the possible induction of apoptosis as a result of the shRNA/siRNA-mediated downregulation of LRP in A549 and HeLa cells.
 - Immunofluorescence microscopy – visualisation of nuclear morphological changes associated with the cell undergoing programmed cell death.
 - Caspase-3 activation assay – to determine if the activity of the apoptosis-associated protein, caspase-3, becomes upregulated in response to decreased LRP expression.
- Identification of the possible decrease in cellular proliferation as a result of the shRNA/siRNA-mediated downregulation of LRP using a BrdU assay.

2. CHAPTER 2 – MATERIALS AND METHODS

2.1. Cell lines and cell culture maintenance

2.1.1. Cell lines

The following cell lines were utilised within this study:

- Human lung carcinoma cells (A549) – An adherent human alveolar basal epithelial cell line, derived from the explanted tumour of a 58-year old Caucasian male.
- Human cervix carcinoma cells (HeLa) – An adherent human epithelial cell line from cervical carcinoma, derived from Mrs Henrietta Lacks in 1951.

2.1.2. Cell culture maintenance

The above mentioned cell lines were obtained from the American Type Culture Collection (ATCC) and were maintained and grown in cell culture plates (Corning) at 37 °C with 95% air and 5% CO₂ in DMEM (Celtic Molecular Diagnostics) media supplemented with 10% (v/v) heat inactivated FCS (BIOCOM biotech) and 1% (v/v) antibiotic penicillin and streptomycin (Gibco®).

2.2. Plasmids and plasmid amplification

2.2.1. Plasmids

The following plasmids were utilised within this study:

pCI-neo-GFP (appendix 1.1 – Figure A1)

pti-H1 (appendix 1.2 – Figure A2)

pTZ57R/T shRNA-1, 4 and 7 (appendix 1.3 – Figure A3)

pTZ57R/T shRNA-1.1 (appendix 1.3 – Figure A3)

2.2.2. Plasmid amplification

Plasmid amplification allows for the rapid production of large amounts of plasmid DNA to be utilised for experimentation. The abovementioned plasmids (section 2.2.1) were amplified in the following way.

2.2.2.1. Production of chemically competent *E. coli* XL1-Blue

The production of chemically competent *E. coli* XL1-Blue ensures that these bacteria are susceptible to the uptake up plasmid DNA. Chemically competent *E. coli* XL1-Blue were produced as follows:

A single *E. coli* XL1-Blue colony was picked from a freshly streaked LB plate (appendix 1.4.4), inoculated into 2 mL of LB liquid medium (appendix 1.5) and incubated at 37 °C overnight with shaking. 1 mL of this culture was then inoculated into 100 mL of liquid LB medium and incubated at 37 °C with shaking until the OD₆₀₀ had reached approximately 0.25 - 0.3. The culture was chilled on ice for 15 min and subsequently centrifuged (4 000 x rpm, 4 °C, 10 min). The supernatant was then discarded and the pellet resuspended in 30 mL ice cold 0.1 M CaCl₂ (appendix 1.6). This solution was incubated on ice for 30 min, centrifuged as above, the supernatant discarded and the pellet resuspended in 6 mL of ice cold 0.1 M CaCl₂ containing 15% glycerol (appendix 1.7). 200 µL aliquots of this solution were then stored at -80 °C.

2.2.2.2. Transformation of plasmids into *E. coli* XL1-Blue

Transformation refers to a procedure resulting in uptake of plasmid DNA by chemically competent bacteria. The transformation of plasmid DNA into *E. coli* XL1-Blue was completed as follows:

100 ng of plasmid DNA (either pCI-neo-GFP, pti-H1 or pTZ57R/T shRNA-1.1) was added to 50 µL of chemically competent *E. coli* XL1-Blue that had been thawed on ice. This solution was incubated on ice for 30 min, heat shocked at 42 °C for 45 s and

subsequently incubated on ice for 2 min. The transformed bacteria were then spread on LB plates containing 100 µg/mL ampicillin (appendix 1.4.1) and incubated at 37 °C overnight. The plates were stored at 4 °C.

2.2.2.3. Plasmid isolation

Plasmid isolation is a procedure utilised to extract plasmid DNA from bacteria. The Plasmid DNA Purification technique was employed for the isolation of pCI-neo-GFP and pti-H1 plasmid DNA as well as the isolation of pTZ57R/T shRNA-1, 4 and 7 plasmids to be utilised for DNA sequencing. The Endotoxin-free plasmid DNA purification technique was used for the isolation of pTZ57R/T shRNA-1.1 plasmid DNA to be utilised for transfections.

2.2.2.3.1. Plasmid DNA Purification (mini-prep)

The pCI-neo-GFP, pti-H1 and pTZ57R/T shRNA-1, 4 and 7 plasmid DNA was isolated using the Plasmid DNA Purification kit (Macherey-Nagel) according to the following protocol:

A single *E. coli* XL1-Blue colony containing the plasmid of interest (either pCI-neo-GFP and pti-H1 or pTZ57R/T shRNA-1, 4 and 7) was picked from a freshly streaked LB plate (containing 100 µg/mL ampicillin) or LB plate (containing 50 µg/mL ampicillin, Xgal and IPTG), respectively, inoculated in 5 mL liquid LB medium containing 100 µg/mL ampicillin and incubated at 37 °C overnight with shaking. The solution was then centrifuged (11 000 x g, 30 s, RT), the supernatant discarded and the pellet resuspended completely in 250 µL of buffer A1. 250 µL of buffer A2 was added, the solution gently mixed by inversion and incubated at RT for no longer than 5 min until the lysate appeared clear. 300 µL of buffer A3 was added and the solution gently mixed by inversion, following which, it was centrifuged (11 000 x g, 5 min, RT). The supernatant was then decanted into the NucleoSpin® Plasmid/Plasmid (NoLid) Column in a collection tube, this was centrifuged (11 000 x g, 1 min, RT) and the flow through discarded. 600 µL of buffer A4 was then added to the NucleoSpin® Plasmid/Plasmid (NoLid) Column, centrifuged (11 000 x g, 1 min, RT)

and the flow through discarded. The NucleoSpin® Plasmid/Plasmid (NoLid) Column was then recentrifuged (11 000 x g, 2 min, RT) to dry the silica membrane and the collection tube was discarded. 50 µL of buffer AE was added to the NucleoSpin® Plasmid/Plasmid (NoLid) Column, which had been placed in a clean collection tube, incubated at RT for 1 min and subsequently centrifuged (11 000 x g, 1 min, RT). The NucleoSpin® Plasmid/Plasmid (NoLid) Column was discarded and the isolated plasmid DNA stored at -20 °C.

2.2.2.3.2. Endotoxin-free plasmid DNA purification (maxi-prep)

The pTZ57R/T shRNA-1.1 plasmid DNA was isolated using the Endotoxin-free plasmid DNA purification kit supplied by Macherey-Nagel according to the following protocol:

A single *E. coli* XL1-Blue colony containing the pTZ57R/T shRNA-1.1 plasmid was picked from a freshly streaked LB-Amp plate, inoculated in 5 mL liquid LB medium containing 100 µg/mL ampicillin and incubated for approximately 8 h at 37 °C with shaking. This culture was diluted (1:1 000) in 300 mL liquid LB medium and incubated at 37 °C with shaking for 12-16 h, until the OD₆₀₀ = 8. This solution was centrifuged (6 000 x g, 10 min, 4 °C), the supernatant discarded completely and the pellet resuspended in 12 mL of Buffer RES-EF. 12 mL of Buffer LYS-EF was added, mixed gently by inversion and the solution incubated for 5 min at RT. 12 mL of Neutralization Buffer NEU-EF was added to the suspension, the solution inverted 10-15 times and the lysate incubated on ice for 5 min. 35 mL of Equilibration Buffer EQU-EF was applied to a column filter inserted into a NucleoBond® Xtra Column and was allowed to empty by gravity flow. The cell lysate solution was inverted several times to ensure homogeneity, loaded into the column and allowed to clear by gravity flow. 10 mL of Buffer FIL-EF was then added, the column allowed to empty by gravity flow and the flow through discarded. 90 mL of Buffer ENDO-EF was added to the NucleoBond® Xtra Column and was allowed to empty by gravity flow, following which 45 mL of Buffer WASH-EF was added and also allowed to empty by gravity flow (the flow through was discarded between each step). 15 mL of prewarmed (to 50 °C) Elution Buffer ELU-EF was then added to the NucleoBond®

Xtra Column and the flow through collected in a clean 50 mL Falcon tube. 0.7 volumes of RT isopropanol was added to the eluent, the solution vortexed and allowed to incubate at RT for 2 min. The plunger from a 30 mL syringe was removed and a NucleoBond® Finalizer attached to the outlet. The eluent-isopropanol solution was then added to the syringe, the plunger inserted, the solution gently pushed through the NucleoBond® Finalizer and the flow through discarded. The NucleoBond® Finalizer was removed from the syringe, the plunger removed and the NucleoBond® Finalizer was reattached to the syringe. 5 mL of endotoxin-free 70% ethanol was filled into the syringe, the plunger inserted, the solution pressed gently through the NucleoBond® Finalizer and the flow through discarded. The NucleoBond® Finalizer filter membrane was then dried by strongly passing air through the syringe and collecting residual moisture on a tissue (the NucleoBond® Finalizer was removed from the syringe, the plunger removed and the NucleoBond® Finalizer was reattached to the syringe each time air was pressed through). Finally, 500 µL of Buffer TE-EF was added to a 1 mL syringe attached to the NucleoBond® Finalizer, the plasmid DNA eluted drop by drop by slowly inserting the plunger, and stored at -20 °C.

2.3. Determination of cell surface LRP/LR levels

The amount of LRP/LR on the surface of A549 and HeLa cells was determined using flow cytometry according to the following protocol:

A549 and HeLa cells were detached using trypsin/EDTA (Separations), centrifuged (4 000 x rpm, 5 min, RT) and fixed for 10 min in 4% paraformaldehyde (appendix 3.1) at 4 °C. After fixing, cells were pelleted by centrifugation (4 000 x rpm, 10 min, 4 °C), the supernatant discarded and the pellet resuspended in 1 mL Epics™ Sheath Fluid (Beckman Coulter). 500 µL of the cell suspension was then transferred into a second eppendorf tube (to be used as a control), the two cell suspensions centrifuged (4 000 x rpm, 10 min, 4 °C) and the supernatant discarded. The control pellet was resuspended in 100 µL Epics™ Sheath Fluid and the experimental pellet in 100 µL of a 30 µg/ml solution of the anti-LRP specific primary antibody IgG1-iS18 in Epics™ Sheath Fluid; these were incubated at RT for 1 h. The cells were subsequently washed

in 200 μ L Epics™ Sheath Fluid, centrifuged (5 000 x rpm, 1 min, RT) and the supernatant discarded (this step was repeated thrice). The control and experimental pellets were resuspended in 100 μ L of a 30 μ g/ml solution of the Goat Anti-Human IgG-FITC secondary antibody (Beckman Coulter) in Epics™ Sheath Fluid and incubated for 1 h at RT in the dark. Following incubation, cells were washed with 200 μ L Epics™ Sheath Fluid, centrifuged (5 000 x rpm, 1 min, RT) and the supernatant discarded (this step was repeated thrice). The pellets were then resuspended in 500 μ L Epics™ Sheath Fluid and 10 000 cells from each solution were analysed using a fluorescence activated cell scanner (Beckman Coulter).

2.4. Determination of total LRP levels

2.4.1. Cell lysis

Cell lysis is a process in which the cell membrane is disrupted chemically, mechanically or physically, resulting in the release of cellular contents such as proteins, DNA and RNA. A549 and HeLa cells were lysed according to the following protocol:

The medium from confluent A549 and HeLa cells was discarded, the cells washed with DPBS (Gibco®) and 500 μ L of lysis buffer (appendix 2.1) added. The cells were detached using cell scrapers (BD Biosciences), the cell suspension transferred to eppendorf tubes and incubated on ice for 15 min. Following incubation, the cell lysates were centrifuged (14 000 x rpm, 2 min, RT) and the supernatant stored at -20 °C.

2.4.2. BCA assay

The Bicinchoninic acid (BCA) assay is a biochemical assay utilised to determine the amount of protein in a particular sample. This assay is based on the reduction of Cu^{2+} to Cu^+ by proteins, resulting in the formation of a purple coloured product, which can be measured spectrophotometrically at 562 nm. Known amounts of bovine serum albumin (BSA) are used as standards to create a calibration curve from which the

amount of protein contained in the samples can be determined. The BCA assay (Sigma-Aldrich) was completed according to the following protocol:

Various amounts (0, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL) of BSA were prepared and 25 μ L of each standard in addition to the prepared cell lysates (diluted 1:5 in dH₂O) were loaded in triplicate in the wells of a 96 well plate. The two BCA reagents (copper (II) sulfate and bicinchoninic acid) were mixed in a 1:50 ratio and 200 μ L of this solution added to each well (containing the standards and cell lysates). The plate was then incubated at 37 °C for 30 min and the absorbance of each well measured using a microtiter plate reader at 562 nm. The amount of protein contained in the cell lysates was determined using the constructed calibration curve.

2.4.3. Preparation of protein samples for SDS-PAGE

Protein samples were diluted with dH₂O to 1mg/mL mixed in a 1:5 ratio with 5 X sample loading dye (appendix 2.2) and stored at 4 °C.

2.4.4. SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique used to separate proteins according to their molecular weight. SDS is an anionic detergent that results in the denaturation of secondary and tertiary protein structures. Additionally, SDS binds to unfolded proteins and confers a net negative charge along the length of the polypeptide. The proteins from the cell lysates were subjected to SDS-PAGE according to the following protocol:

Aliquots each containing 5 μ g of total cellular proteins in sample loading dye were heated at 95 °C for 5 min before being stacked and resolved (230 V, 230 mA) on a 5% stacking (appendix 2.3.7.2) and 12% separating (appendix 2.3.7.1) sodium dodecyl sulphate-polyacrylamide gel, respectively.

2.4.5. Transfer of proteins from the gel to PVDF membrane (western blotting)

The resolved sodium dodecyl sulphate-polyacrylamide gel was placed above a 0.45 μm PVDF membrane (Separations), which had been activated in ethanol, subsequently soaked in transfer buffer (appendix 2.3.8) and sandwiched between 6 filter papers wetted in transfer buffer. The proteins resolved on gels were electro-transferred (350 mA) for 45 min onto the PVDF membrane.

2.4.6. Immunodetection of LRP and β -actin expression levels

Immunodetection is a technique making use of antibodies to detect the presence of a specific antigen, and can be used for the analysis of protein expression. Immunodetection of LRP and β -actin expression levels was completed according to the following protocol:

Following transfer, the PVDF membrane was blocked with 3% BSA in PBS-Tween (appendix 2.6) for 1 h at RT with gentle shaking and subsequently incubated in a 1:10 000 dilution of either the LRP specific primary antibody IgG1-iS18 (Affimed Therapeutics) or the β -actin detection antibody Anti- β -Actin–Peroxidase (Sigma-Aldrich) in 3% BSA in PBS-Tween for 1h.

In the case of LRP detection, the membrane was then washed three times for 10 min each in PBS-Tween (appendix 2.5) with shaking and subsequently incubated in a 1:10 000 dilution of a peroxidase-conjugated secondary antibody anti-human IgG-POD (Santa Cruz Biotechnology) in 3% BSA in PBS-Tween for 1 h in the dark with shaking.

The membranes (both for LRP and β -actin detection) were then finally washed three times for 10 min each in PBS-Tween with shaking.

The expression of the protein of interest was detected using supersignal the west pico chemiluminescent substrates (Separations) mixed in a 1:1 ratio. The protein bands were visualised by exposure to X-ray film (Separations), which was subsequently developed and fixed using developer (Sigma-Aldrich) and fixer (Sigma-Aldrich) solutions, respectively.

2.4.7. Densitometric analysis

To quantitatively determine the relative LRP expression levels, densitometric analysis was completed. Subsequent to immunodetection, an image of the X-ray film was obtained using the GS-800 Calibrated Densitometer and densitometric analysis completed using the Quantity One – 4.5.2 software. The band intensities were calculated and background noise subtracted, producing a calibration curve. The area under the curve indicates IOD (Integrated Optical Density) and this parameter was used to compare the levels of LRP expression in A549 and HeLa cells.

2.5. Determination of A549 and HeLa transfectability

A549 and HeLa cells were transfected with a pCI-neo-GFP plasmid encoding the green fluorescent protein (GFP) using *TransIT®-LT1* Transfection Reagent (Invitrogen); the visualisation of green fluorescence using an immunofluorescence microscope would indicate that the aforementioned cells were transfectable.

2.5.1. Transfection of A549 and HeLa cells with pCI-neo-GFP

Transfection refers to the process by which foreign nucleic acids are introduced into mammalian cells. A549 and HeLa cells were transfected with pCI-neo-GFP according to the following protocol:

4×10^5 A549 and 3.5×10^5 HeLa cells were plated in 6 cm plates (Corning) containing sterile microscope coverslips. 24 h later, 7.5 μ L of prewarmed (to RT) and vortexed *TransIT®-LT1* reagent was diluted in 250 μ L DMEM in a sterile eppendorf tube, and this gently mixed. 2.5 μ g of the pCI-neo-GFP plasmid was then added to the

diluted *TransIT*®-LT1 reagent, gently mixed and this solution allowed to incubate at RT for 15 min. Following this incubation, the *TransIT*®-LT1 reagent – DNA complex was added drop wise to the previously plated A549 and HeLa cells, which were then further incubated for 24 h in a humidified incubator at 37 °C with 95% air and 5% CO₂.

2.5.2. Immunofluorescence microscopy

The microscope coverslips were removed from the cell culture plates, fixed in 2% paraformaldehyde (appendix 4.1) for 15 min, rinsed in several changes of PBS and blocked in 0.25% Triton X-100 containing 0.5% BSA in PBS (appendix 4.2) for 10 min. The coverslips were then incubated for 5 min in a 1:100 dilution of a 2 mg/mL stock solution of the nuclear stain Hoechst 33342 (Sigma-Aldrich) and subsequently rinsed in several PBS changes. 50 µL of Gel Mount™ (Sigma-Aldrich) was placed on a clean microscope slide, the coverslips mounted cell side down and allowed to dry for approximately 3 h. These slides were stored in the dark at 4 °C until viewed on an immunofluorescence microscope at 100 X magnification.

2.6. Production of shRNA

shRNA directed against human LRP mRNA target sequences were produced to potentially downregulate the expression of this protein in A549 and HeLa cells. Prof. Marco Weinberg kindly designed the shRNAs, and these were produced together with fellow M. Sc. student Miss Danielle Gonsalves.

2.6.1. Design of oligonucleotides

shRNAs-1, 4 and 7 were designed (shRNA-4 and -7 homologous to sequences previously used in murine studies¹⁶⁹, and the shRNA-1 sequence was identified using The RNAi Consortium - <http://www.broadinstitute.org/rnai/trc>) to be expressed from the H1 RNA Pol III promoter. Each shRNA “guide strand” sequence was designed to be 21 nucleotides in length. The shRNA expression constructs were designed with the guide strand on the 3’ arm, a poly T termination signal, and includes a full H1

promoter sequence to constitute a complete expression cassette (appendix 5.1). To prepare the shRNA cassettes, the H1 RNA Pol III promoter was used as a template in a nested PCR, whereby the sequences corresponding to the shRNAs were incorporated into two reverse primers (one for the primary PCR and one for the secondary PCR). The same forward primer, which is complementary to the start of the H1 promoter, was used in both the primary and secondary PCRs. The PCR products therefore contained the full H1 promoter sequence that precedes the sequences coding for the shRNAs. The PCR products coding for the shRNA expression constructs contained a single terminal 3' A-overhang resulting from *Taq* polymerase activity and a final extension step and were therefore suitable for TA cloning.

2.6.2. Nested PCR amplification of oligonucleotides

The oligonucleotides were amplified in two rounds (nested PCR reaction) according to the following protocol.

2.6.2.1. Primary PCR

- Template DNA – pTZ57R/T shRNA-1, 4 and 7
pti-H1 plasmid
- Forward Primer – pTZ57R/T shRNA-1, 4 and 7
5' GATCTCTAGAGCGAACGCTGACGTCATCAA
- Reverse Primer – pTZ57R/T shRNA-1
5' CCATTTGGGTCAGGAATGGCAACAATTGCACGAGCGGGTCCGAGTG
GTCTCATAC
- Reverse Primer – pTZ57R/T shRNA-4
5' CTTCTGGGTCAGGAGAAGGCTGCCTGGATCTGGCGGGTCCGAGTG
GTCTCATAC

- Reverse Primer – pTZ57R/T shRNA-7
5' GAATTTGGGTCAGGAATTCCTCCTTGGTCACTGCCGGGTCCGAGTG
GTCTCATAC

50 pg pti-H1 plasmid, 0.05 μ mol forward primer, 0.05 μ mol respective reverse primer, 200 μ M of each dNTP (A, T, G and C) (Fermentas), 2.5 mM $MgCl_2$ (Fermentas), 1.25 units of *Taq* polymerase (Fermentas), 10 μ L of 1 X *Taq* polymerase buffer (Fermentas) and nuclease free water (Fermentas) to make the reaction up to 50 μ L was combined on ice in a sterile nuclease-free 200 μ L PCR tube, and subsequently placed in a thermocycler. The solutions were initially denatured for 3 min at 95 °C, 30 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 60 °C and extension for 30 s at 72 °C was completed before a final extension step for 15 min at 75 °C. The reaction 1 PCR product was visualised by electrophoresis on a 1% agarose gel (appendix 5.5) and stored at -20 °C.

2.6.2.2. Secondary PCR

- Template DNA – pTZ57R/T shRNA-1, 4 and 7
Respective PCR product from reaction 1
- Forward Primer – pTZ57R/T shRNA-1, 4 and 7
5' GATCTCTAGAGCGAACGCTGACGTCATCAA
- Reverse Primer – pTZ57R/T shRNA-1
5' AAAAAAGCTCGTGCAATTGTTGCCATTTGGGTCAGGAATG
- Reverse Primer – pTZ57R/T shRNA-4
5' AAAAAAGCCAGATCCAGGCAGCCTTCCTGGGTCAGGAGAA
- Reverse Primer – pTZ57R/T shRNA-7
5' AAAAAAGGCAGTGACCAAGGAGGAATTTGGGTCAGGAATT

Reaction 2 constituents and cycling parameters as described above (section 2.6.2.1). The reaction 2 PCR product was visualised by electrophoresis on a 1% agarose gel and stored at -20 °C.

2.6.3. PCR clean-up

A single band of DNA needed to be isolated from the gel since multiple bands were visualised when the PCR products were electrophoretically resolved. For this, the Wizard® SV Gel and PCR Clean-Up System (Promega) was utilised according to the following protocol:

Following electrophoresis, the band of interest was excised from the gel, placed in a 1.5 mL microcentrifuge tube, and dissolved in 10 µL Membrane Binding Solution per 10 mg gel by vortexing and incubation at 65 °C. The dissolved gel was then transferred into a SV Minicolumn inserted in a Collection Tube, incubated at RT for 1 min, centrifuged (16 000 x g, 1 min, RT) and the flow through discarded. 700 µL of Membrane Wash Solution was then added, centrifuged as before and the flow through discarded. 500 µL of Membrane Wash Solution was added, centrifuged as before and the flow through discarded. The assembly was then recentrifuged (as before) with the microcentrifuge lid open to allow for the evaporation of residual ethanol. 50 µL of Nuclease-Free Water was added to the SV Minicolumn, which had been transferred to a clean 1.5 mL microcentrifuge tube, incubated at RT for 1 min, centrifuged as above and the SV Minicolumn discarded. The cleaned-up PCR products were visualised by electrophoresis on a 1% agarose gel (appendix 5.2 – Figure A5) and stored at -20 °C.

2.6.4. Ligation reaction

A ligation reaction refers to the insertion of a DNA sequence of interest into a vector (plasmid), yielding circular double stranded DNA. The cleaned up PCR product was ligated into a TA cloning vector using the InsTAclone™ PCR Cloning Kit (Fermentas) according to the following protocol:

0.17 pmol of the pTZ57R/T vector, 6 µL of 5 X Ligation Buffer, 0.52 pmol of PCR product, 5 Weiss units of T4 DNA Ligase and nuclease-free water to make the reaction volume up to 30 µL was combined in a nuclease-free 200 µL microcentrifuge tube on ice and vortexed briefly. This ligation mixture was incubated at RT for 1 h and stored at -20 °C.

2.6.5. Transformation reaction

The transformation of the plasmids (pTZ57R/T shRNA-1, 4 and 7) obtained from the ligation reaction (section 2.6.4) into *E. coli* XL1-Blue was completed using the InsTAclone™ PCR Cloning Kit (Fermentas) according to the following protocol:

The day before transformation, a single colony of *E. coli* XL1-Blue from a freshly streaked LB plate was inoculated into 2 mL of C-medium and incubated at 37 °C overnight with shaking. 1.5 mL of pre-warmed (to 37 °C) C-medium was added to 150 µL of the overnight bacterial culture and this was incubated at 37 °C for 20 min with shaking. The bacterial cells were pelleted by centrifugation (16 000 x g, 1 min, RT) and the supernatant discarded. The pellet was resuspended in 300 µL of T-solution (T-solutions A and B mixed in a 1:1 ratio), incubated on ice for 5 min, centrifuged as before and the supernatant discarded. The pellet was resuspended in 120 µL of T-solution and incubated on ice for 5 min. 50 µL of this solution was then transferred to clean microcentrifuge tubes containing 2.5 µL of the respective ligation mixture, which had been chilled on ice for 2 min, the solution mixed and subsequently incubated on ice for 5 min. The cells containing the DNA were then immediately plated onto pre-warmed (to 37 °C) LB plates containing 50 µg/mL ampicillin, Xgal and IPTG to allow for blue/white selection (appendix 5.3 – Figure A6)

2.6.6. Plasmid isolation

Isolation of pTZ57R/T shRNA-1, 4 and 7 plasmids from 10 white colonies each from *E. coli* XL1-Blue which had been plated on LB plates containing 50 µg/mL

ampicillin, Xgal and IPTG was completed according to the protocol described in section 2.2.2.3.1.

2.6.7. Plasmid DNA sequencing

The 10 samples each of isolated pTZ57R/T shRNA-1, 4 and 7 plasmid DNA was sequenced by Inqaba Biotech and the resulting chromatograms viewed using Chromas Lite 2.01. The plasmid DNA insert sequences were then compared to their complementary target sequences. One isolation of the pTZ57R/T shRNA-1 plasmid (labelled as pTZ57R/T shRNA-1.1) was identical to its target sequence and was therefore utilised in the following experiments.

2.6.8. Retransformation of pTZ57R/T shRNA-1.1

The pTZ57R/T shRNA-1.1 plasmid was then retransformed into *E. coli* XL1-Blue according to the protocol outlined in section 2.2.2.2.

2.6.9. Isolation of pTZ57R/T shRNA-1.1

pTZ57R/T shRNA-1.1 plasmid DNA was isolated from a single colony of *E. coli* XL1-Blue according to the protocol outlined in section 2.2.2.3.2.

2.7. Transfection of cells with pTZ57R/T shRNA-1.1 and determination of LRP downregulation

2.7.1. Transfection

A549 and HeLa cells were transfected with pTZ57R/T shRNA-1.1 according to the following protocol:

24 h before transfection, 4×10^5 A549 and 3.5×10^5 HeLa cells were plated in the wells of a 6 well plate. 7.5 μ L of prewarmed (to RT) and vortexed *TransIT*®-LT1 reagent was added to 250 μ L of DMEM in a sterile eppendorf tube, and this solution

gently mixed. 2.5 µg of the pTZ57R/T shRNA-1.1 plasmid was then added to the diluted *TransIT*®-LT1 reagent, gently mixed and allowed to incubate at RT for 15 min. Following this incubation, the *TransIT*®-LT1 reagent-DNA complex was added drop wise to the previously plated A549 and HeLa cells, which were then allowed to incubate for 72 h in a humidified incubator at 37 °C with 95% air and 5% CO₂.

2.7.2. Determination of LRP downregulation

72 h post-transfection, A549 and HeLa cells were lysed, their protein content quantified, electrophoresis samples prepared, proteins stacked and resolved on a 5% stacking and 12% separating sodium dodecyl sulfate polyacrylamide gel, respectively, resolved proteins transferred from the gel to a 0.45 µm PVDF membrane, pTZ57R/T shRNA-1.1 transfected and non-transfected LRP expression levels determined by immunodetection and pTZ57R/T shRNA-1.1 transfected LRP expression levels compared to non-transfected controls using densitometry as described above in sections 2.4.1, 2.4.2, 2.4.3, 2.4.4, 2.4.5, 2.4.6, and 2.4.7, respectively.

2.8. Purchase of siRNA directed against LRP mRNA, transfections and determination of LRP expression levels

Since transfecting A549 and HeLa cells with the pTZ57R/T shRNA-1.1 plasmid did not result in a significant downregulation of LRP expression in A549 and HeLa cells, siRNA targeting the open reading frame of the human LRP gene (ON-TARGET^{plus}® siRNA – Human LAMR1) was purchased from Thermo Scientific Dharmacon® and utilised in all subsequent experiments. Additionally, non-targeting siRNA (ON-TARGET^{plus}® control siRNA – Non-Targeting siRNA 1) to be used as a positive control as well as the recommended transfection reagent (DharmaFECT® 1 transfection reagent) was purchased from this company.

Although both shRNA and siRNA can be used to bring about similar outcomes (reduction in mRNA and protein expression), they are intrinsically different molecules¹⁷⁰. siRNA are chemically synthesised 21 nt molecules, while shRNA are vector based and make use of the cellular machinery for their expression.

2.8.1. Transfection of A549 and HeLa cells with siRNA-LAMR1

4×10^5 A549 and 3.5×10^5 HeLa cells were seeded in the wells of a 6 well plate. 24 h later, 150 μmol (for A549 cells) or 200 μmol (for HeLa cells) of siRNA-LAMR1 and 6 μL of DharmaFECT® 1 transfection reagent were each concurrently diluted in DMEM, each to a final volume of 200 μL , the contents of each tube was gently mixed and allowed to incubate for 5 min at RT. The diluted siRNA-LAMR1 solution was then added to the diluted DharmaFECT® 1 transfection reagent, mixed gently and incubated for 20 min at RT. The culture medium was removed from the plated A549 and HeLa cells and 1600 μL of DMEM containing 10% (v/v) FCS added to the siRNA-reagent solution, which the cells were then grown in for 72 h in a humidified incubator at 37 °C with 95% air and 5% CO₂.

2.8.2. Determination of LRP expression levels

72 h post-transfection, A549 and HeLa cells were lysed, their protein content quantified, electrophoresis samples prepared, proteins resolved on a 12% sodium dodecyl sulfate polyacrylamide gel, resolved proteins transferred from the gel to a 0.45 μm PVDF membrane, siRNA-LAMR1 transfected and non-transfected LRP expression levels determined by immunodetection and siRNA-LAMR1 transfected LRP expression levels compared to non-transfected controls using densitometry as described above in sections 2.4.1, 2.4.2, 2.4.3, 2.4.4, 2.4.5, 2.4.6, and 2.4.7, respectively.

2.9. Examination of cellular viability

An MTT assay was utilised to assess cellular viability. MTT is converted to formazan crystals by the enzyme mitochondrial succinate dehydrogenase, present only in viable cells. The absorbance of the formazan crystals is therefore directly proportional to cellular viability.

2.9.1. Transfection of A549 and HeLa cells with siRNA-scr and siRNA-LAMR1

A549 and HeLa cells were seeded in the wells of a 96 well plate and transfected with siRNA-LAMR1 (appendix 6.1). Additionally cells were transfected with 50 μ mol of the ON-TARGET^{plus}® control siRNA – Non-Targeting siRNA 1 (here designated siRNA-scr) and incubated in 8 mM PCA (Sigma-Aldrich) (appendix 6.2) for negative and positive control purposes, respectively. 72 h post-transfect/incubation, 10 μ g of MTT (Separations) (appendix 6.3) was added to each well and the cells allowed to incubate (as before) for 2 h. The culture medium was subsequently removed, the formazan crystals in each well dissolved in 200 μ L DMSO (Monitoring and Control Laboratories) and the absorbance of this solution measured using a microtiter plate reader at 570 nm.

2.9.2. Incubation of A549 and HeLa cells with siRNA-scr and DharmaFECT® 1 transfection reagent

0.6×10^4 A549 and 3×10^3 HeLa cells were seeded in the wells of a 96 well plate. 24 h later, cells were incubated with 50 μ mol siRNA-scr, 0.2 μ L of DharmaFECT® 1 transfection reagent or both (all in DMEM containing 10% (v/v) FCS) to a final volume of 100 μ L in a humidified incubator at 37 °C with 95% air and 5% CO₂. 72 h later, 10 μ g of MTT was added to each well and the cells allowed to incubate (as before) for 2 h. The culture medium was subsequently removed, the formazan crystals in each well dissolved in 200 μ L DMSO and the absorbance of this solution measured using a microtiter plate reader at 570 nm.

2.10. Examination of apoptotic nuclear morphology

A549 and HeLa cells were seeded in the wells of a 6 well plate containing microscope coverslips and transfected with siRNA-LAMR1 as indicated above in section 2.8.1. Additionally cells were transfected with siRNA-scr and incubated in 8 mM PCA for negative and positive control purposes, respectively. 72 h post-transfection, the microscope coverslips were removed, the cells stained with Hoechst 33342 for

visualisation of nuclear morphology and subsequently prepared for immunofluorescence microscopy according to the protocol outlined in section 2.5.2.

2.11. Examination of caspase-3 activity

A549 and HeLa cells were seeded in the wells of a 6 well plate and transfected with siRNA-LAMR1 as indicated above in section 2.8.1. Additionally cells were transfected with siRNA-scr and incubated in 8 mM PCA for negative and positive control purposes, respectively. 72 h post-transfection, cell lysates were analysed for caspase-3 activity using a colorimetric Caspase-3 Assay Kit (Sigma-Aldrich) according to the following protocol.

A549 and HeLa cells were detached using trypsin/EDTA and resuspended in DMEM containing 1% (v/v) penicillin/streptomycin and 10% (v/v) FCS. 10^7 cells from each sample was centrifuged (600 x g, 10 min, 4 °C), the supernatant discarded, the pellet washed in 1 X PBS, centrifuged as above and the supernatant discarded. Each pellet was then resuspended in 1 X Lysis buffer, incubated on ice for 20 min and centrifuged (20 000 x g, 10 min, 4 °C). 5 µL of each lysate was then added to the wells of a flat bottom 96 well plate containing 2 mM of the caspase-3 substrate Ac-DEVD-pNA and 1 X Assay Buffer was added to a total volume of 100 µL. The plate was incubated in a humidified incubator at 37 °C with 95% air and 5% CO₂ for 24 h and the absorbance of each well subsequently measured using a microtiter plate reader at 405 nm.

2.12. Assessment of cellular proliferation

A549 and HeLa cells were seeded in the wells of a 96 well plate and transfected with siRNA-LAMR1 (appendix 6.1). Additionally cells were transfected with siRNA-scr and incubated in 100 µg/mL Doxycycline (Sigma-Aldrich) (appendix 7.1) for negative and positive control purposes, respectively. 72 h post-transfection, the percentage of proliferative A549 and HeLa cells in each sample was determined using a BrdU Cell Proliferation Assay (Calbiochem®) according to the following protocol:

72 h post-transfection, 20 μ L of the BrdU Label solution (diluted 1:2 000 in DMEM containing 1% (v/v) penicillin/streptomycin and 10% (v/v) FCS) was added to each well and the plate allowed to incubate overnight (~14 h) in a humidified incubator at 37 °C with 95% air and 5% CO₂. The contents of the plate was then removed, 200 μ L of Fixative/Denaturing solution added to each well and the plate incubated for 30 min at RT. The contents of the plate was removed, 100 μ L of diluted (1:100 in Dilution Buffer) 100 X Anti-BrdU antibody added to each well and the plate incubated for 1 h at RT. The plate was washed thrice with 1 X Wash Buffer and the subsequently blotted dry on paper towels. 100 μ L of diluted (1:1 000 in Conjugate Diluent) Peroxidase Goat Anti-Mouse IgG HRP Conjugate antibody was added to each well, incubated for 30 min at RT and subsequently washed thrice (as before). The entire plate was then flooded with dH₂O, the contents of the wells removed and the plate dried by tapping gently on paper towels. 100 μ L of the substrate solution was added to each well, and incubated in the dark at RT for 15 min. 100 μ L of Stop Solution was then added to each well (in the same order as the Substrate Solution) and the absorbance of each well immediately measured using a microtiter plate reader at 540 nm.

2.13. Statistical analysis

Data was statistically analysed with a Dunnett's test using GraphPad InStat. Results were considered statistically significant when the p-value was less than 0.05.

3. CHAPTER 3 – RESULTS

3.1. Determination of cell surface LRP/LR and total LRP levels on A549 and HeLa cells

3.1.1. Determination of cell surface LRP/LR levels

The amount of LRP/LR on the surface of A549 and HeLa cells was determined by flow cytometry; the aforementioned cells were incubated with the anti-LRP specific primary antibody IgG1-iS18 and subsequently with the Goat anti-human IgG-FITC secondary antibody (section 2.3).

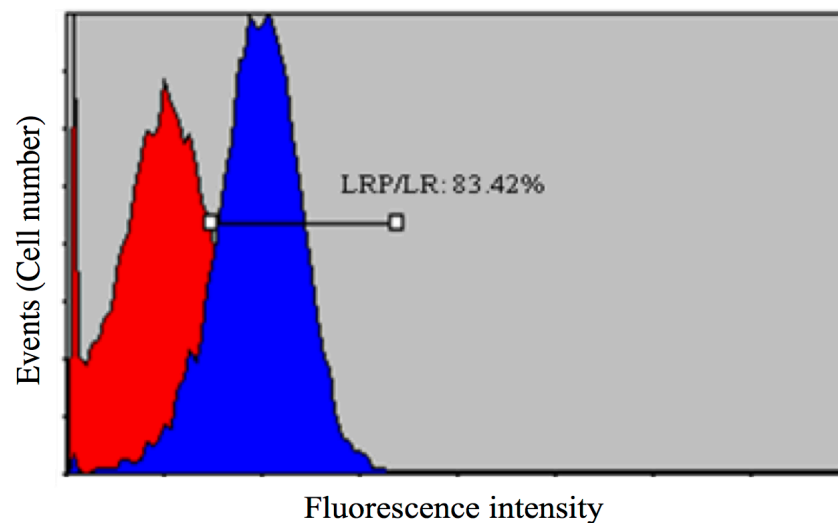
The resulting histograms (Figure 3.1) demonstrate that 83% of A549 cells and 80% of HeLa cells display LRP/LR on their surface.

3.1.2. Determination of total LRP levels

Since this protein has numerous cellular locations (namely the cell surface, the cytoplasm, the perinuclear compartment and the nucleus) the total level of LRP in A549 and HeLa cells was assessed using western blotting and quantitatively determined using densitometric analysis (section 2.4).

Western blot analysis revealed a band at a molecular weight of 37 kDa, representative of the 37 kDa laminin receptor precursor (LRP) (Figure 3.2), confirming its expression in the aforementioned cell lines (β -actin was used as a loading control). Additionally, densitometric analysis of the western blot signals revealed a non-significant ($p > 0.05$) difference between the LRP expression levels of A549 and HeLa cells (Figure 3.2). The 67 kDa form of the receptor was not detected in the western blots (data not shown).

a) A549 cells



b) HeLa cells

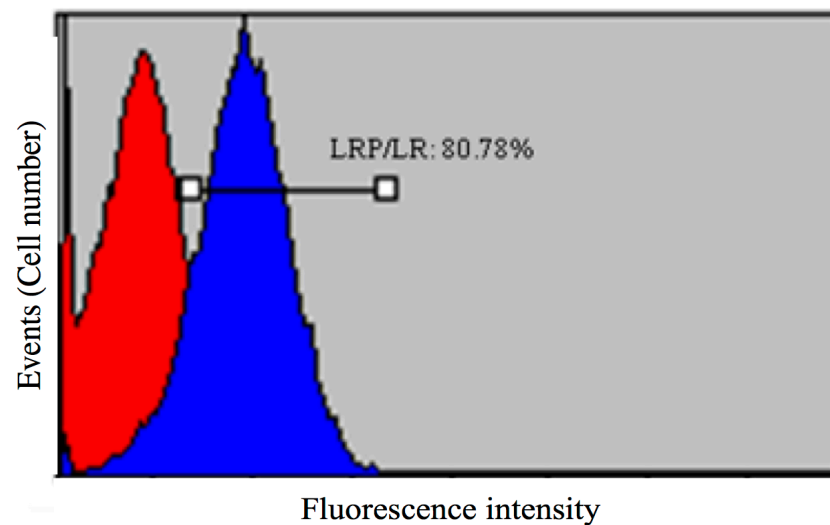


Figure 3.1 – Flow cytometric analysis of surface LRP/LR levels on A549 and HeLa cells. Red and blue peaks are representative of non-labelled and cells incubated with the anti-LRP IgG1-iS18 antibody and subsequently with the goat anti-rabbit IgG human ads-FITC secondary antibody, respectively. 83% and 80% of a) A549 and b) HeLa cells, respectively, displayed LRP on their surface.

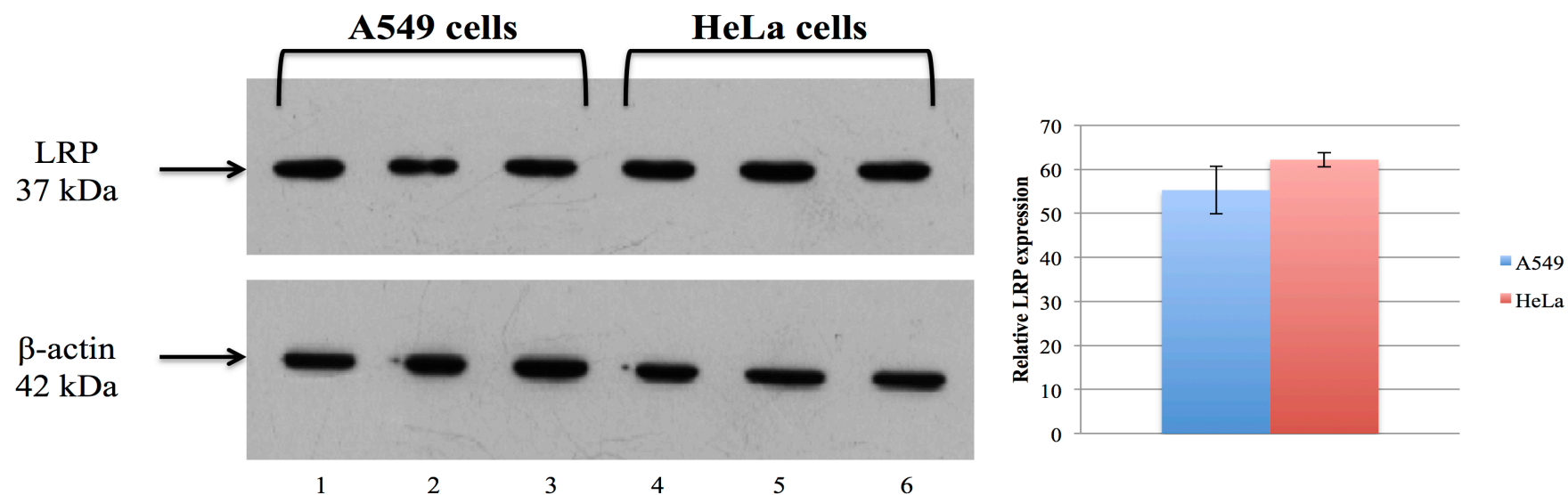


Figure 3.2 – Determination of total cellular LRP levels in A549 and HeLa cells. Proteins were extracted, separated on a 12% SDS-PAGE and western blotting performed using primary antibodies directed against LRP or β-actin (used as a loading control). Densitometric analyses of western blot signals reveal that A549 (lanes 1-3) and HeLa (lanes 4-6) cells express similar levels of LRP ($p > 0.05$).

3.2. Determination of A549 and HeLa cell transfectability

The introduction of plasmid DNA and siRNA into A549 and HeLa cells, to potentially decrease their expression of LRP, was imperative for the completion of this study; hence, the transfectability of the aforementioned cells was determined. A549 and HeLa cells were transiently transfected with a plasmid encoding for the green fluorescent protein (pCI-neo-GFP), stained with the fluorescent nuclear dye Hoechst 33342 and subsequently viewed using immunofluorescence microscopy (section 2.5).

The resulting images (Figure 3.3) reveal both blue and green fluorescence; the presence of green fluorescence indicates produced green fluorescent protein, therefore signifying that the A549 and HeLa cells are transfectable.

3.3. Determination of LRP downregulation in A549 and HeLa cells post-transfection with pTZ57R/T shRNA-1.1

The level of LRP expression in A549 and HeLa cells post-transfection with the produced pTZ57R/T shRNA-1.1 plasmid was determined using western blotting and densitometric analysis (section 2.7).

Densitometric analysis of the obtained western blot signals (Figure 3.4) revealed that pTZ57R/T shRNA-1.1 transfected A549 and HeLa cells exhibited non-significant ($p > 0.05$) differences in their expression of LRP, compared to non-transfected cells (which had been set to 100%) (β -actin was used as a loading control).

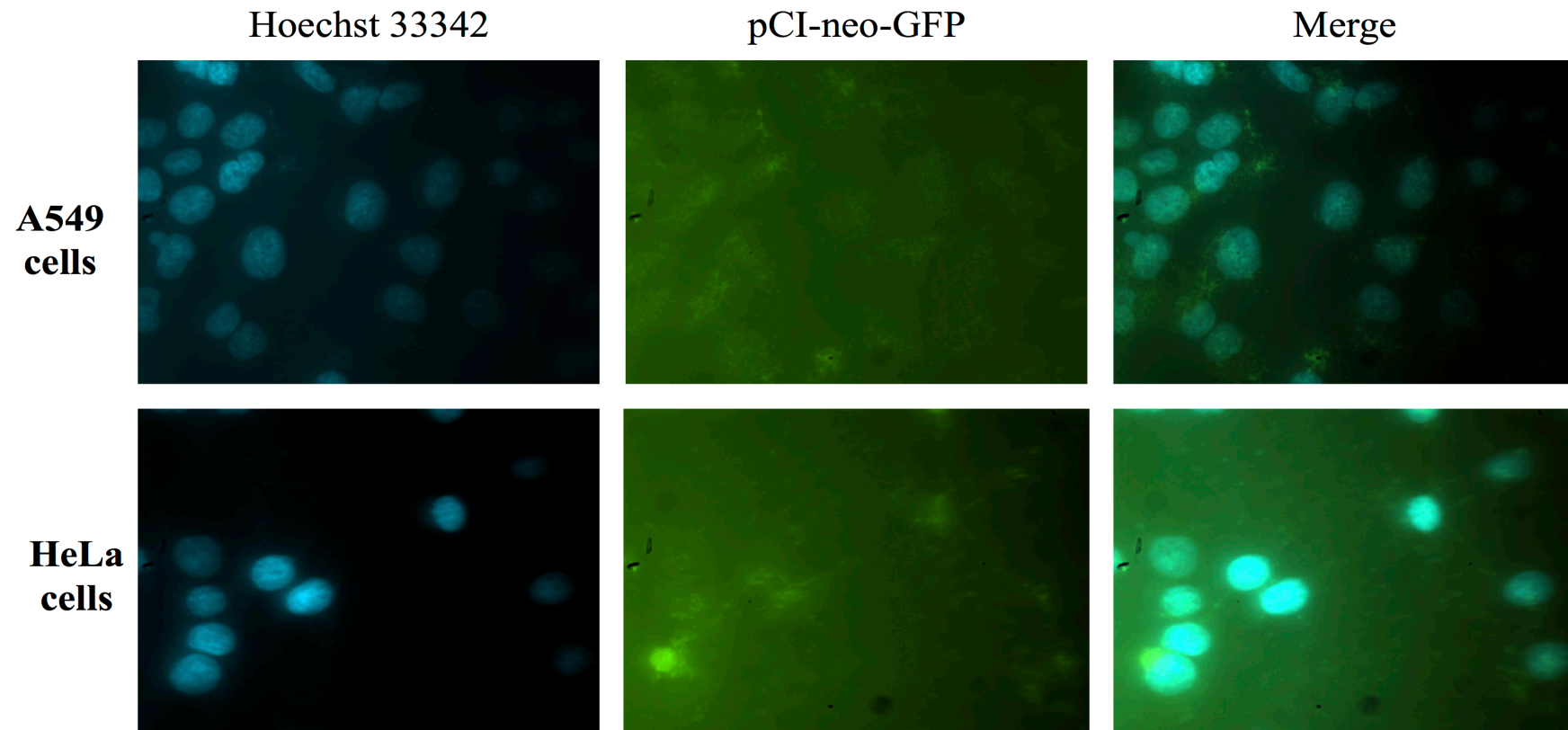


Figure 3.3 – Determination of A549 and HeLa cell transfectability. 24 h post-transfection of A549 and HeLa cells with a plasmid encoding for the green fluorescent protein (pCI-neo-GFP), cells were stained with the fluorescent nuclear dye Hoechst 33342 and viewed on an immunofluorescence microscope. The resultant images show both blue and green fluorescence, indicating that these cell lines are transfectable.

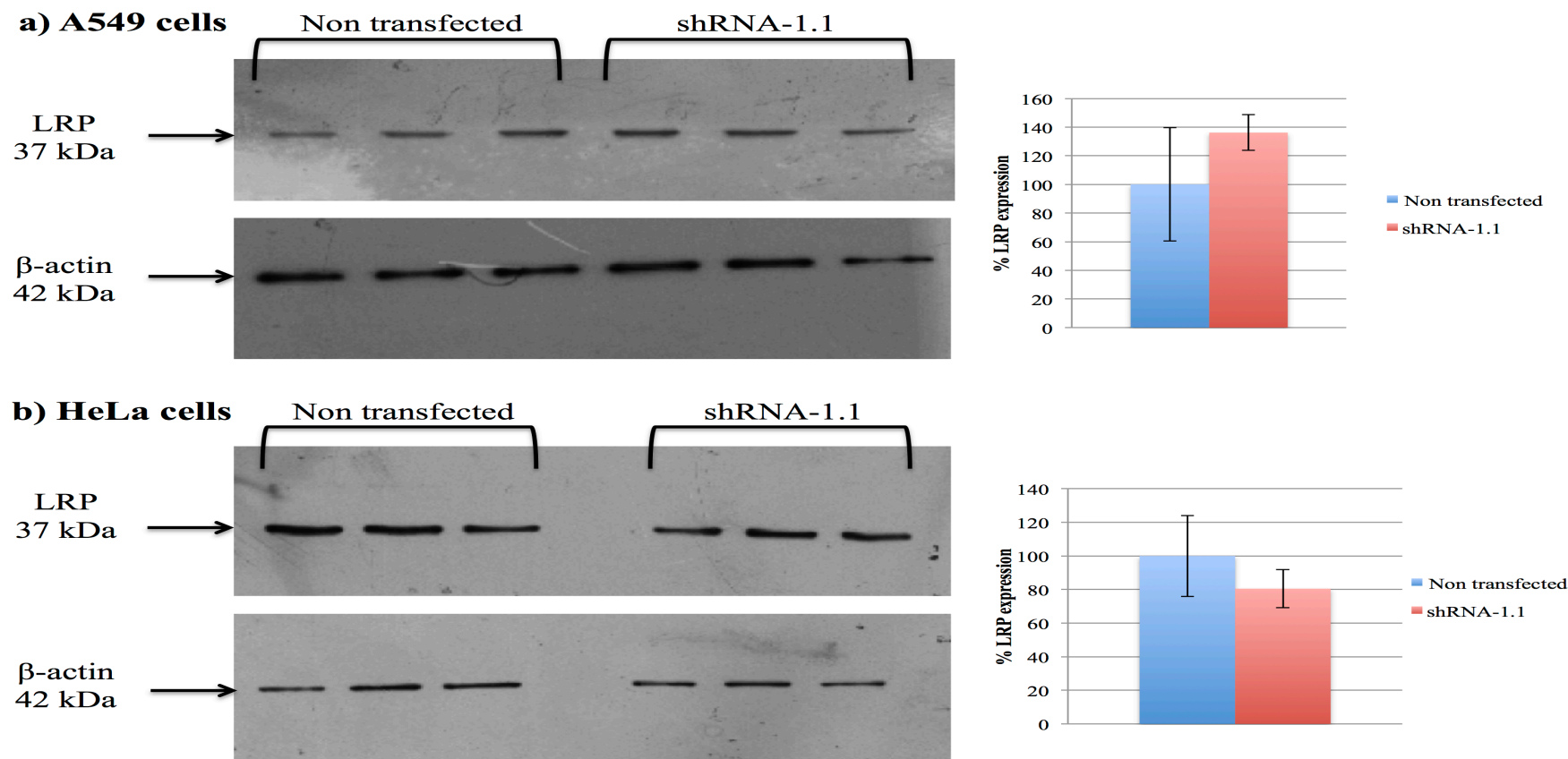


Figure 3.4 – LRP expression in A549 and HeLa cells post-transfection with shRNA-1.1. The expression levels of LRP in A549 and HeLa cells was investigated 72 h post-transfection with shRNA-1.1. Densitometric analysis of western blot signals revealed non-significant ($p > 0.05$) differences in LRP expression in a) A549 and b) HeLa cells, respectively, compared to non-transfected cells.

3.4. Determination of LRP downregulation in A549 and HeLa cells post-transfection with siRNA-LAMR1

The level of LRP expression in A549 and HeLa cells post-transfection with the purchased siRNA-LAMR1 was determined using western blotting and densitometric analysis (section 2.8).

Densitometric analysis of the western blot signals (Figure 3.5) revealed that siRNA-LAMR1 transfected A549 and HeLa cells displayed a significant (** $p < 0.01$) 83% and 60% reduction in LRP expression, respectively, compared to non-transfected cells (which had been set to 100%) (β -actin was used as a loading control).

3.5. Determination of the effect of the siRNA-mediated downregulation of LRP expression on cellular viability

The effect of the siRNA-mediated downregulation of LRP expression on cellular viability in the aforementioned cell lines was determined using an MTT assay. Cells were incubated with 8 mM PCA (a compound known to decrease cellular viability through the induction of apoptosis¹⁷¹) as a positive control and transfected with a non-targeting siRNA (siRNA-scr) as a negative control (section 2.9.1). Non-transfected, 8 mM PCA and siRNA-LAMR1 values were compared to siRNA-scr values, which had been set to 100%.

siRNA-LAMR1 treated A549 and HeLa cells revealed a significant (* $p < 0.05$, ** $p < 0.01$) 13% and 18% reduction in cellular viability, respectively, compared to siRNA-scr treated cells (Figure 3.6).

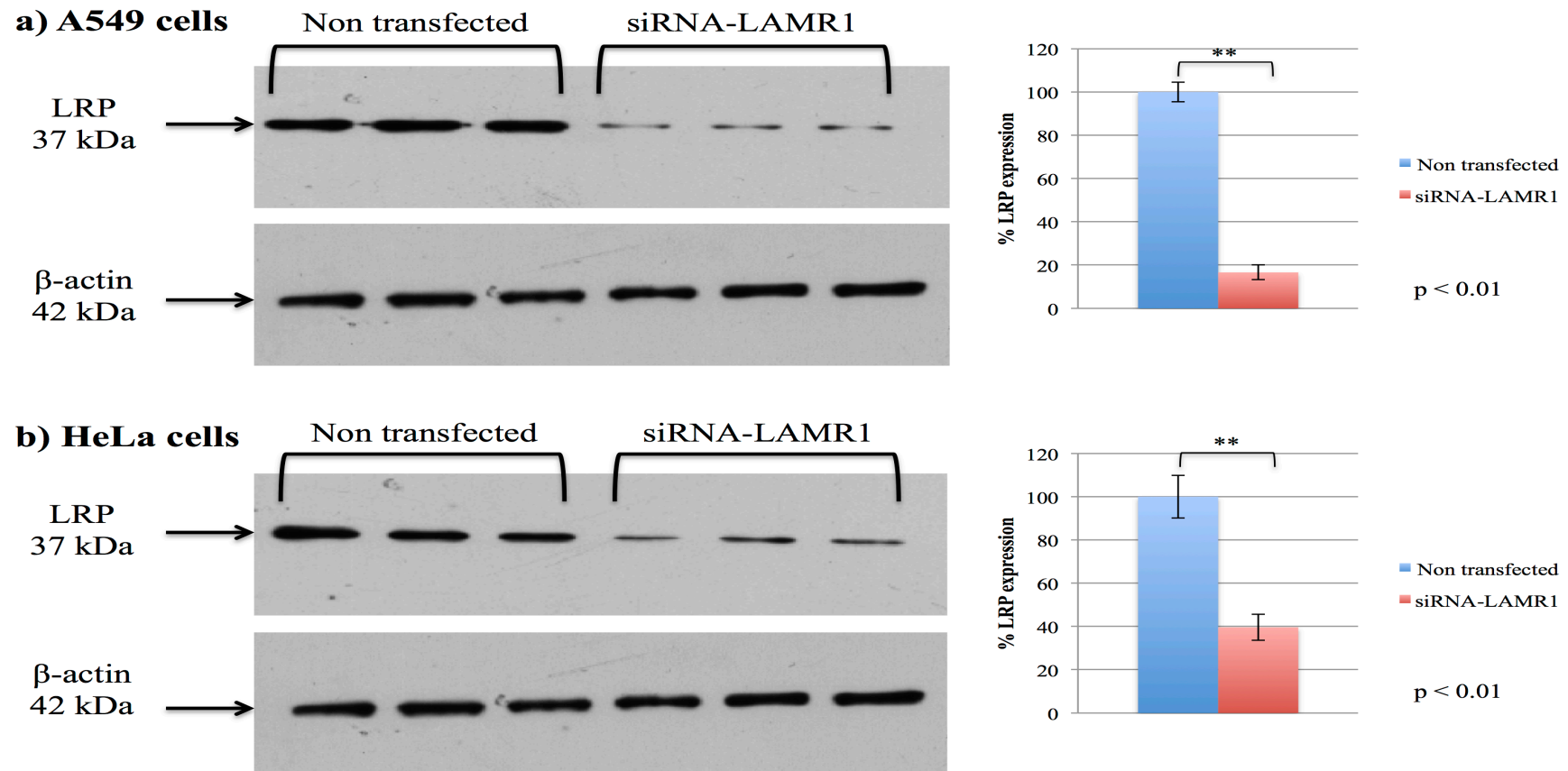
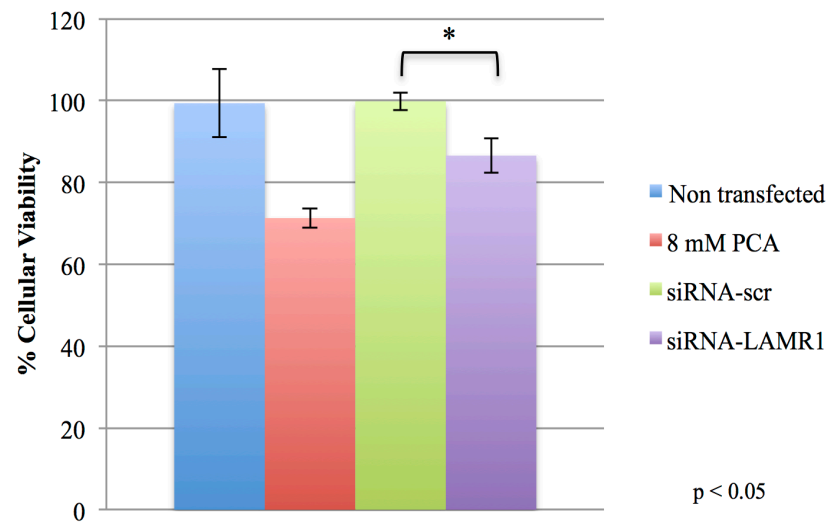


Figure 3.5 - siRNA-mediated downregulation of LRP expression in A549 and HeLa cells. The expression level of LRP in A549 and HeLa cells was investigated 72 h post-transfection with siRNA-LAMR1. Densitometric analysis of western blot signals revealed a significant (** $p < 0.01$) 83% and 60% reduction in LRP expression in a) A549 and b) HeLa cells, respectively, compared to non-transfected controls.

a) A549 cells



b) HeLa cells

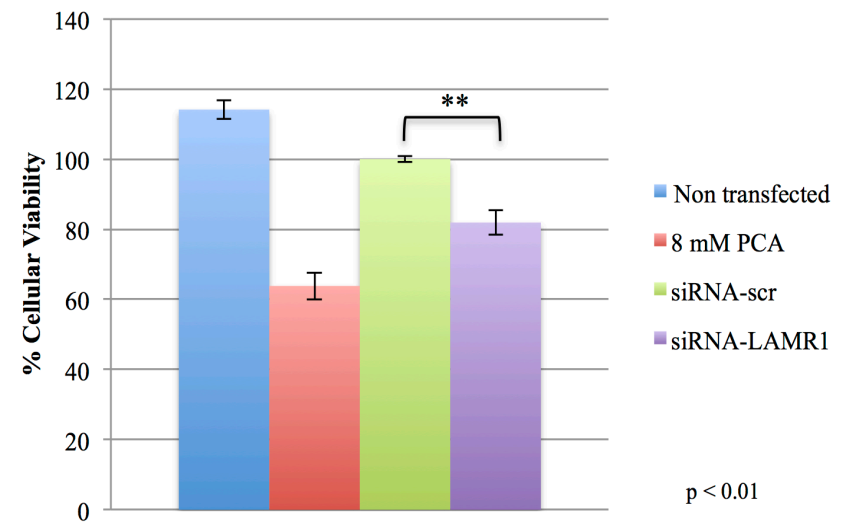


Figure 3.6 – The effect of the siRNA-mediated downregulation of LRP expression on cellular viability in A549 and HeLa cells. The viability of A549 and HeLa cells was analysed 72 h post-transfection using an MTT assay. siRNA-LAMR1 treated a) A549 and b) HeLa cells reveal a significant (* $p < 0.05$, ** $p < 0.01$) 13% and 18% reduction in cellular viability, respectively, compared to siRNA-scr treated cells (set to 100%) (8 mM PCA used as positive control).

Additionally, siRNA-scr treated cells exhibited a non-significant 14% decrease in cellular viability, compared to non-transfected HeLa cells (Figure 3.6b). Incubating HeLa cells with siRNA-scr, DharmaFECT® 1 transfection reagent or both was used to determine the causative agent of the observed reduction in cellular viability. An MTT assay was completed and the values obtained for the abovementioned samples were compared to control cells (containing no additions), which had been set to 100% (as indicated in section 2.9.2).

This assay indicates that the DharmaFECT® 1 transfection reagent was responsible for causing the observed non-significant reduction in cellular viability (Figure 3.7).

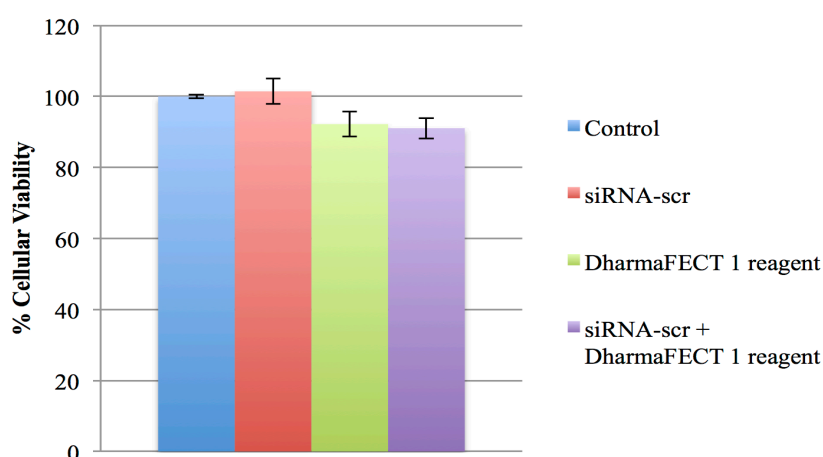


Figure 3.7 - The effect of siRNA-scr and DharmaFECT® 1 reagent on cellular viability. HeLa cells were incubated with siRNA-scr, DharmaFECT® 1 reagent or both; 72 h later, their viability was assessed using an MTT assay. siRNA-scr treated cells display similar viability to control cells (incubated for 72 h in DMEM containing 10% (v/v) FCS), while the DharmaFECT® 1 and siRNA-scr + DharmaFECT® 1 treated cells display an 8% and 9% decrease in cellular viability, respectively, compared to control cells ($p > 0.05$).

3.6. Assessment of the effect of the siRNA-mediated downregulation of LRP expression on nuclear morphology

The nuclear morphology of A549 and HeLa cells was assessed 72 h post-transfection with siRNA-LAMR1. Additionally, the aforementioned cell lines were incubated with 8 mM PCA (an apoptosis inducer¹⁷¹) and transfected with siRNA-scr for positive and negative control purposes, respectively. The cells were then stained with the fluorescent nuclear dye Hoechst 33324 and viewed by immunofluorescence microscopy (section 2.10).

siRNA-LAMR1 treated A549 and HeLa cells reveal constricted nuclei, which appear to have lost their integrity, indicating a loss their nuclear morphology, compared to siRNA-scr treated cells (Figure 3.8 – white arrows). The nuclei of cells incubated with 8 mM PCA appear constricted; this was expected, as this compound is a known apoptosis inducer¹⁷¹. Before transfection/incubation, the cell number was consistent; we therefore suggest that the toxicity of PCA was responsible for cell death, resulting in decreased cell numbers observed in the corresponding micrographs.

3.7. Examination of the effect of the siRNA-mediated downregulation of LRP expression on caspase-3 activity

The activity of the apoptosis-associated effector protein, caspase-3, in response to the siRNA-mediated downregulation of LRP expression in A549 and HeLa cells was assessed using a Caspase-3 activation assay (section 2.11). Additionally, the aforementioned cell lines were incubated with 8 mM PCA (an apoptosis inducer known to increase caspase-3 activity¹⁷¹) and transfected with siRNA-scr for positive and negative control purposes, respectively.

siRNA-LAMR1 treated A549 and HeLa cells reveal a significant (** $p < 0.01$, *** $p < 0.001$) 9% and 83% upregulation in the activity of caspase-3, respectively, compared to siRNA-scr treated cells (set to 100%) (Figure 3.9). The increased activity of caspase-3 in A549 and HeLa cells incubated with 8 mM PCA was expected since this compound is an inducer of apoptosis known to increase the activity of the aforementioned protein¹⁷¹.

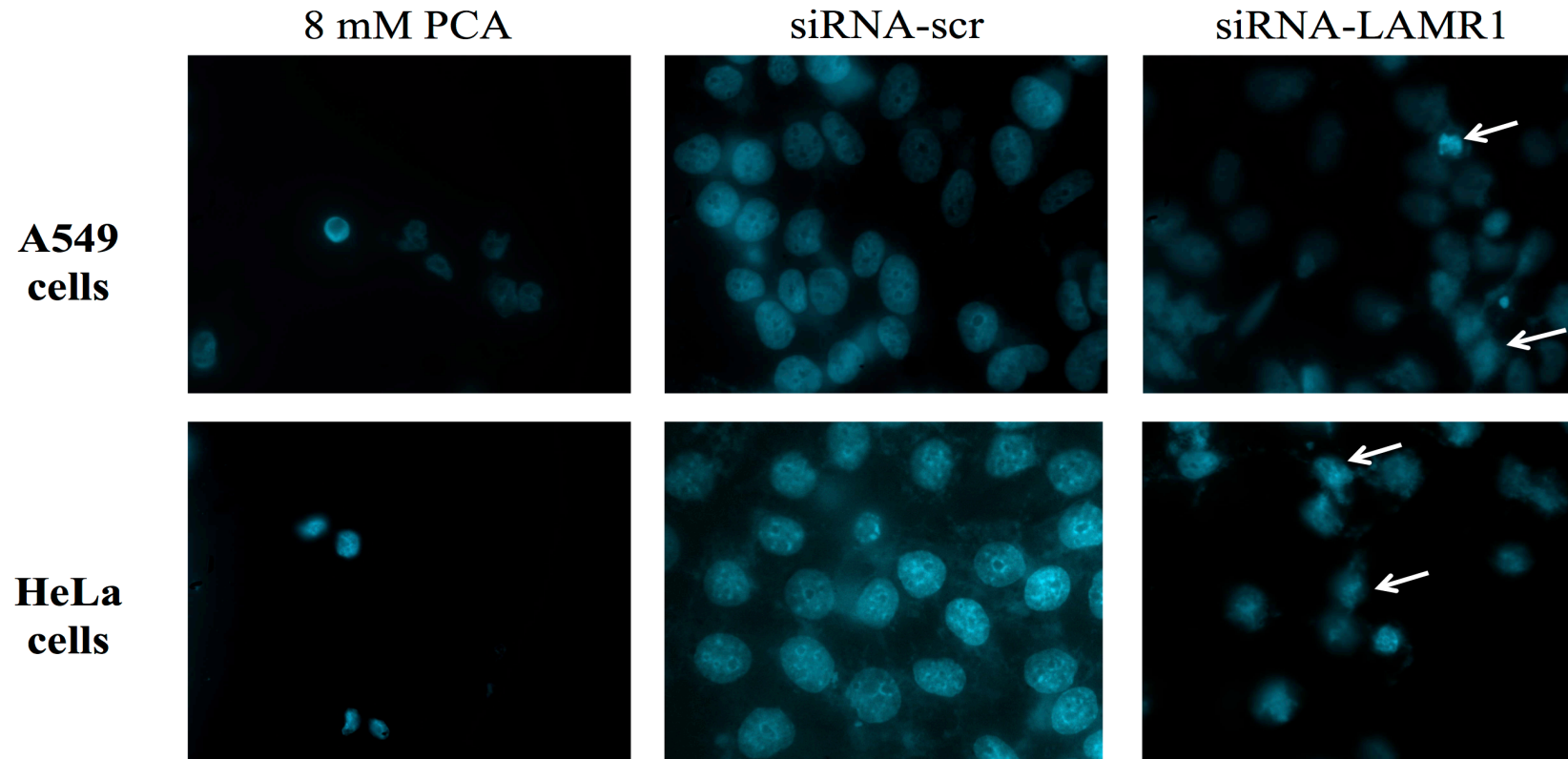
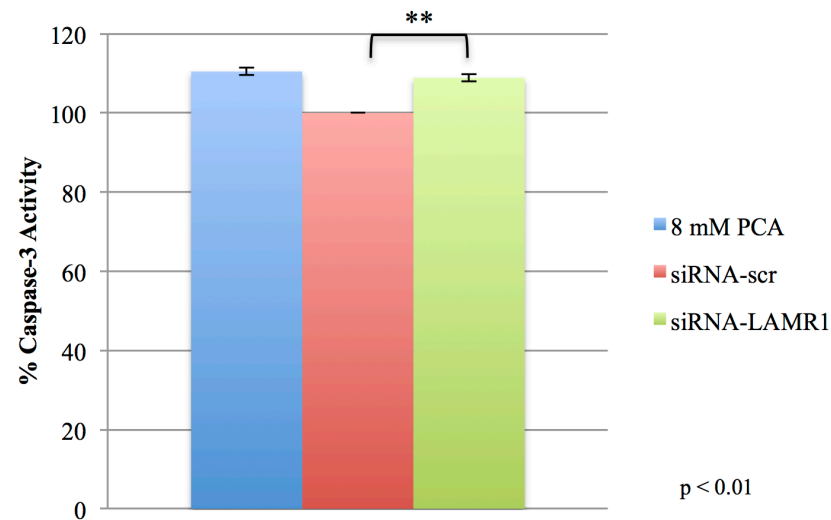


Figure 3.8 – The effect of the siRNA-mediated downregulation of LRP expression on nuclear morphology. 72 h post-transfection, A549 and HeLa cells were stained with Hoechst 33342 and viewed by immunofluorescence microscopy. siRNA-LAMR1 treated A549 and HeLa cells exhibit constricted nuclei and decreased nuclear integrity (indicated by the white arrows), compared to siRNA-scr treated cells (8 mM PCA used as positive control).

a) A549 cells



b) HeLa cells

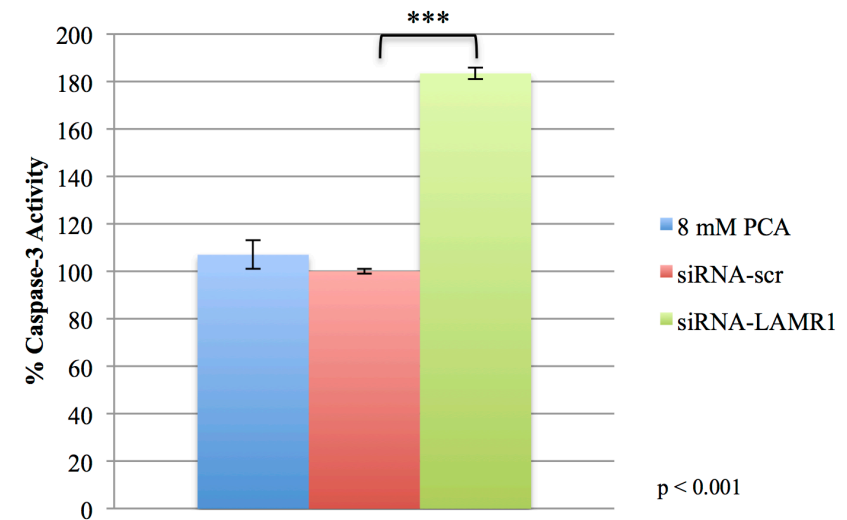


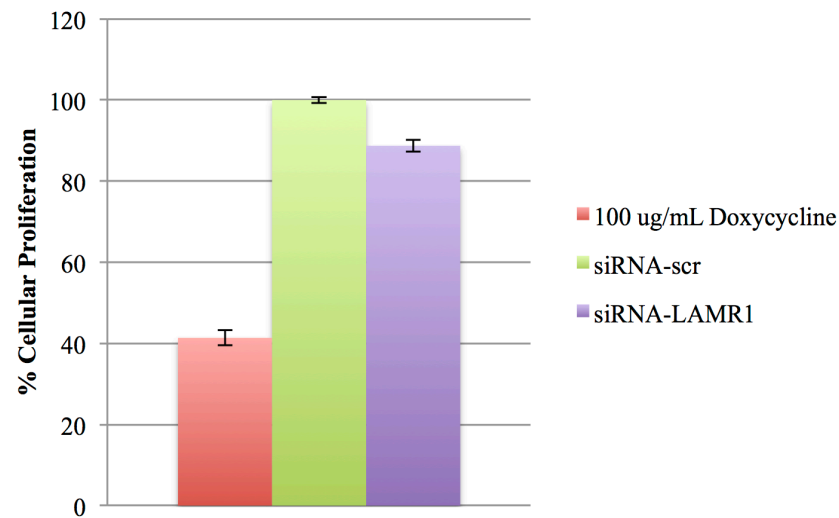
Figure 3.9 – The effect of the siRNA-mediated downregulation of LRP expression on caspase-3 activity. The activity of the apoptosis-associated protein, caspase-3, in A549 and HeLa cells was analysed 72 h post-transfection. siRNA-LAMR1 treated a) A549 and b) HeLa cells reveal a significant (* $p < 0.01$, *** $p < 0.001$) 9% and 83% upregulation in caspase-3 activity, respectively, compared to the siRNA-scr treated cells (set to 100%) (8 mM PCA used as a positive control).

3.8. Examination of the effect of the siRNA-mediated downregulation of LRP expression on cellular proliferation

The proliferation of A549 and HeLa cells in response to the siRNA-mediated downregulation of LRP expression was assessed using a BrdU Cell Proliferation Assay (section 2.12). Additionally, the aforementioned cells were incubated with 100 µg/mL Doxycycline (a known inhibitor of cellular proliferation ¹⁷²) and transfected with siRNA-scr for positive and negative control purposes, respectively.

siRNA-LAMR1 treated A549 and HeLa cells revealed a non-significant ($p > 0.05$) 11% and 13% reduction in cellular proliferation, respectively, compared to siRNA-scr treated cells (set to 100%) (Figure 3.10). The decreased level of cellular proliferation observed in A549 and HeLa cells incubated with 100 µg/mL Doxycycline was expected as this compound is a known inhibitor of the aforementioned process ¹⁷².

a) A549 cells



b) HeLa cells

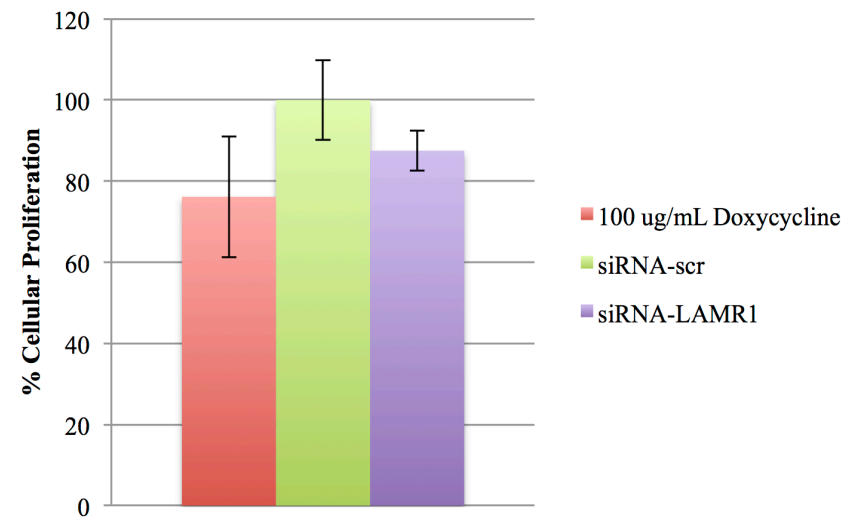


Figure 3.10 – The effect of the siRNA-mediated downregulation of LRP expression on cellular proliferation. The percentage of actively proliferative A549 and HeLa cells was analysed 72 h post-transfection. siRNA-LAMR1 treated a) A549 and b) HeLa cells reveal a non-significant ($p > 0.05$) 11% and 13% reduction in cellular proliferation, respectively, compared to siRNA-scr treated cells (set to 100%) (100 μ g/mL Doxycycline used as a positive control).

4. Chapter 4 – Discussion

The role of the laminin receptor in cancer progression has been a topic of great interest for many years and has therefore been extensively investigated. Numerous studies have implicated cell surface LRP/LR in the progression of cancer – this receptor is overexpressed on the surface of a number of cancerous cell lines, giving them the ability to metastasise and invade surrounding tissues^{131; 132}. Given that LRP/LR is involved in a number of cellular processes and is found in numerous cellular locations (the cell surface⁸⁹, the cytoplasm^{8; 75; 88}, the perinuclear compartment^{75; 90; 91} and the nucleus^{90; 91}), additional roles of this receptor in cancer progression have been suggested.

4.1. Cell surface LRP/LR and total LRP levels

To confirm the expression of LRP/LR in A549 and HeLa cells, cell surface LRP/LR (Figure 3.1) and total LRP (Figure 3.2) levels was investigated using flow cytometry and western blotting, respectively. Flow cytometry measured the number of A549 and HeLa cells in the population that expressed LRP/LR on their surface, and was not indicative of the level of LRP/LR on the surface of these cells. A high percentage of A549 and HeLa cells were shown to display cell surface LRP/LR, as expected from previous studies¹³². In addition, total LRP expression was confirmed in both A549 and HeLa cells, and densitometric analyses of the western blot signals revealed that these cells express similar total levels of LRP.

The 67 kDa laminin receptor is formed from the 37 kDa laminin receptor precursor^{81; 82}, with the exact mechanism of this conversion currently still elusive. However, some studies have suggested that unglycosylated 37 kDa LRP becomes acylated at Ser2 through the action of fatty acids; and this acylation is a critical step in the conversion of 37 kDa LRP to 67 kDa LR^{83; 84}. We detect LRP/LR on the cell surface because it was discovered that both 37 kDa LRP and 67 kDa LR are present in cellular plasma membrane fractions, albeit 67 kDa LR in lower concentrations than its 37 kDa isoform⁸⁹. Furthermore, the low concentration of 67 kDa LR in cellular cytoplasmic

extractions renders it undetectable by western blot analysis ⁸⁹; we therefore detect only 37 kDa LRP from these extractions.

4.2. LRP silencing

To determine the role played by LRP/LR in the tumorigenic lung and cervical cancer cells, it was essential for LRP expression to be decreased. This necessitated the introduction of foreign nucleic acids (in a process known as transfection) into these cells. The foreign nucleic acids would integrate into the RNAi system, causing the degradation of LRP mRNA, consequently decreasing LRP expression. Since each cell line possesses a different level of transfectability, the transfection efficiency of the A549 and HeLa cells was determined. To accomplish this, these cells were transfected with a plasmid encoding for the green fluorescent protein (pCI-neo-GFP), stained with the nuclear stain Hoechst 33342 and subsequently viewed by immunofluorescence microscopy. The observance of both blue and green fluorescence (Figure 3.3) indicated that A549 and HeLa cells are both transfectable. Since green fluorescence was observed surrounding each nuclei for both the A549 and HeLa cells, we concluded that their transfection efficiency was 100%. This finding allowed for further experimentation.

A549 and HeLa cells were transfected with the pTZ57R/T shRNA-1.1 plasmid produced within this study, which encodes for shRNA directed against LRP mRNA, to possibly downregulate LRP expression. However, subsequent densitometric analyses of the obtained western blot signals revealed non-significant differences in the expression levels of LRP in the aforementioned cell lines (Figure 3.4). The same transfection protocol and reagent was used to introduce both the pCI-neo-GFP and the pTZ57R/T shRNA-1.1 plasmid DNA into the A549 and HeLa cells, and since the introduction of the pCI-neo-GFP plasmid was successful, the efficacy of the transfection method and reagent was not questioned. The ability of shRNA to successfully downregulate the expression of a particular protein is not only dependent on its ability to enter the cells, but also on its overall design, specificity and target sequence. The LRP target sequence for shRNA-1.1 was generated using The RNAi Consortium, and was chosen because of the high probability it boasted to bring about knockdown effects. Prof. Marco Weinberg designed the oligonucleotides with

emphasis placed on the efficacy of the resultant shRNA. Additionally, the sequence specificity of the shRNA was also investigated, and since the obtained sequence was identical to the target sequence, it was assumed that strong complementary binding between the shRNA and LRP mRNA would occur, resulting in mRNA degradation and hence protein downregulation. Taking all this information into account, the reason/s for the unsuccessful downregulation of LRP expression upon transfection of A549 and HeLa cells with the pTZ57R/T shRNA-1.1 plasmid remains elusive.

Following the unsuccessful downregulation of LRP expression using the produced pTZ57R/T shRNA-1.1 plasmid, siRNA directed against LRP mRNA was purchased and utilised further. siRNA-LAMR1 was chosen because it targeted the open reading frame of the human LRP gene. LRP expression was found to be significantly decreased in A549 and HeLa cells 72 h post-transfection with siRNA-LAMR1, confirmed by densitometric analysis of the western blot signals (Figure 3.5). The level of LRP expression in A549 and HeLa cells was significantly (** $p < 0.01$) reduced (80% and 60%, respectively), demonstrating the efficacy of the siRNA.

The reduction in the expression of 37 kDa LRP was possible since both the shRNA and siRNA sequences targeted 37 kDa LRP mRNA. It is not possible to use RNAi to reduce the expression of 67 kDa LR as it is not a direct product of transcription and translation, rather it is thought to be produced by the post-transcriptional modification of 37 kDa LRP^{83; 84}. However, since it is understood that the 37 kDa form is a precursor for the 67 kDa form⁸⁴, it could be assumed that a reduction in the expression of LRP would consequently reduce the expression of LR.

4.3. Cellular viability

The effect of the downregulation of LRP expression on the viability of cancerous cells (an important characteristic of the disease) was then investigated. LRP downregulation was found to correlate with a significant (* $p < 0.05$, ** $p < 0.01$) reduction in the viability of A549 and HeLa cells (Figure 3.6), indicating a crucial role for LRP this process. Since the maintenance of cellular viability is imperative for the survival and propagation of cancerous cells, the suggestion that LRP is involved in this process further implicates this protein in the disease. A non-significant

reduction in cellular viability was however also observed in siRNA-scr treated HeLa cells compared to non-transfected cells (Figure 3.6b) and the DharmaFECT® 1 transfection reagent was shown to be the causative agent in this decrease (Figure 3.7). This finding correlates to previous research completed by Thermo Scientific into this topic, where it is understood that a 20% reduction in cellular viability is a successful non-toxic transfection (Literature Code: 00078-06-E-02-U).

4.4. Apoptosis

Further experiments were performed to determine if apoptosis (a form of programmed cell death) was induced in A549 and HeLa cells as a result of LRP downregulation, and hence was responsible for the observed decrease in cellular viability in these cells. The presence of apoptotic A549 and HeLa cells post-transfection were firstly identified by visible disruptions in their nuclear morphology (Figure 3.8) and secondly by the significant increase in the activity of the apoptosis-associated protein caspase-3 (Figure 3.9).

Since the laminin receptor is physiologically functional in the perinuclear compartment ^{75; 90; 91} and the nucleus ^{90; 91}, it was not surprising that the downregulation of this receptor had an effect on nuclear integrity. Since LRP is present in the nucleus, and involved in the maintenance of nuclear structures ⁹⁰, it could be suggested that the downregulation of LRP causes nuclear instability which may result in the induction of apoptosis.

To further demonstrate apoptosis in the aforementioned cells, the activity of caspase-3 was investigated. Caspase-3 is an effector protein functional at the end stage of apoptosis, where its activation is responsible for bringing about the observable changes in the cell related to this form of cell death. The identification of increased caspase-3 activity was therefore used as a marker for apoptosis. The activity of caspase-3 was higher in siRNA-LAMR1 treated HeLa cells compared to siRNA-LAMR1 treated A549 cells, indicating that the downregulation of LPR expression was more effective in inducing apoptosis in HeLa cells than in A549 cells. It is unclear whether the increased activity of caspase-3 is as a consequence of upstream

events, which would initiate apoptosis in the cell, or through an inhibitory interaction directly with LRP. Further experiments must be performed to elucidate this.

Both the loss of nuclear morphology and the increased activity of apoptosis-associated protein, caspase-3, are indicative of apoptosis, and the results of this study therefore are suggestive of a critical role played by the laminin receptor in the blockage of this form of cell death.

4.5. Cellular proliferation

The proliferative state of A549 and HeLa cells post-transfection were investigated to determine if the observed significant decrease in cellular viability could also be attributed to a reduction in cell division. siRNA-LAMR1 treated cells revealed a non-significant ($p > 0.05$) reduction in cellular proliferation in A549 and HeLa cells (11% and 13%, respectively) compared to siRNA-scr treated cells (Figure 3.10). The results were not significant, however, if this experiment was repeated, the standard deviations could be reduced and significant results possibly obtained. Since LRP is physiologically involved in cellular proliferation, the reduction of its expression would cause a disruption in this process. However, from these results, it can also be suggested that this receptor may play a role in allowing the aberrant proliferation of cancerous cells.

The role of LRP in the maintenance of cellular viability and the stimulation of cellular proliferation has been reported in previous studies ⁷⁵, additionally, the induction of apoptosis in cancerous cells in response to LRP downregulation has also been demonstrated ⁷². This study has confirmed the role of LRP in the maintenance of cellular viability and the induction of apoptosis in A549 and HeLa cells. Furthermore, this study has revealed that the induction of apoptosis is mediated by both a loss in nuclear integrity and significantly increased activity of caspase-3 in these cells.

4.6. Targeting LRP/LR for cancer treatment

The implications of the expression of LRP in cancer progression are vast – increased invasion ^{131; 132}, metastasis ^{131; 132} and cellular proliferation ⁷⁵ as well as decreased

cellular viability ⁷⁵ and apoptosis ⁷² are processes mediated by this protein. Targeting LRP can therefore be developed as a potential alternative therapeutic for the treatment of the abovementioned processes implicated in cancer progression. Additionally, targeting LRP for the possible treatment of the abovementioned processes have been achieved in a number of cancerous cell lines. This therefore suggests that an LRP related therapy could potentially be utilised for the treatment of numerous distinct cancer types. It must however be stressed that this receptor is involved in many physiological processes in normal cells, including cell growth, differentiation, movement and attachment ⁸⁶, and the downregulation of LRP in these cells would result in undesirable homeostatic disruptions. The targeting of LRP solely to cancerous cells is therefore imperative for its use as a therapeutic alternative.

5. Chapter 5 – Conclusion and Future Work

5.1. Conclusion

This study has shown that the downregulation of LRP expression in A549 and HeLa cells resulted in a significant decrease in cellular viability. Additionally, it was demonstrated that this decrease in cellular viability was mediated by the induction of apoptosis (identified by visualisation of the loss in their nuclear integrity as well as the significantly increased activity of the apoptosis-associated protein caspase-3) and possibly through the inhibition of cellular proliferation. This finding is illustrated in Figure 5.1.

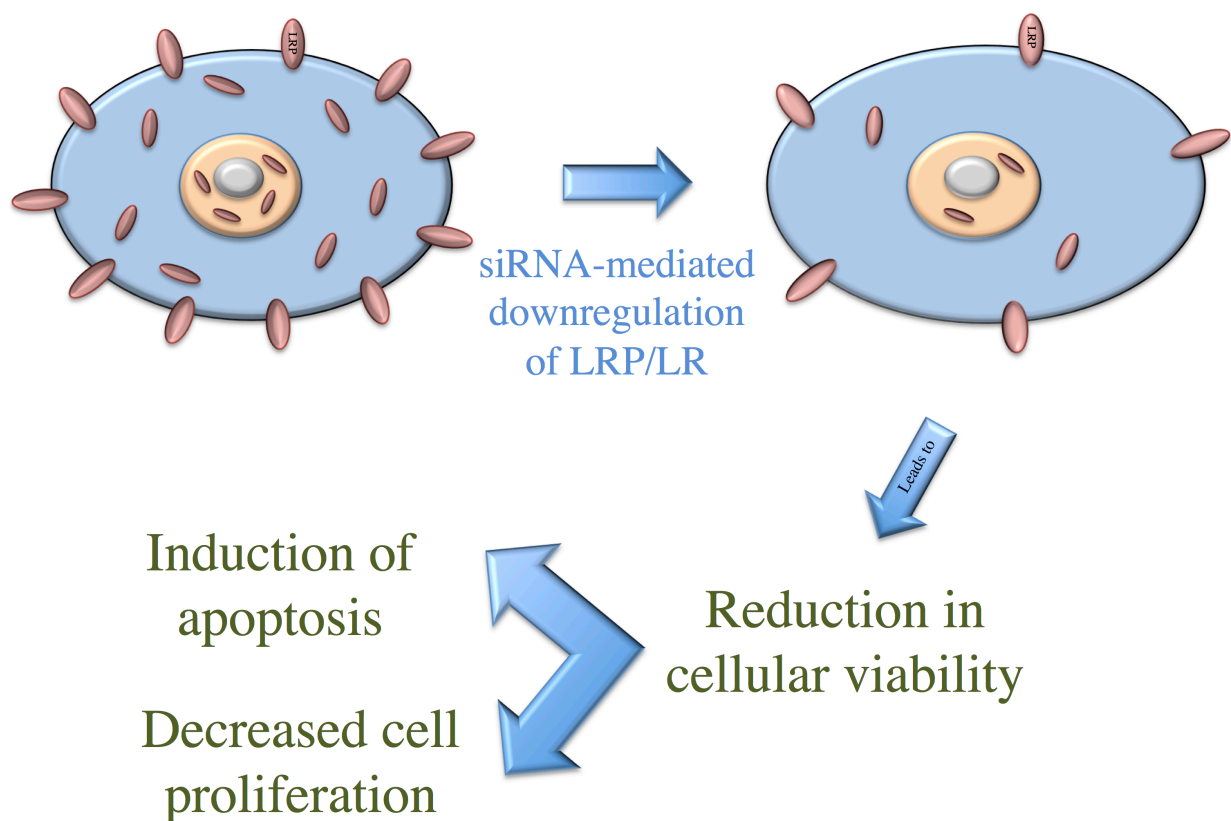


Figure 5.1 – Conclusion of the study. The siRNA-mediated downregulation of LRP expression caused a decrease in cellular viability, resultant from the induction of apoptosis and possibly the inhibition of cellular proliferation.

The findings of this study indicate that targeting LRP mRNA could possibly be used as an alternative therapeutic strategy for the treatment of cancer.

5.2. Future Work

The following experiments can be suggested from the findings of this study.

Research from a number of reports has been accumulated to suggest crucial roles for LRP/LR in the maintenance of the viability of cancerous cells, the promotion of metastasis, the blockage of apoptosis and the stimulation of cellular proliferation. It would be interesting to determine if targeting LRP/LR with a single specific tool (shRNA, siRNA, antibodies etc.) could cause all the aforementioned cancer related processes to become disrupted.

Even though it is possible that LRP/LR targeted therapies for cancer can be beneficial for the treatment of this disease, it also has the potential to be extremely problematic in non-cancerous tissues. Therefore, it would be necessary to determine the effects of LRP downregulation on normal tissues. Additionally, research into therapies involved in targeting LRP/LR exclusively on cancerous tissues is necessary – the use of viral delivery vectors could be successful in this endeavour.

Moreover, targeting LRP/LR could be investigated in animal models using nude mice and if successful, the effectiveness of these therapies in humans could be further assessed in clinical trials.

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Appendix

1. Plasmids

1.1. pCI-neo

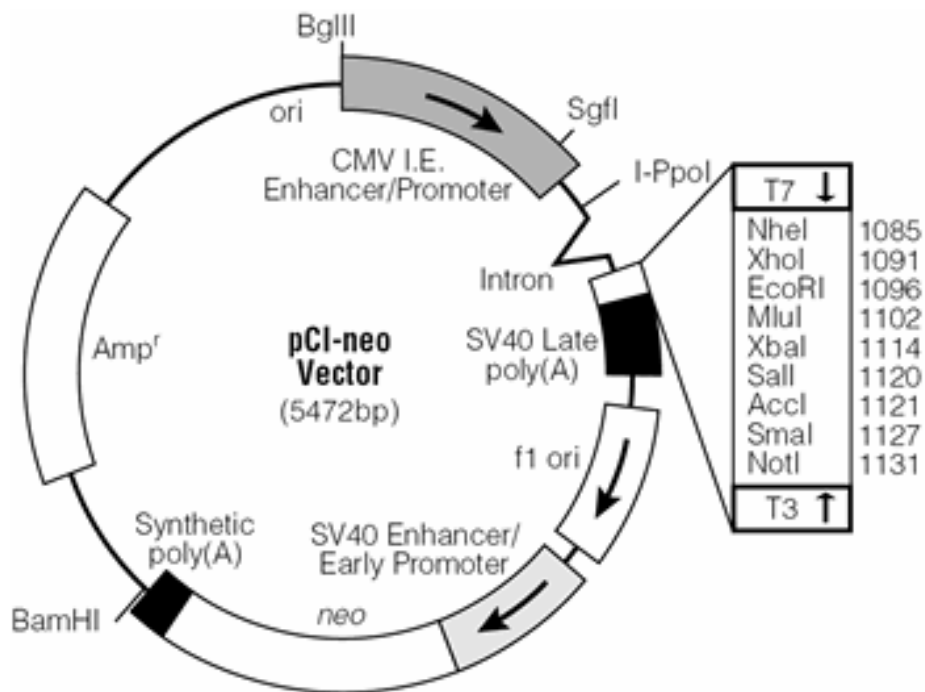


Figure A1 – pCI-neo plasmid map.

1.2. pti-H1

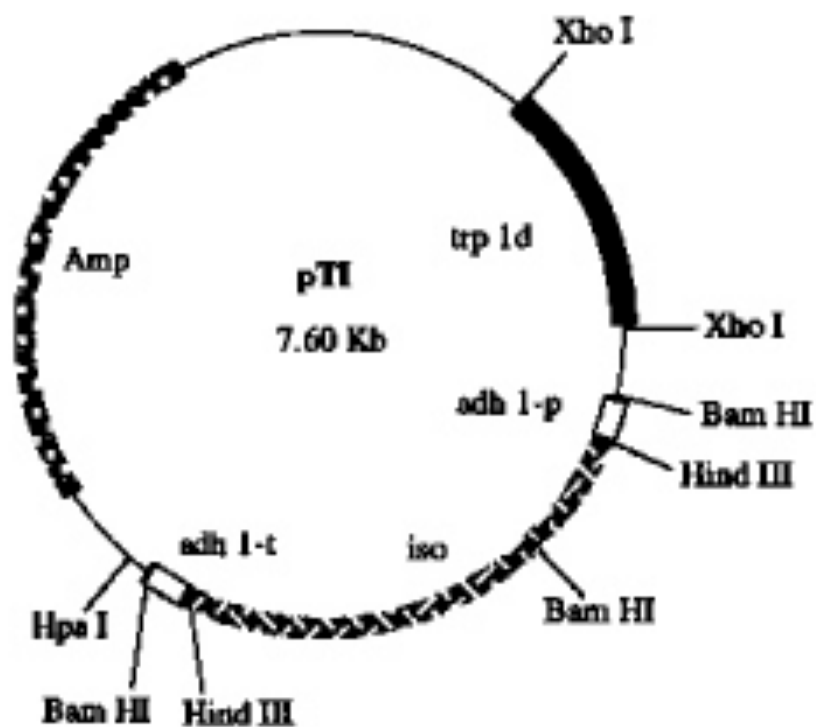


Figure A2 – pti-H1 plasmid map.

1.3. pTZ57R/T

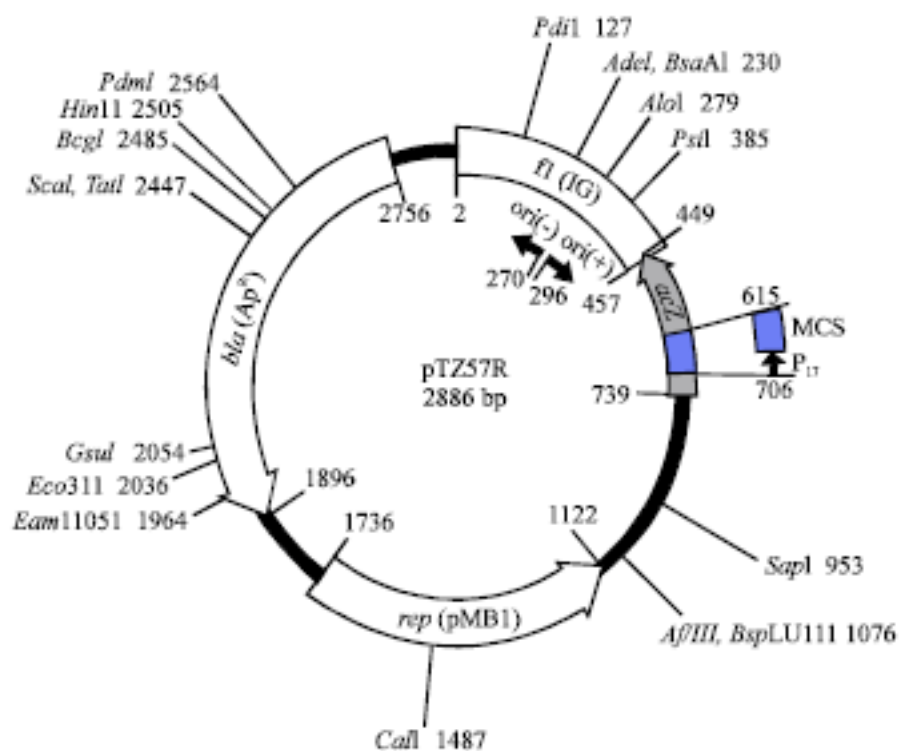


Figure A3 - pTZ57R/T plasmid map.

1.4. LB, LB-Amp and LB-Amp/Xgal/IPTG plates

1.4.1. 20 mg/mL Ampicillin

Dissolve 0.02% (w/v) ampicillin dH₂O and store in 1mL aliquots at -20 °C.

1.4.2. 40 mg/mL Xgal

Dissolve 0.4% (w/v) Xgal in DMSO and store in 1 mL aliquots in foil wrapped eppendorf tubes at -20 °C.

1.4.3. 100 mM IPTG

Dissolve 238 mg IPTG in 10 mL dH₂O and store in 1 mL aliquots at -20 °C

1.4.4. LB-plates

Dissolve the following in dH₂O

10% (w/v)	Tryptone
5% (w/v)	Yeast extract
0.086 M	NaCl
15% (w/v)	Agar

Autoclave to sterilize, cool to ~55 °C and pour ~10 mL into a petri dish. Allow agar to set (~20 min at RT) and incubate upside down at 37 °C overnight. Store plates at 4 °C.

1.4.5. LB-Amp plates

Dissolve the following in dH₂O

10% (w/v)	Tryptone
5% (w/v)	Yeast extract
0.086 M	NaCl
15% (w/v)	Agar

Autoclave to sterilise, cool to ~55 °C. Add either 2.5 µL or 5 µL of a 20 mg/mL stock solution of ampicillin to make the final ratio 50 µg/mL or 100 µg/mL, respectively. Pour ~10 mL into a petri dish. Allow agar to set (~20 min at RT) and incubate upside down at 37 °C overnight. Store plates at 4 °C.

1.4.6. LB-Amp/Xgal/IPTG plates

Spread 40 µL of 40 mg/mL Xgal and 40 µL of 100 mM IPTG on pre-warmed (to RT) LB-Amp (50 µg/mL) plates and incubate at 37 °C for 1 h before use.

1.5. LB liquid medium

Combine the following in dH₂O

10% (w/v)	Tryptone
5% (w/v)	Yeast Extract
0.1 M	NaCl

Autoclaved to sterilise and store at 4 °C

1.6. 0.1 M Calcium Chloride (CaCl₂)

Dissolve 1.1 g CaCl₂ in 100 mL dH₂O and store at 4 °C

1.7. 0.1 M CaCl₂ containing 15% glycerol

Combine

1.1 g	CaCl ₂
15 mL	Glycerol

Make up volume to 100 mL with dH₂O and store at 4 °C

2. Determination of total LRP levels

2.1. Lysis buffer

Combine the following in dH₂O:

10 mM	Tris-HCl
100 mM	NaCl
10 mM	EDTA
0.5% (v/v)	Nonident-P40
0.05% (w/v)	Deoxycholate

Store solution at 4 °C

2.2. 5 X Sample loading dye

Combine the following in dH₂O:

10% (w/v)	SDS
10 mM	β-mercapto-ethanol
20% (v/v)	Glycerol
0.2 M	Tris-HCl (pH 6.8) (appendix 2.3.4)
0.05%	Bromophenolblue

Store at 4 °C (-20 °C long term)

2.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

2.3.1. 1 X SDS running buffer

Combine the following in dH₂O:

0.2 M	Tris
0.19 M	Glycine
3 mM	SDS

Store at RT

2.3.2. 40% Acrylamide:Bis-Acrylamide

Combine Acrylamide and Bis-Acrylamide (19:1) in dH₂O.

Heat to dissolve, filter and store in dark bottles at 4 °C

2.3.3. Separation buffer

Combine the following in dH₂O:

1.5 M Tris

pH to 8.8 with HCl

Store at 4 °C

2.3.4. Stacking buffer

Combine the following in dH₂O:

500 mM Tris

pH to 6.8 with HCl

Store at 4 °C

2.3.5. 10% SDS

Dissolve 10% (w/v) SDS in dH₂O and store at RT

2.3.6. 1% APS

Dissolve 1% (w/v) APS in dH₂O and store at 4 °C

2.3.7. 12% Separating and 5% Stacking gel

2.3.7.1. 12% Separating gel

Combine the following in dH₂O:

29.9% (v/v)	40% Acrylamide:Bis-Acrylamide
25% (v/v)	Separation buffer
1% (v/v)	10% SDS
1% (v/v)	1% APS
0.4% (v/v)	TEMED

2.3.7.2. 5% Stacking gel

Combine following in dH₂O:

12.5% (v/v)	40% Acrylamide:Bis-Acrylamide
12.5% (v/v)	Stacking buffer
1% (v/v)	10% SDS
1% (v/v)	1% APS
0.1% (v/v)	TEMED

2.3.7.3. Pouring gels

Pour the 12% separation gel, overlay with ethanol until polymerised. Remove the ethanol, pour the 5% stacking gel and insert the comb.

2.3.8. Transfer Buffer

Combine the following in dH₂O:

20 mM	Tris
150 mM	Glycine
20%	Methanol

Store at 4 °C

2.4. 1 X PBS

Combine the following in dH₂O:

137 mM NaCl

12 mM Phosphate

2.7 mM KCl

pH 7.4

Store at RT

2.5. PBS-Tween

Add 0.001% Tween to 1 X PBS

Store at 4 °C

2.6. 3% BSA in PBS-Tween

Dissolve 3% BSA in PBS-Tween

Store at 4 °C

3. Determination of cell surface LRP/LR levels

3.1. 4% paraformaldehyde

Dissolve 4% paraformaldehyde in 0.1 M PBS.

Heat solution at 65 °C while stirring and add 1N NaOH drop wise until the solution clears. Cool, filter and store solution at 4 °C

4. Transfection of A549 and HeLa cells with pCI-neo-GFP

4.1. 2% paraformaldehyde

Dissolve 2% paraformaldehyde in 0.1 M PBS.

Heat at 65 °C while stirring and add 1N NaOH drop wise until the solution clears.

Cool, filter and store solution at 4 °C

4.2. 0.25% Triton X-100 containing 0.5% BSA in PBS

Dissolve the following in 1 X PBS

0.25% Triton X-100

0.5% BSA

Store at 4 °C

5. Production of shRNA

5.1. shRNA design

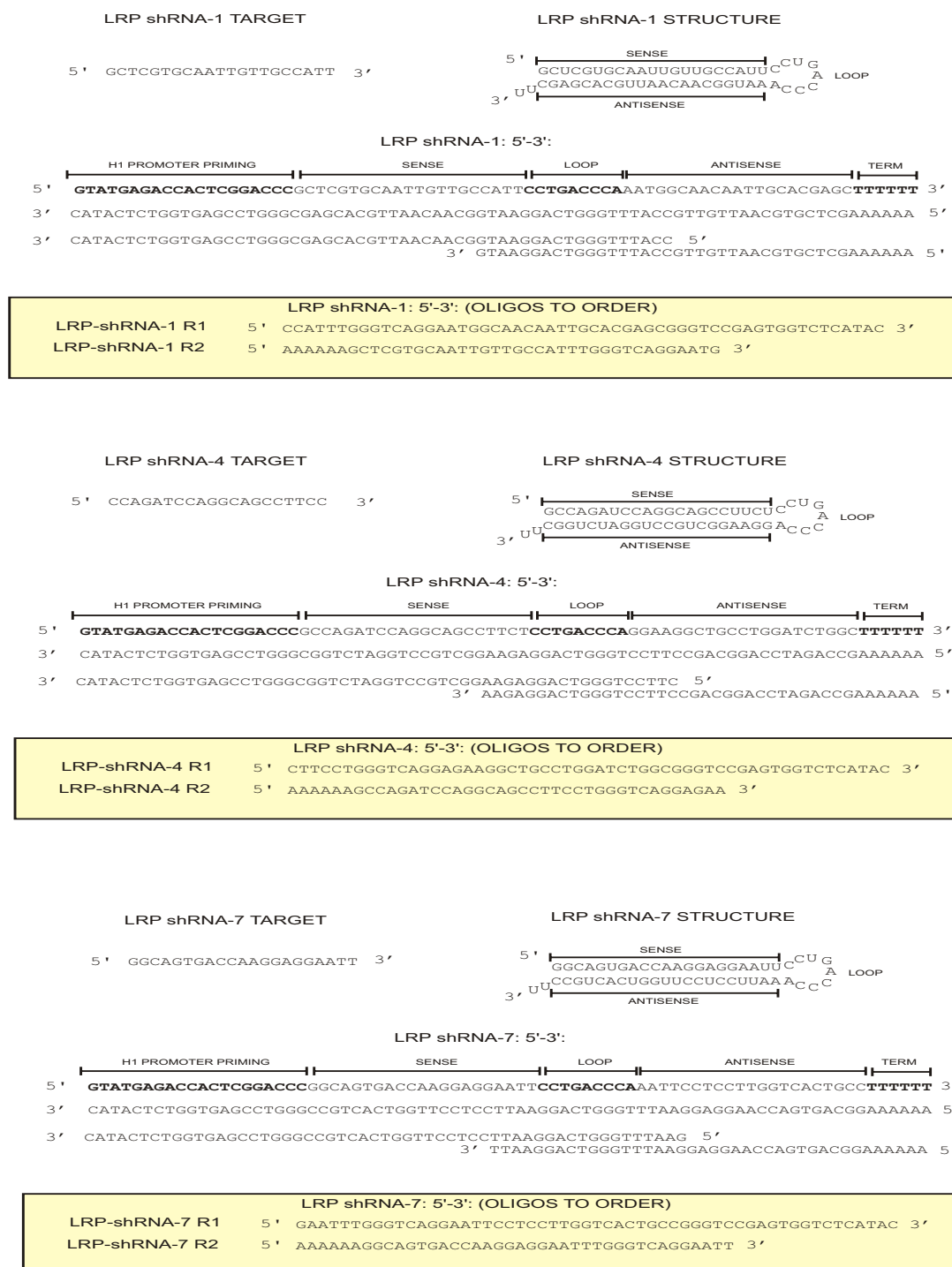


Figure A4 – Design of shRNA-1, 4 and 7 oligonucleotides. The design and target sequences of shRNA-1, 4 and 7.

5.2. PCR amplification of oligonucleotides

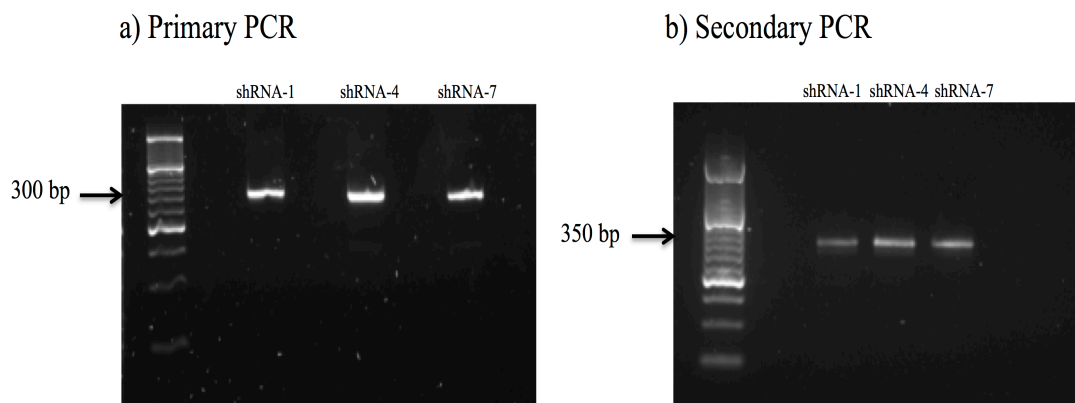


Figure A5 – Nested PCR amplification of oligonucleotides. a) Primary and b) secondary PCR products resolved on 1% agarose gels displaying 300 and 350 bp fragments, respectively.

5.3. Transformation of pTZ57R/T shRNA-1, 4 and 7 plasmids and blue/white selection

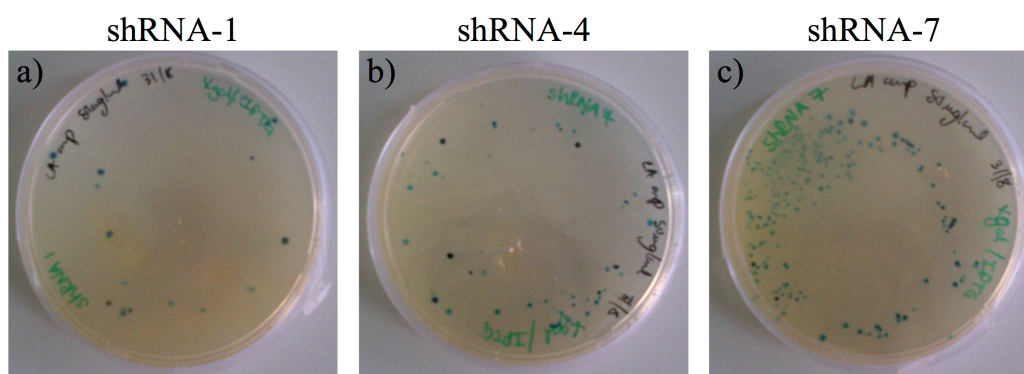


Figure A6 – Transformation of pTZ57R/T shRNA-1, 4 and 7 into *E. coli* XL1-Blue. a) shRNA-1, b) shRNA-4 and c) shRNA-7 transformed into *E. coli* XL-Blue and plated on LB-amp, Xgal, and IPTG plates, allowing for blue/white selection.

5.4. 1 X TBE

Combine the following in dH₂O:

0.09 M Tris

0.09 M Boric Acid

0.003 M EDTA

Store at RT

5.5. 1% Agarose gel

Dissolve 0.5 g agarose in 50 mL 1 X TBE buffer.

Heat until the agarose has completely dissolved.

Add GelStar™ (Lonza) in a 1:10 000 ratio, pour into gel mould, insert comb and allow to solidify.

6. Examination of cellular viability

6.1. Transfection per well (96 well plate format)

0.6×10^4 A549 and 3×10^3 HeLa cells were seeded in the wells of a 96 well plate. 24 h later, 150 μ mol (for A549 cells) or 200 μ mol (for HeLa cells) of siRNA-LAMR1 and 0.2 μ L of DharmaFECT® 1 transfection reagent were each concurrently diluted in DMEM to a final volume of 10 μ L, the contents of each tube was gently mixed and allowed to incubate for 5 min at RT. The diluted siRNA-LAMR1 solution was then added to the diluted DharmaFECT® 1 transfection reagent, mixed gently and incubated for 20 min at RT. The culture medium was removed from the plated cells (either A549 or HeLa) and 80 μ L DMEM containing 10% (v/v) FCS added to the siRNA-reagent solution which the cells were then grown in for 72 h in a humidified incubator at 37 °C with 95% air and 5% CO₂.

6.2. 8 mM PCA

Dissolve 8 mM PCA in DMEM containing 10% (v/v) FCS and store at 4 °C

6.3. 1 mg/mL MTT

Dissolve 1 mg of MTT in 1 mL PBS and store in a dark bottle at 4 °C.

7. Assessment of cellular proliferation

7.1. 100 µg/mL Doxycycline

Dissolve 100 µg of Doxycycline in 1 mL DMEM containing 10% (v/v) FCS and store at 4 °C