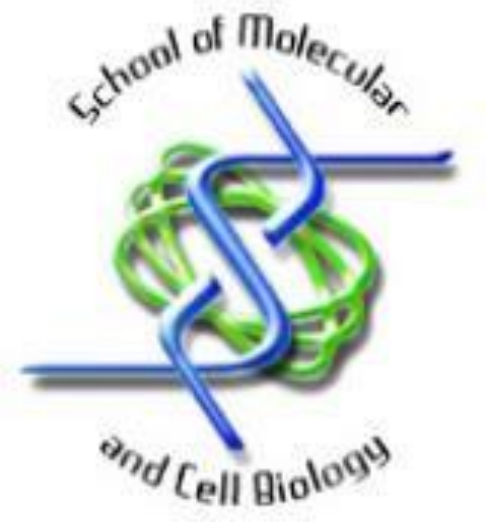

Assessing the role of LRP/LR on the viability of pancreatic cancer and neuroblastoma cells through siRNA-mediated LRP/LR down-regulation

Dissertation by: Carryn Jude Chetty

Person number: 363350

Submitted to: The Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science, 2015

DECLARATION

I, Carryn Jude Chetty (363350), am a student registered for the degree of Master of Science in the academic year 2015.

I hereby declare the following:

- I am aware that plagiarism is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where explicitly indicated otherwise and acknowledged.
- I have not submitted this work before for any other degree or examination at this or any other university.
- The information used in the dissertation has not been obtained by me while employed by, or working under the aegis of, any person or organisation other than the university.
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Signature:

Date:

5/11/2015

Dedicated to Braxter (28.03.2002 – 16.08.2012) and Furby (05.02.2005 – 16.06.2014).

Gone but never forgotten.

ABSTRACT

Over the decades, cancer has become a global burden with alarmingly high incidence and mortality rates in both economically developed and developing countries. Characteristically, tumour cells exhibit an over-expression of the 37kDa/67kDa laminin receptor (LRP/LR) in comparison to their normal cell counterparts, with this receptor being implicated in several tumourigenic processes – importantly for the present study, the maintenance of cellular viability and the evasion of apoptosis. This present study aimed to elucidate the role of LRP/LR on the cellular viability of pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells. Flow cytometry revealed that both of these tumourigenic cell lines exhibited LRP/LR on their surface, with further analysis using median fluorescence intensity values showing that IMR-32 cells contain about 70% more cell-surface LRP/LR than AsPC-1 cells. Additionally, Western blotting and densitometry suggested that IMR-32 cells contained about 63% more total LRP/LR than AsPC-1 cells. Western blot analysis also revealed that targeting the mRNA of the 37kDa LRP using a LRP-specific siRNA (siRNA-LAMR1) in AsPC-1 and IMR-32 cells led to significant down-regulation of 90% and 71% in LRP expression, respectively. Consequently, MTT assays showed that LRP knockdown led to reductions of 82% and 65% in the viability of AsPC-1 and IMR-32 cells, respectively. Moreover, use of an alternative LRP-specific siRNA (esiRNA-RPSA) confirmed the specificity and excluded an off-target effect of siRNA-LAMR1 for LRP. BrdU assays revealed that knockdown of LRP reduced the proliferation of AsPC-1 and IMR-32 cells by 76% and 44%, respectively. Confocal microscopy indicated nuclear morphological changes suggestive of apoptosis as the form of cell death occuring in both cell lines after LRP down-regulation. This observation was confirmed using Annexin-V assays, which revealed that AsPC-1 cells underwent 44% more apoptosis than IMR-32 cells post LRP knockdown. Furthermore, caspase-3 activity assays revealed that both cell lines experienced apoptotic induction after siRNA-mediated down-regulation of LRP. Caspase-8 and -9 activity assays suggested that post LRP knockdown, IMR-32 cells undergo apoptosis solely via the extrinsic pathway, whilst AsPC-1 cells use both the intrinsic and extrinsic apoptotic pathways, possibly through a retaliatory feedback loop. Overall, LRP/LR is critical for the maintenance of the tumour cellular viability, making the receptor a promising therapeutic target and proposing the potential use of siRNA technology for treatment of pancreatic cancer and neuroblastoma.

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"For I know the plans I have for you, declares the Lord. Plans to prosper you and not to harm you. Plans to give you hope and a future."– Jeremiah 29:11

RESEARCH OUTPUTS

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- Chetty, C.J., Khumalo, T., Da Costa Dias, B., Reusch, U., Knackmuss, S., Little, M. and Weiss, S.F.T. (2014) Anti-LRP/LR specific antibody IgG1-iS18 impedes adhesion and invasion of liver cancer cells. *PLoS ONE.* **9**(5): e96268. doi:10.1371/journal.pone.0096268

Review articles (Joint first author, not forming part of MSc dissertation):

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Conference outputs:

Poster presentations (forming part of MSc by dissertation):

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Other poster presentations:

- Chetty, C.J., Khumalo, T., Da Costa Dias, B., Reusch, U., Knackmuss, S., Little, M. and Weiss, S.F.T. (2014) The role of the 37kDa/67kDa laminin receptor in the metastasis of leukaemia and liver cancer cells. *South African Society of Biochemistry and Molecular Biology (SASBMB).* 6-9 July 2014

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α	Alpha
β	Beta
γ	Gamma
µl	Microlitre
ml	Millilitre
µg	Microgram
mg	Milligram
M	Molar
mM	Micromolar
min	Minutes
nm	Nanometre
ºC	Degrees Celcius
A	Amps
V	Volts
%	Percentage

LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
AIF	Apoptosis-inducing factor
Apaf-1	Apoptotic protease activating factor-1
APC	Allophycocyanin
APS	Ammonium persulfate
ATCC	American type culture collection
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
CAT	Chloramphenicol acetyltransferase
CDK	Cyclin-dependent kinase
CO_2	Carbon dioxide
DD	Death domain
DED	Death effector domain
DISC	Death induced signalling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EMEM	Eagle Minimum Essential Medium
FACS	Fluorescene Activated Cell Scanning
FADD	Fas-associated death domain

LIST OF ABBREVIATIONS (cont.)

FAK	Focal adhesion kinase
FISH	Fluorescent *in situ* hybridization
FITC	Fluorescein isothiocyanate
FCS	Fetal calf serum
HRP	Horseradish peroxidase
IAP	Inhibitor of apoptosis
IgG	Immunoglobulin G
kDa	Kilodaltons
LRP/LR	Laminin receptor precursor/ laminin receptor
MAPK	Mitogen-activated protein kinase
MFI	Median fluorescence intensity
mIBG	Meta-iodobenzylguanidine
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PAGE	Polyacrylamide gel electrophoresis
PE	Phycoerythrin
PI3K	Phosphoinositide 3-kinase
PBS	Phosphate buffered saline
PCA	Protocatechuic acid
PrP	Prion protein
PS	Phosphatidyl serine
PVDF	Polyvinylidene fluoride
RB	Retinoblastoma
RISC	RNA-induced silencing complex
Rpm	Revolutions per minute

LIST OF ABBREVIATIONS (cont.)

RLUC	Renilla luciferase
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RPSA	Ribosomal protein SA
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA
TEMED	Tetramethylethylenediamine
TMB	Tetramethyl benzidene
TNFR	Tumour necrosis factor receptor
VEGF	Vascular endothelial growth factor
7-AAD	7-amino-actinomycin D

CHAPTER 1: INTRODUCTION

1.1 Cancer – A worldwide burden

1.1.1 Epidemiology, diagnosis and treatment

Over the years, cancer incidence has increased rapidly throughout the world, especially in economically developed countries where it has been implicated as the leading cause of death[1]. Similarly, economically developing countries are also burdened with the disease, where it was identified to be the second leading cause of death[1]. It is understood that majority of cancer types portray a non-discriminative nature and therefore may manifest in people of any gender, race, age and economic status[2].

According to the World Cancer Research Fund (WCRF), 14.1 million incidences of cancer were reported worldwide in the year 2012 alone and based on this pattern of occurrence, it was estimated that 24 million new diagnoses will be encountered by the year 2035. Additionally, the WCRF has identified the 20 most commonly diagnosed cancer types globally for the year 2012 (Fig.1). The present study focused on two particular cancer types – pancreatic cancer and neuroblastoma – ranked twelfth and seventeenth most commonly diagnosed cancer types in the year 2012 contributing to 2.4% and 1.7% of the total number of cases diagnosed, respectively (http://www.wcrf.org/cancer_statistics/). These alarming statistics indicate the urgency and importance of developing novel therapeutic strategies to combat cancer.

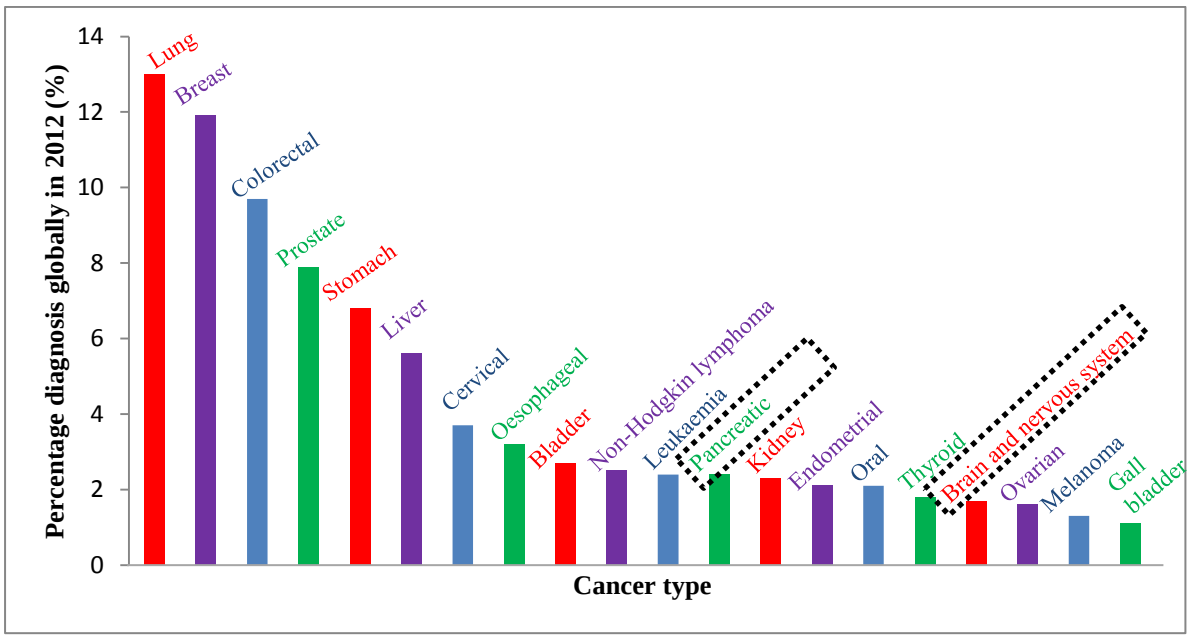

Figure 1: Percentage diagnosis of the 20 most common cancer types globally in the year 2012 (Adopted from: http://www.wcrf.org/cancer_statistics/).

Owing to the fact that several cancer types are lifestyle-related, deaths occurring thereof may be preventable[3]. It is also pivotal that diagnosis occurs early enough to allow cancer cells to be rapidly eradicated before metastasis and the formation of secondary, tertiary and quaternary tumours occurs – where treatment becomes more difficult and significantly less effective[3].

To date, some of the most common means of cancer diagnosis entail the use of biopsies, genetic testing, fluorescent *in situ* hybridization (FISH), X-rays, ultrasound and magnetic resonance imaging (MRI) scans[4]. Treatment usually involves chemotherapy, radiation therapy, organ transplants or surgery and oftentimes, a combination of these therapeutic strategies are used for effective treatment[5]. Additionally, deeper insight into the characteristic behaviour and functioning of cancerous cells enabled the development of more advanced therapeutics such as gene therapy, viral therapy and immunotherapy[4] (Fig.2). It is also suggested that certain vaccines may prove to be efficient as therapeutic agents for cancer and there is scope for the development of novel vaccines to target different cancer types once more knowledge is gained with regards to molecular mechanisms[4].

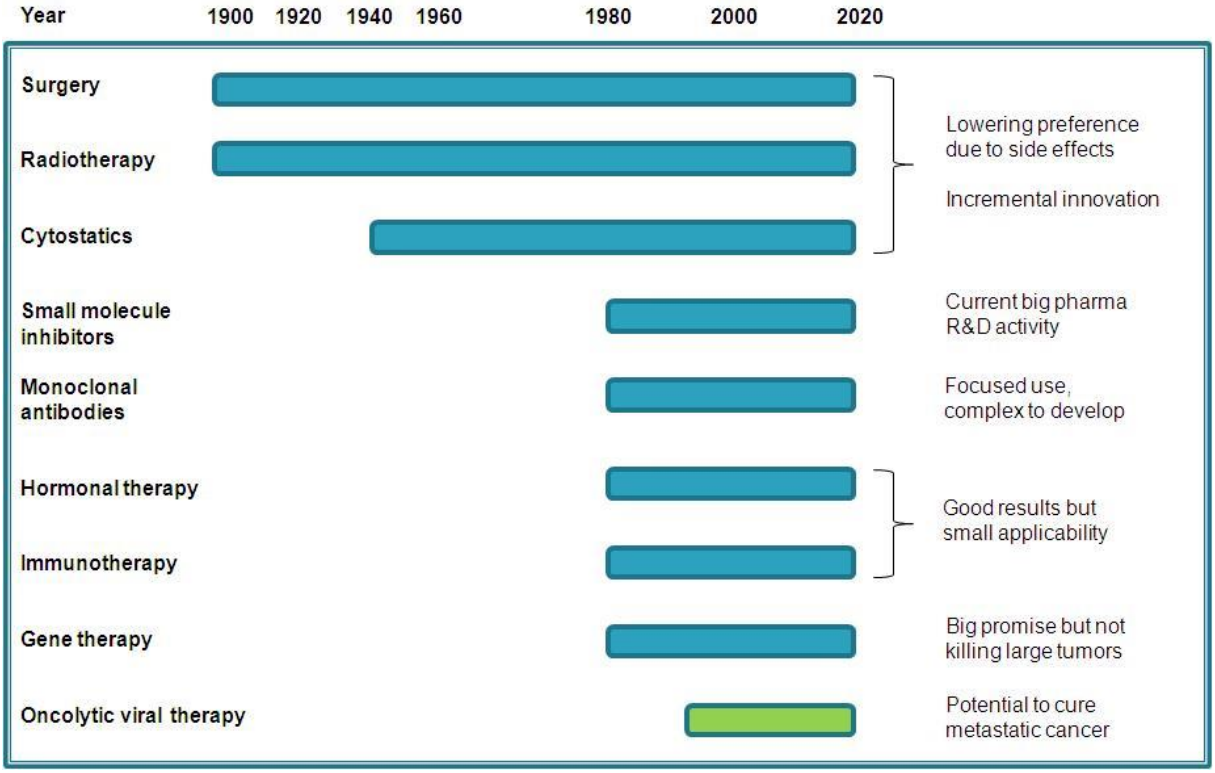

Figure 2: Commonly-used cancer therapies over the past 100 years (http://oncolyticvirus.wordpress.com/2010/03/04/100-years-of-cancer-therapies-and-oncolytic-viruses).

1.1.2 Pancreatic cancer

1.1.2.1 Characterization

Pancreatic cancer is ranked amongst the most aggressive cancer types, being the seventh highest cause of cancer deaths worldwide, contributing to 6% of total deaths due to cancer annually (www.cancerresearchuk.org). It is initiated when cells in the pancreas proliferate and multiply uncontrollably, thereby forming a tumour[6]. This cancer type is metastatic, meaning that it has the ability to invade secondary organs throughout the body[7]. Of the several types of pancreatic cancer that exist, pancreatic adenocarcinoma (originating in the region of the pancreas that is responsible for the production of digestive enzymes) is the most common – resulting in about 85% of pancreatic cancer cases[8]. Less aggressive in comparison to pancreatic adenocarcinoma are neuroendocrine tumours, which originate in the region of the pancreas that produces hormones and are responsible for 1-2 in every 100 pancreatic cancer cases[9].

1.1.2.2 Signs, symptoms and risk factors

Symptoms of pancreatic adenocarcinoma are rare in the early stages of the disease, and symptoms that allow the disease to be clearly diagnosed only develop in the advanced stages[8]. Such symptoms include loss of appetite[10], uncontrolled weight loss[10], darkly-coloured urine[11], abdominal cramps or pain[12], lightly-coloured stools[11], back pains and Jaundice[12]. One of the major contributing factors to the aggressiveness of pancreatic cancer is the difficulty in detection of the disease due to the late occurrence of symptoms, thereby allowing the disease to metastasize to other organs[13]. One of the main risk factors for pancreatic cancer is age, where the disease is rare before the age of 40 and majority of cases are found to be in people exceeding the age of 70[14]. Other risk factors include smoking[15], some genetic ailments[16], diabetes[14] and obesity[10], with the consumption of certain food types such as processed and red meats posing a minor increase in risk[17, 18].

1.1.2.3 Staging

Staging of pancreatic cancer usually occurs after a CT scan is performed[19]. The most commonly used system of staging is the AJCC-UICC (American Joint Committee on Cancer – Union for International Cancer Control) system[20]. This system classifies four main stages of pancreatic cancer, namely stages T1,T2,T3 and T4 (Fig.3) – based on the size of the tumour and metastasis to the lymph nodes and other secondary organs[20]. It is understood

that at stages T1 and T2, the tumour is considered to be resectable, meaning it can be surgically removed[20]. At stages T3 and T4, the tumour is either borderline resectable or unresectable depending on the degree of metastasis that has occurred[20].

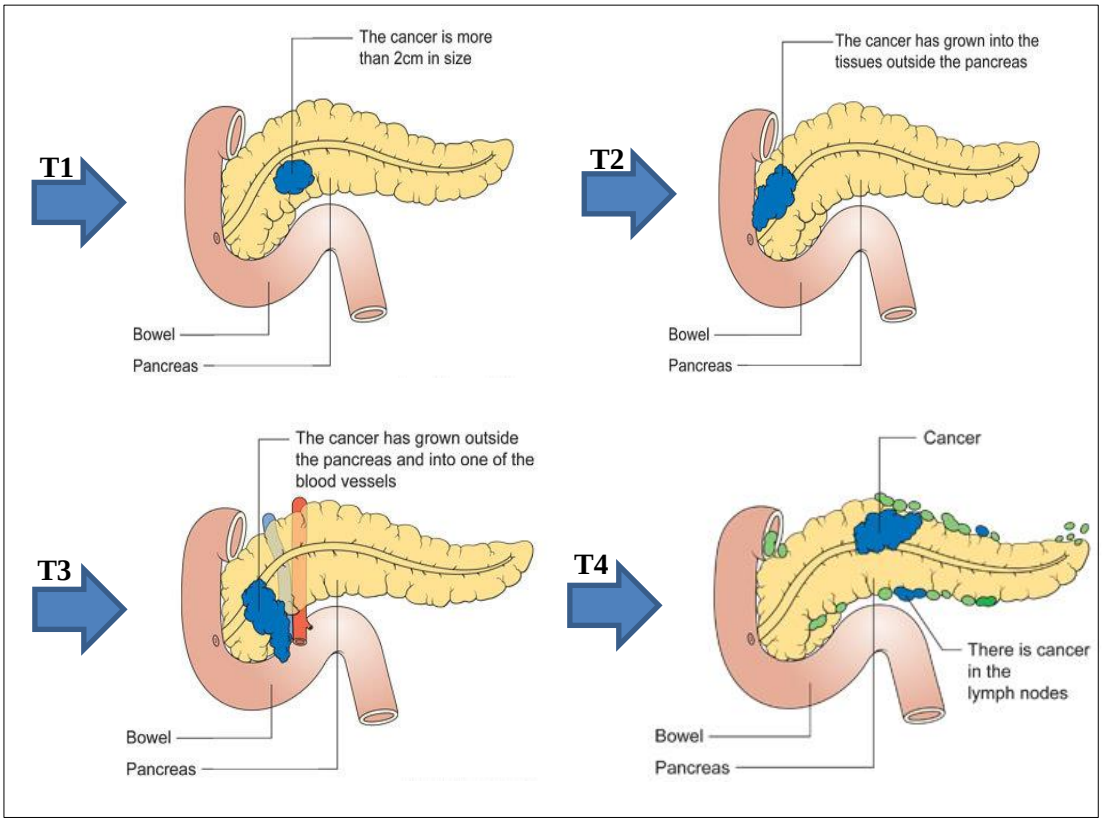

Figure 3: The stages of pancreatic cancer. At stages T1 and T2, the tumour is mainly localized in the tissues of the pancreas and the tumour is considered to be resectable by surgery. At stages T3 and T4, metastasis and distant metastasis occurs and renders the tumour unresectable – particularly when the cancer has spread to the lymph nodes (Adopted from: http://www.cancerresearchuk.org/about-cancer/type/pancreatic-cancer/treatment/the-stages-of-pancreatic-cancer).

1.1.2.4 Treatment and prevention

Commonly used treatment strategies for pancreatic cancer include surgery[21], chemotherapy[22], radiation therapy[23] and palliative treatments (which treats the symptoms of the disease and not the disease-causing mechanisms)[24]. Often, a combination of these therapies is used in pancreatic cancer treatment[25]. It is recommended that regular screening should be carried out via the use of MRI imaging and endoscopic ultrasounds in order to allow for early detection and diagnosis of pancreatic cancer[26, 27], since surgery is the only possible cure for this cancer type[21]. Unconfirmed but suggested preventative measures entail abstinence from smoking[28], maintenance of a healthy weight[28] and alterations in dietary consumption – decreasing red and processed meats[17] and increasing whole grains, vegetables and fruits[29].

1.1.3 Neuroblastoma

1.1.3.1 Characterization

Neuroblastoma is widely considered to be the most commonly occurring cancer type during infancy – contributing to 6-10% of all known childhood cancers[30, 31]. Moreover, about 50% of neuroblastoma cases are prevalent in children below the age of two years old, with an annual global mortality rate of 10 per million children within this age group[30, 31].

Little is known about the causative factors for neuroblastoma and most cases are non-familial[32]. This cancer type is characterized as a neuroendocrine tumour, meaning that it may originate in any element of the neural crest within the sympathetic nervous system[33]. The adrenal glands serve as the most common region of origin of neuroblastoma, evident in about 60% of incidences where the disease is widespread and in about 40% of cases where the tumour is localized[34]. Additionally, the abdomen and the chest have also been implicated as regions where neuroblastoma may develop - contributing to about 30% and 19% of all neuroblastoma cases, respectively[34]. The neck and pelvic regions have also been identified as areas in which neuroblastoma may originate, however these regions are much less frequently implicated[34].

1.1.3.2 Signs, symptoms and risk factors

Early and effective diagnosis of neuroblastoma is an ongoing challenge since initial symptoms of the disease are not clearly identifiable and additionally, about 50-60% of neuroblastoma cases exhibit metastases which occurs asymptomatically[34]. When present, some of the more prominent symptoms include pain in the joints, fever, fatigue and loss of appetite[35] – however, the location of the primary tumour and regions of metastasis largely determine the symptoms that are experienced (Fig.4). Several possible risk factors for neuroblastoma development have been investigated but with inconclusive outcomes, parental factors such as smoking, excessive consumption of alcohol, exposure to certain chemicals during pregnancy as well as the use of hormone therapies and medicinal preparations[36, 37, 38].

It is noteworthy that neuroblastoma has been categorized into three main risk groups, namely low-, intermediate- and high-risk[39, 40]. This categorization is based on factors such as age, degree of metastasis, and certain genetic and microscopic features of the tumour cells[39, 40]. Moreover, low-risk neuroblastoma cases are usually effectively treated with surgery, whereas

treatment of high-risk neuroblastoma cases poses increasing difficulty even with the most advanced therapeutic strategies available[39, 40].

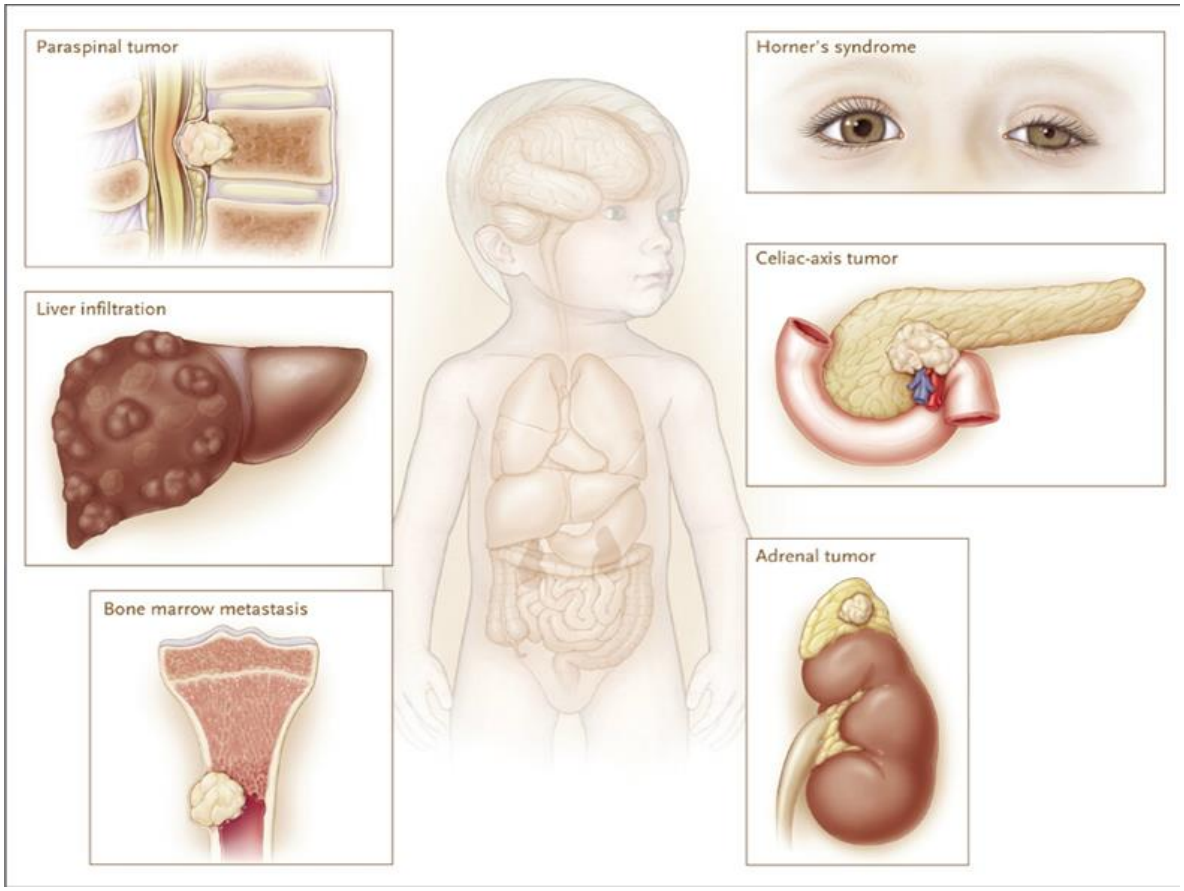

Figure 4: Possible locations of neuroblastoma tumours. The adrenal glands are the most common region in which neuroblastoma tumours originate. Tumours may also develop in the spinal region, causing inability to stand, walk and crawl. Pallor can be experienced as a result of neuroblastoma tumours that infiltrate the bone marrow. Tumours developing in abdominal regions such as the liver and celiac artery may lead to constipation and swollen belly. Swelling, bruising and Horner's syndrome may develop as a result of neuroblastoma tumours originating in the bones around the eyes (http://www.mitchellhuthfund.org.uk/#!neuroblastoma/c21kz).

1.1.3.3 Diagnosis

Among the most common neuroblastoma diagnostic tools is the measurement of catecholamine levels in urine, since about 90% of neuroblastoma cases present with increased catecholamine levels[41]. Other diagnostic methods include the use of microscopic techniques, as well as scans – in particular, a mIBG (meta-iodobenzylguanidine) scan[33, 42]. In essence, mIBG is an analogue of norepinephrine and can therefore be incorporated into neurons of the sympathetic nervous system in place of this neurotransmitter[42]. By use of radio-ionization of mIBG, about 90-95% of neuroblastoma tumours can be detected [42].

1.1.3.4 Staging

Following detection, neuroblastoma is staged according to its location in the body using the International Neuroblastoma Staging System (INSS)[43, 44], described in figure 5 below.

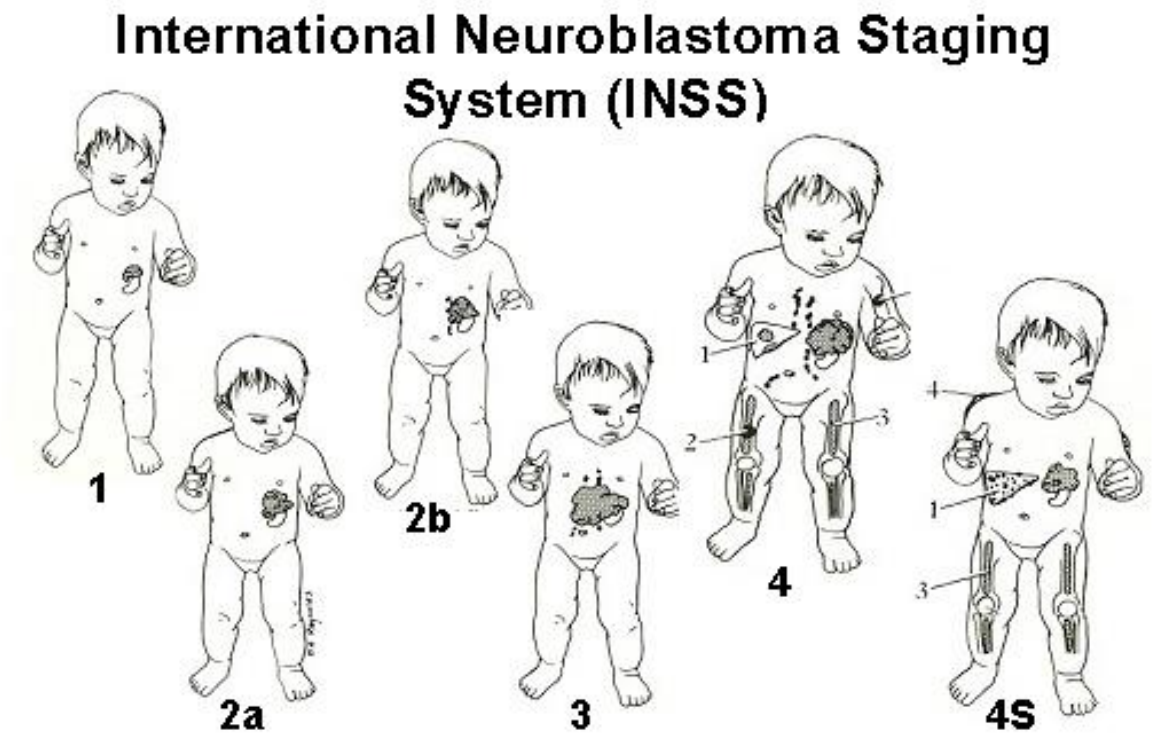

Figure 5: The stages of neuroblastoma. In stage 1, the tumour is localized in the area of origin. In stages 2a and 2b, the tumour starts to spread unilaterally to the ipsilateral and contralateral lymph nodes. Stage 3 involves infiltration of the tumour across the midline with or without involvement of regional lymph nodes, whilst stage 4 entails dissemination to distant lymph nodes and regions such as the liver, bone and bone marrow, amongst others. Stage 4S neuroblastoma occurs only in patients below the age of one year old, with dissemination solely limited to the liver, skin or bone marrow (http://www.nant.org/Patients_and_Families/neuroblastoma.php).

1.1.3.5 Treatment

Early-stage neuroblastoma tumours are often easily and effectively eradicated due to the tumour being localized and are therefore curable by surgery alone. In contrast, advanced-stage neuroblastoma tumours occurring in children older than 18 months old are increasingly difficult to treat even with aggressive therapeutic strategies. Treatment is usually administered dependent on the risk category, with low-risk neuroblastoma tumours often being observed without any treatment or cured solely with surgery – with a success rate of about 90%[44]. Intermediate-risk neuroblastoma is often treated with a combination of chemotherapy and surgery, and this mode of treatment yields a cure rate of about 70-90%[45]. High-risk neuroblastoma is treated with intensive multimodal therapies – involving

surgery[46], radiation and chemotherapy[46], hematopoietic stem cell[32, 47] and bone marrow transplants[47], differentiation agents and immunotherapy[48] – with a cure rate of only about 30%. Gaining insight into causes and prevention of neuroblastoma remains challenging.

1.2 Cancer – A cellular understanding

1.2.1 Overview of cancer cells

Cancer can be defined as abnormal cellular growth resulting from the uncontrolled proliferation of cells[49] and diminished levels of apoptosis or programmed cell death[50]. These events may occur as a result of the accumulation of genetic mutations or they may be due to the action of several external factors such as tobacco smoking and exposure to radiation, amongst others[51]. Essentially, tumourigenesis refers to the process of transformation of a normal cell to a cancerous state, facilitated by cellular alterations such as epigenetic and unstable genetic modifications[52]. Critical contributing factors to the process of tumourigenic transformation are cellular alterations, collectively termed "the hallmarks of cancer"[53] (Fig.6).

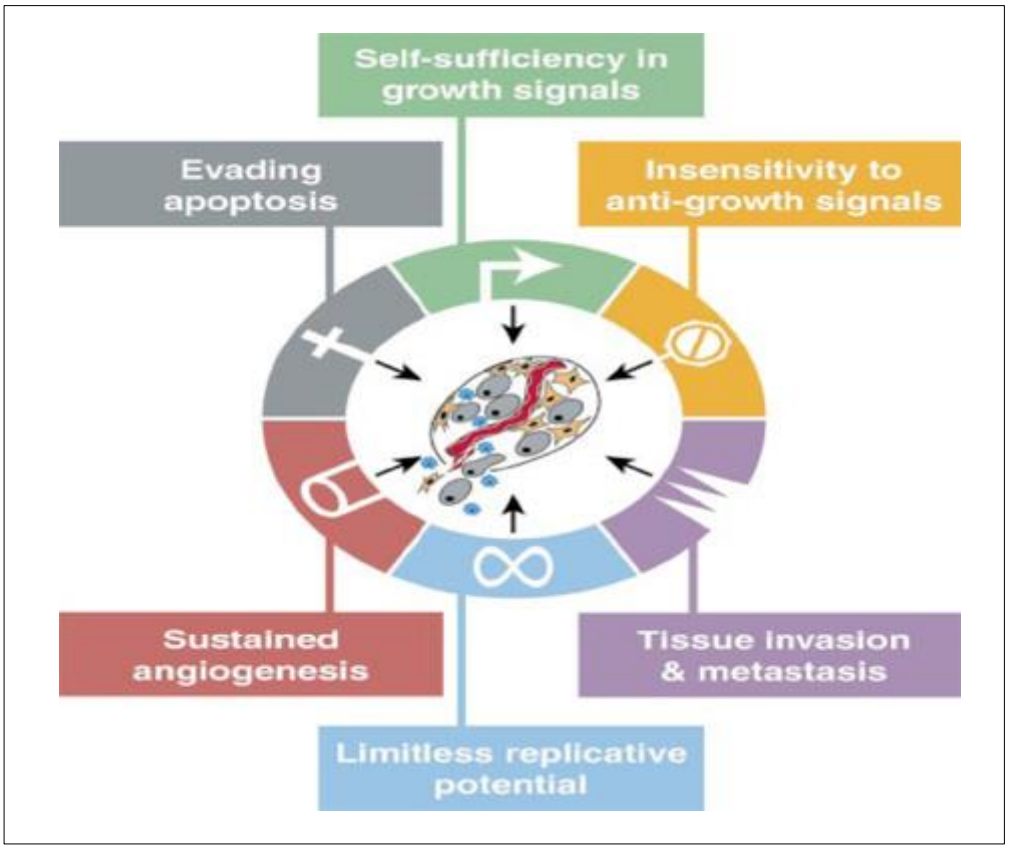

Figure 6: The hallmarks of cancer. These six alterations are essential for the transformation of a cell from a normal state to a tumourigenic state[53].

1.2.2 The hallmarks of cancer

The transformation of a normal cell to a neoplastic state is a highly complex and specialized process, requiring the acquisition of six essential traits (Fig.6) – elaborated on, below.

1.2.2.1 Self-sufficiency in growth signals

Often considered to be the most essential capability acquired by tumour cells is the ability of these cells to sustain proliferative signalling[53]. In contrast to cancerous cells, normal cells are able to maintain a state of homeostasis with regards to cell structure, function and number. They do this by regulation of the production and release of signals that stimulate growth of the cells and additionally, these signals direct entry of cells into the cell cycle as well as the progression of these cells through the cell cycle[53].

Tumour cells, on the other hand, have the ability to facilitate deregulation of these growth signals thus compromising the homeostatic balance[53]. Both autocrine and paracrine signalling mechanisms have been implicated in growth-signal deregulation and the result of this is uncontrolled, continuous cell proliferation[54, 55]. It is understood that proliferation of tumour cells may be sustained due to their potential for growth in the absence of growth factors[54, 55]. These neoplastic cells have gained the ability to independently produce their own growth factors and alternatively, they can also transmit signals that stimulate normal cells within the tumour vicinity to supply them with an array of growth factors[54, 55]. Moreover, somatic mutations in proteins such as B-Raf and PI3-kinase may result in the disruption of proper signalling via the MAP-kinase[56] and PI3K[57, 58] signalling pathways, respectively – thereby promoting uncontrolled and continued tumour cell proliferation.

1.2.2.2 Insensitivity to anti-growth signals

Apart from the sustenance of growth-promoting signals, tumour cells also need to evade growth suppression signals in order to continuously grow and proliferate[53]. Critical to the processes that govern growth suppression is the action of tumour suppressor genes[53]. Although several tumour suppressors have been found to play various roles in tumour inhibition, two key tumour suppressor genes – namely retinoblastoma (RB) and p53 – have been identified as critical in the control and governance of cell fate[53].

The essential function of the RB protein is to govern whether or not a cell qualifies to proceed through the cell growth and cell division cycles by integration of and response to intracellular and extracellular signals[59, 60]. Defective RB proteins therefore allow cancer

cells to proliferate uncontrollably due to a lack of proper regulatory functions provided by the RB pathway[60, 61]. On the other hand, p53 functions to halt tumour progression upon receipt of intracellular abnormality and stress signals[62]. Essentially, p53 is a pro-apoptotic member which responds to excessive damage to the genome and unfavourable cellular conditions by induction of apoptosis[62]. Tumour cells have the ability to evade the action of p53, thereby avoiding apoptosis and allowing sustained proliferation[62].

1.2.2.3 Tissue invasion and metastasis

When a cell undergoes transformation from a normal to a neoplastic state, it acquires the potential to alter the expression of certain molecules[53]. Particularly, cell-cell adhesion molecules (CAMs) such as E-cadherin have been found to be selectively down-regulated in tumour cells[63]. This is because E-cadherin facilitates the formation of sheets of epithelial cells and is able to keep the cells within these sheets in a quiescent state, thus down-regulating the expression of this molecule avails cells to undergo tumourigenic transformation[63]. Conversely, specific proteases such as collagenases are selectively up-regulated in tumour cells[64]. This up-regulation promotes the degradation of the extracellular matrix (ECM), allowing cancerous cells to migrate to and invade secondary organs via basal lamina degradation[64].

1.2.2.4 Limitless replicative potential

It is widely understood that normal cells are only able to undergo a limited number of growth and division cycles. In contrast, tumourigenic cells gain the ability to go through limitless cycles of growth and division – a phenomenon known as immortalization[53]. Extensive evidence suggests a key role for telomeres in the facilitation of immortalization[65]. Telomeres comprise several tandem repeats and they are responsible for protecting the ends of chromosomes from DNA damage[65]. Telomerase is a DNA polymerase enzyme that is responsible for the addition of these telomere repeats to the ends of chromosomes, thereby preventing DNA damage from occurring[65, 66]. Moreover, the length of telomeric DNA governs the number of replicative cycles a cell can go through before degradation occurs[65]. Therefore, cancerous cells exhibit an up-regulation of telomerase expression in comparison to normal cells, thus preventing telomere shortening and allowing cells to continually proliferate by avoiding senescence and apoptosis[65, 66].

1.2.2.5 Sustained angiogenesis

Similar to normal cells, neoplastic cells require a sustained source of oxygen, nutrients and blood in order for their growth and survival[53, 67]. Additionally, tumour cells also require a means of removal of carbon dioxide and metabolic wastes[53, 67]. These requirements are achieved in tumour cells via the deregulated expression of an angiogenic inducer known as vascular endothelial growth factor (VEGF)[68]. VEGF assists with the initiation and promotion of blood vessel formation, thus creating the vasculature required for the tumour to acquire nutrients and oxygen and also for waste disposal purposes[69].

1.2.2.6 Evading apoptosis

Apoptosis is a mechanism used to induce cell death in response to cellular stress and damage to DNA, thereby maintaining the homeostatic balance within the cellular environment[70]. However, tumour cells are able to evade the process of apoptosis and in this way, the tumour is able to proliferate and progress[70]. As previously described, the loss of or the deregulated expression of the p53 tumour suppressor enables cancerous cells to avoid undergoing the process of apoptosis and proliferate uncontrollably to form aggressive tumours[62]. Additionally, tumourigenic cells up-regulate the expression of certain anti-apoptotic factors and down-regulate the expression of pro-apoptotic factors in order to evade apoptosis[71,72]. Essentially, the evasion of apoptosis has been implicated as a major contributing factor in the aggressiveness of an array of cancer types[53], thus it is essential to target the mechanisms that govern this hallmark with regards to cancer therapeutics – this being central to the present study.

1.2.3 Apoptosis

This form of cell death was first described in the early 1800's by Carl Vogt, however the term 'apoptosis' came into effect only in the 1970's – named by John Kerr[73]. Apoptosis is now understood to be a process of well-ordered, physiological programmed cell death that serves essential functions in embryonic development and the maintenance of tissue homeostasis[50]. In contrast to apoptosis is a form of catastrophic, non-programmed cell death known as necrosis[50]. Apoptosis is a highly-regulated and important process from infancy throughout adulthood, but deregulation of this process often occurs and may lead to detrimental conditions such as autoimmune diseases[74], AIDS[75], neurodegenerative disorders[76], and cancer[50] – the latter being the focus of the present study.

Apoptotic induction can be triggered by external stimuli such as oxidative stress, and also by internal stimuli such as DNA damage[50]. This induction may result in two types of cellular changes: 1) changes in cellular morphology and 2) biochemical alterations[77] (Fig.7). Morphological changes occurring during early apoptosis include DNA fragmentation and condensation of chromatin, whilst late apoptosis exhibits changes such as membrane blebbing, diminished membrane integrity and alterations in the structure of cytoplasmic organelles[77]. Biochemical alterations to the apoptotic cell include activation of caspases, breakdown of DNA and protein, and membrane changes leading to phagocytosis[78]. Loss of membrane integrity as the cell progresses from an early to a late apoptotic phase causes cellular contents to be packed into apoptotic bodies, thereby enabling phagocytosis of dead cells by macrophages without harming other cells[78].

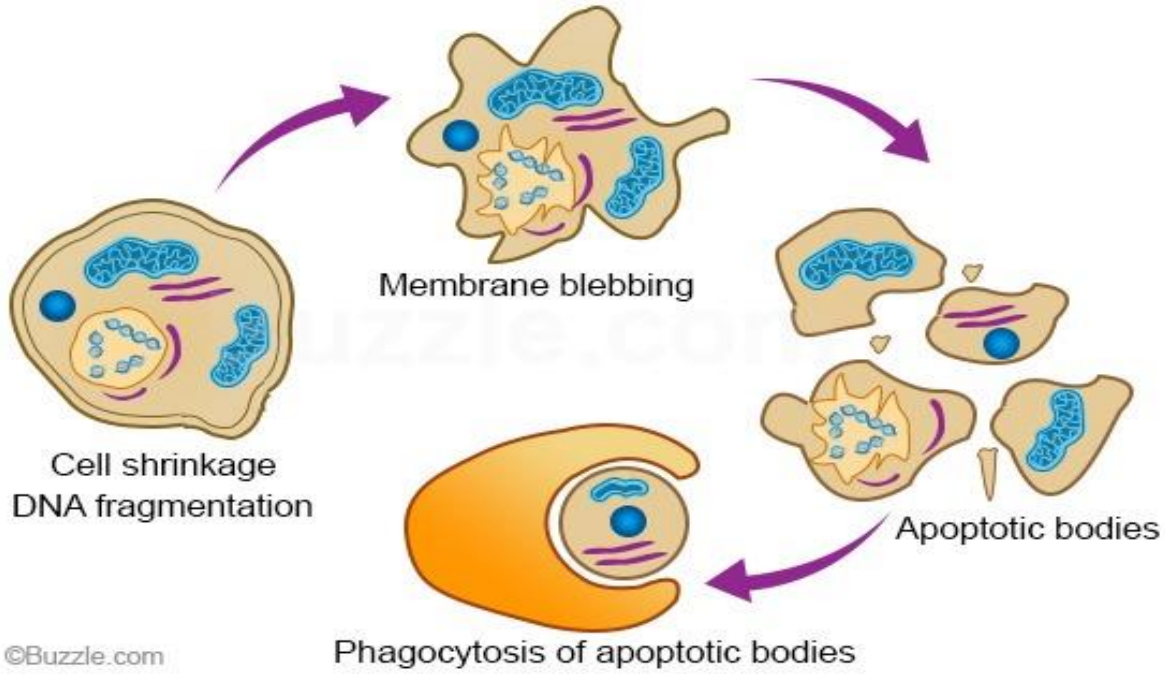

Figure 7: Morphological and biochemical alterations in cells undergoing apoptosis. Upon initiation of apoptosis, cell shrinkage and subsequent DNA fragmentation occurs. This is followed by membrane blebbing, which occurs at later stages of apoptosis and triggers exposure of phosphatidylserine (PS) on the outer cell membrane via a "flipping-out" process. This enables the recognition of dead cells by macrophages, allowing phagocytosis of the resultant apoptotic bodies (http://www.buzzlw.com/articles/how-do-cells-commit-suicide.html).

The process of apoptosis is largely dependent on caspases, which are proteases that specifically function as initiators as well as executioners of apoptosis[79]. This family of proteases is abundant, but pivotal to the mechanism of apoptosis are caspases 8, 9 and 10, serving as initiator caspases, and also caspases 3, 6 and 7, functioning as effector caspases[79]. When synthesized, caspases are inactive and may be activated either by a process of

proteolysis or by transactivation as a result of association with other caspases[79]. Two main pathways that lead to the activation of caspases have been identified, namely the intrinsic (mitochondrial) pathway and the extrinsic (death receptor) pathway[50] (Fig.8). Both of these pathways are elaborated on, below.

1.2.3.1 Intrinsic (mitochondrial-dependent) pathway

The mitochondrial-dependent pathway (Fig.8) can be activated by both internal and external death signals[80]. Internal stimuli originate inside the cell and include DNA damage, hypoxia, chemotherapeutic drugs, oxidative stress and high cytosolic concentrations of calcium ions[80]. External stimuli that can trigger this pathway mainly comprise of cell death signals from the death receptor pathway[80]. Regardless of the stimulus, this pathway is permitted to take place due to mitochondrial membranes exhibiting an increase in permeability[81]. Another major contributing factor to the occurrence of the intrinsic pathway is the release of pro-apoptotic members, in particular cytochrome-c, from the mitochondria into the cytosol[81]. In addition to cytochrome-c, mitochondrial release of apoptotic factors such as Smac, DIABLO, HtrA2 and apoptosis-inducing factor (AIF) also occurs[82].

Regulation of the mitochondrial-dependent pathway occurs via the action of the Bcl-2 protein family[83]. The balance between expression of pro-apoptotic Bcl-2 proteins (Bid, Bik, Bak, Bax, Bad, Bim, Bcl-Xs and Hrk) and anti-apoptotic Bcl-2 proteins (Bfl-1, Bcl-2, Bcl-X_L, Bcl-W and Mcl-1) facilitates apoptotic regulation by promoting and inhibiting the release of cytochrome-c from the mitochondria, respectively[84]. Once present in the cytosol, cytochrome-c interacts with a monomeric molecule known as apoptotic protease activating factor-1 (Apaf-1)[82]. This molecule then undergoes a conformational change followed by oligomerisation in the presence of ATP, subsequently resulting in procaspase-9 recruitment and apoptosome formation[82]. Within the apoptosome, procaspase-9 molecules become activated to caspase-9 molecules and this causes subsequent activation of procaspase-3 to caspase-3 molecules[85]. It is these active caspase-3 molecules that initiate downstream processes that ultimately lead to cell death[85].

1.2.3.2 Extrinsic (death receptor-mediated) pathway

The death receptor-mediated pathway (Fig.8) is initiated when specific cell surface receptors called death receptors become activated upon the binding of their respective death ligands[78]. It is understood that the afore-mentioned death receptors form part of the tumour necrosis

factor receptor (TNFR) superfamily, and such death receptors include TNFR-1 and Fas/CD95[78]. A characteristic feature of death receptors is that they contain a cytosolic death domain (DD)[86].

Binding of the Fas ligand to the Fas receptor promotes the association of the Fas-associated death domain (FADD) proteins with Fas via a process of receptor trimerization[79]. Moreover, FADD also contains a death effector domain (DED) which is able to recruit and interact with procaspase-8 through DED-DED interactions – subsequently forming a death induced signalling complex (DISC)[87]. Procaspase-8 molecules become concentrated within the DISC and transactivation of these molecules can occur seeing that they are in close proximity with each other[80]. In this way, active caspase-8 molecules are produced and can therefore cleave inactive procaspase-3 molecules to form active caspase-3[80]. Ultimately, active caspase-3 facilitates the cleavage of certain downstream substrates in order to cause cell death[80].

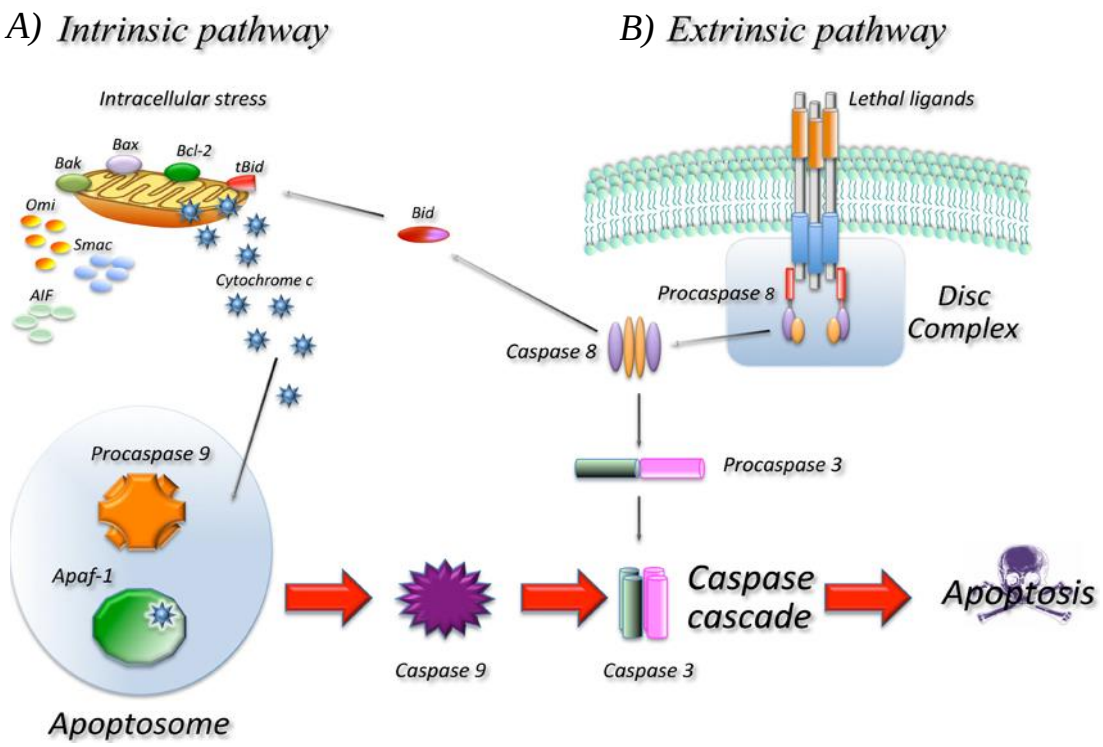

Figure 8: The two main apoptotic pathways occurring in mammalian cells. A) The intrinsic pathway relies on the mitochondrial release of cytochrome-c into the cytosol in response to either internal or external stimuli. Interactions between cytochrome-c and Apaf-1 lead to subsequent recruitment of procaspase-9 molecules, ultimately forming the apoptosome. Active caspase-9 molecules are formed within the apoptosome and facilitate the activation of procaspase-3 molecules to active caspase-3, resulting in downstream events leading to cell death. B) The extrinsic pathway is initiated when Fas binds to and interacts with the FADD proteins of the Fas receptor via a process of receptor trimerization. The DED of FADD associates with the DED of procaspase-8, forming a complex called DISC. DISC allows the transactivation of procaspase-8 molecules in order to form active caspase-8 molecules – thereby assisting in downstream activation of caspase-3 and ultimately causing cell death[88].

It is noteworthy that although these apoptotic pathways are highly controlled and regulated, dysregulation of these may occur and result in several conditions such as neurodegenerative disorders[89] and cancer[90]. In contrast to neurodegenerative disorders, cancer exhibits diminished levels of apoptosis which subsequently facilitates tumourigenesis. The present study targets this property of cancerous cells, thus an elaboration on the association between the lack of apoptotic cell death and enhancement of tumour progression follows.

1.2.4 Relationship between apoptosis and cellular proliferation

By definition, cellular proliferation describes a type of cell division that yields an increase in cell number[91] (Fig.9). Conversely, apoptosis is responsible for the eradication of damaged cells, thus leading to a reduction in cell number[92]. It is therefore evident that these two processes are closely related so as to maintain tissue homeostasis by tightly regulating cell numbers.

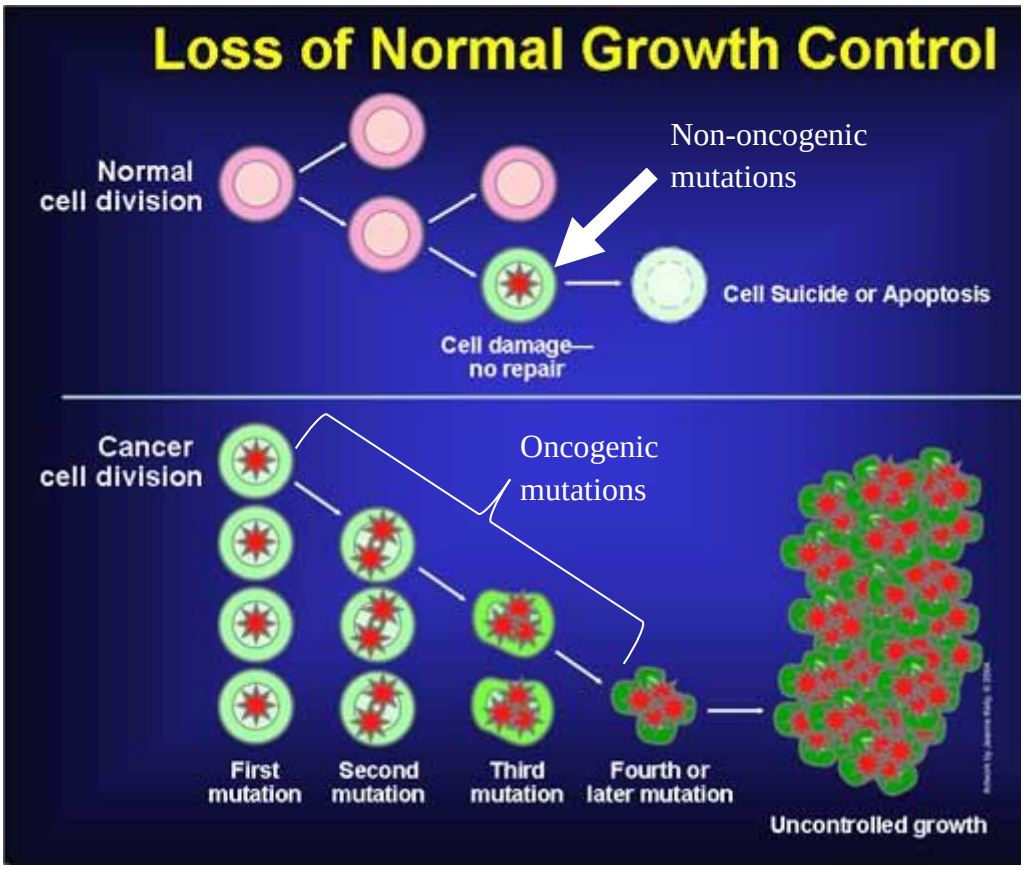

Figure 9: Comparison between normal and tumourigenic cell division. Normal cell division occurs as a result of non-oncogenic mutations that facilitate apoptotic induction in damaged cells. Alternatively, tumourigenic cell division is assisted by oncogenic mutations that result in uncontrolled cellular growth and proliferation (Adapted from: http://www.cancer.gov/cancertopics/cancerlibrary/what-is-cancer).

In order for an organism to grow and function properly, the cell cycle (Fig.10) needs to be adequately completed. Essentially, chromosome duplication and replication in daughter cells are key processes that are responsible for the growth of cells[93]. These processes are highly regulated by the cell cycle[93]. Additionally, cyclin-dependent kinases (CDK's) have been identified as critical proteins that drive the progression of the cell through the stages of the cell cycle[93].

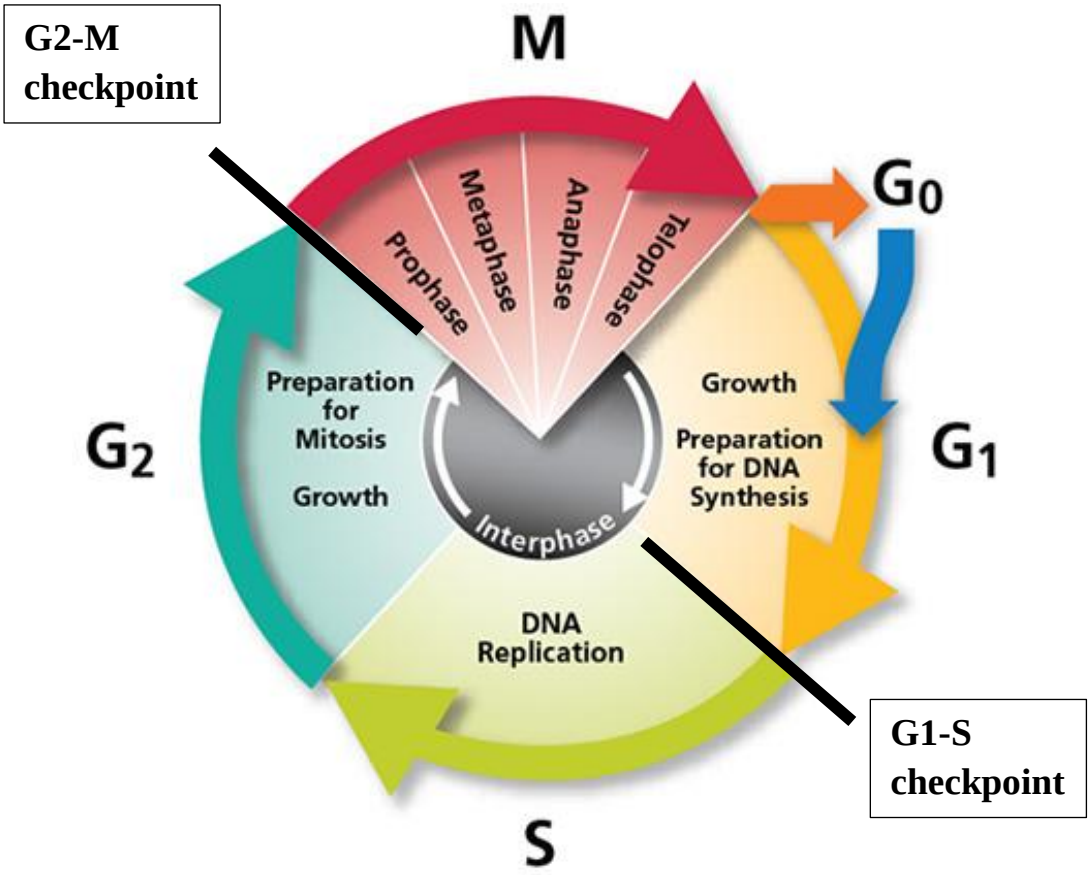

Figure 10: Stages of the cell cycle. Phase G0 (Gap 0) is the stage at which cells are quiescent but metabolically active. Certain growth factors and nutrients serve as extracellular signals which can stimulate entry of inactive quiescent cells into Phase G1 (Gap 1) and subsequent progression to the S-phase. During the S-phase, cells undergo DNA replication which permits entry into the G2 (Gap 2) phase – in which cells increase in size and synthesize proteins required for the M-phase. The M-phase involves mitotic duplication of cells to form identical daughter cells. G1-S and G2-M serve as checkpoints to ensure the orderly progression of cells through critical phases of the cell cycle (Adapted from: http: //www.bdbiosciences.com/research/apoptosis/analysis/index.jsp.)

An array of genes have been found to regulate the cell cycle and importantly, many of these genes have also been implicated in apoptotic regulation[92]. Such genes include c-myc, c-jun, c-fos and p53[92]. Pivotal to the relationship between apoptotic cell death and cellular proliferation is p53[79] (Fig.11), whose essential role is the maintenance of genome integrity by examination of cells in the G1-S and G2-M checkpoints of the cell cycle[94]. This is done

in order to detect unfavourable genome alterations, thus halting the progression of aberrant cells through the cell cycle[94]. Consequently, mutations in p53 impedes proper functioning of the G1-S and G2-M checkpoints, thereby allowing the survival and uncontrolled proliferation of damaged cells – ultimately promoting tumour formation[94].

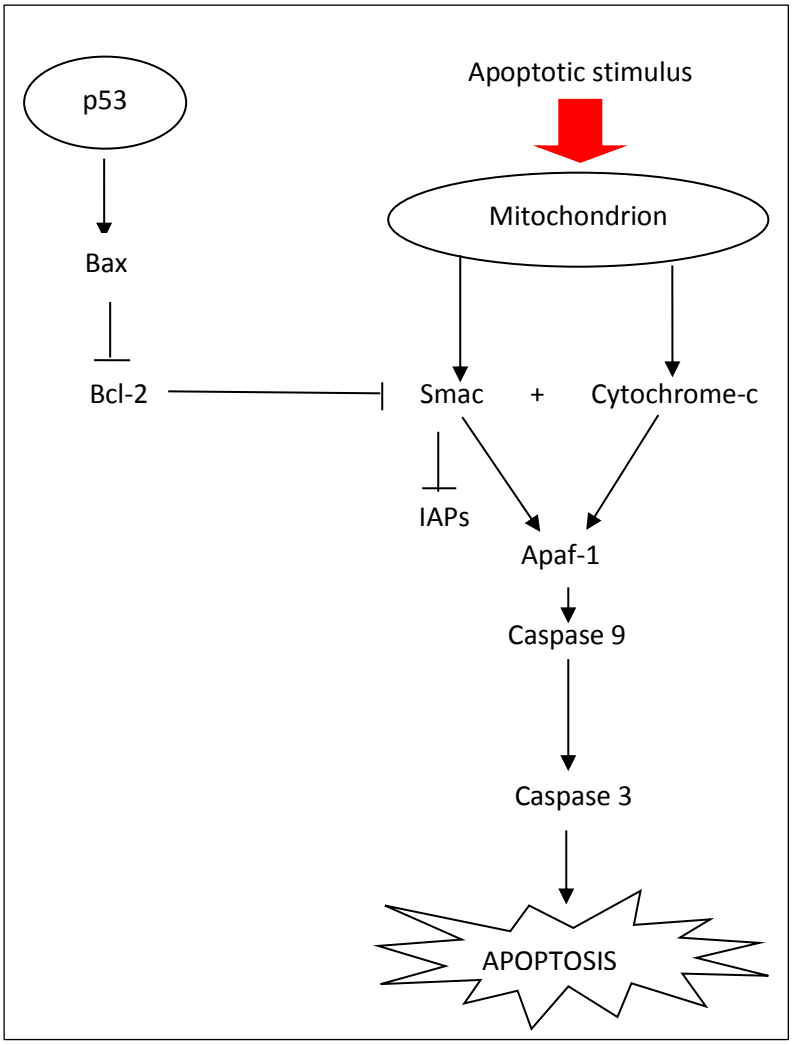

Figure 11: Role of p53 in the induction of apoptosis. Upon detection of an apoptotic stimulus, p53 becomes activated and causes subsequent activation of Bax – a pro-apototic gene. This causes activation of Smac via Bcl-2 inhibition and consequently results in the inactivation of inhibitors of apoptosis (IAPs). Cytochrome-c, a pro-apoptotic molecule, is released from the mitochondrion upon detection of an apoptotic signal. Interactions between Smac and cytochrome-c facilitate apoptotic induction via the intrinsic pathway. Mutations in p53 prevent this apoptotic induction process from taking place and thereby promotes the tumourigenic phenotype (Adopted from: http://www.sigmaaldrich.com/content/dam/sigma-aldrich/life-science/cell-signalling-and/migratecellsignal2/ ATM-p53_signalling_pathway.jpg.)

1.2.5 Role of apoptosis in tumour progression

As previously mentioned, genes are pivotal to the occurrence and regulation of apoptosis, thus making this process of cell death highly susceptible to genetic mutations. The aberrant

expression of certain genes is implicated in disruptions to the homeostatic balance, therefore affording cells the ability to proliferate in an uncontrolled manner[53]. Such genes are discussed below.

1.2.5.1 Bcl-2 protein family

Comprising of both pro- and anti-apoptotic proteins, this family of proteins plays a critical role in apoptotic regulation[95]. However, several cancers exhibit deregulated expression of the genes encoding for these proteins[50]. Over-expression of Bcl-2 has been shown to contribute to the evasion of apoptosis by breast[96], prostate[97] and brain[96] cancers. Studies have additionally suggested that cells that over-express Bcl-2 proteins gain a phenotype that allows them to become multi-drug resistant, thereby aiding cells in tumour formation[98].

1.2.5.2 Tumour necrosis factor (TNF) protein family

Death receptor-mediated apoptosis is often avoided by tumour cells as a result of the acquisition of resistance to key components that induce this apoptotic pathway. Aberrations causing the over-expression of genes coding for inhibitors of the extrinsic pathway, such as the caspase homolog cFlip, have been identified as contributing factors to the occurrence of several types of cancer[99]. Such inhibitors operate by competing with caspases for binding to FADD, subsequently hampering apoptosis and resulting in the formation of a tumour that is Fas-resistant[99]. This resistance is beneficial to tumours by allowing them to evade apoptosis in the presence of the Fas ligand (a major protein of the TNF family)[99] and additionally, Fas-resistance enhances tumour progression by the expression of Fas ligand on the surface of tumourigenic cells – without causing damage to the cells themselves[100].

1.2.5.3 Inhibitor of apoptosis (IAP) protein family

The IAP family consists of several proteins such as Survivin, XIAP, cIAP1 and cIAP2 – all of which have been suggested to interact with specific caspases and inhibit their pro-apoptotic function[101]. Moreover, IAP proteins interact with HtrA2 and Smac/DIABLO, thus leading to caspase activation and the induction of pro-apoptotic events due to the prevention of proper interactions between IAPs and caspases[101]. To date, much is still unknown about the expression of genes encoding IAPs in cancerous cells, but the deregulation of this expression has been implicated in several cancers. Particularly, the over-expression of IAPs such as Apollon has been identified in gliomas[102], whilst XIAP and Survivin have been

found to exhibit over-expression in non-small cell lung cancer[103]. Additionally, Livin is an IAP that shows characteristic over-expression in melanoma cells[104].

It is critically important to note that all of the afore-mentioned processes that govern proper cell functioning, such as apoptosis and cell proliferation, do not occur in isolation but rather depend closely on the interaction of cells with their environment – the extracellular matrix (ECM).

1.2.6 The extracellular matrix (ECM)

Apart from cell-cell associations, cells also communicate via interactions with their surrounding environment –known as the extracellular matrix or ECM[105] (Fig.12). This matrix is complex in nature and serves the principal function of supplying and co-ordinating signals that: regulate proper cell functioning, direct and control cell migration, ensure the adequate receptor-mediated provision of nutrients required by the cell, and facilitate cell contacts[106]. Structurally, two essential domains form the ECM, namely: 1) the basement membrane and 2) the matrix (condensed) layer[105]. The latter is composed of proteoglycans, matrix metalloproteinases (MMPs), and an array of glycoproteins such as fibronectins, integrins, collagens, elastin, and pivotal to the present study, the family of laminins[105, 106].

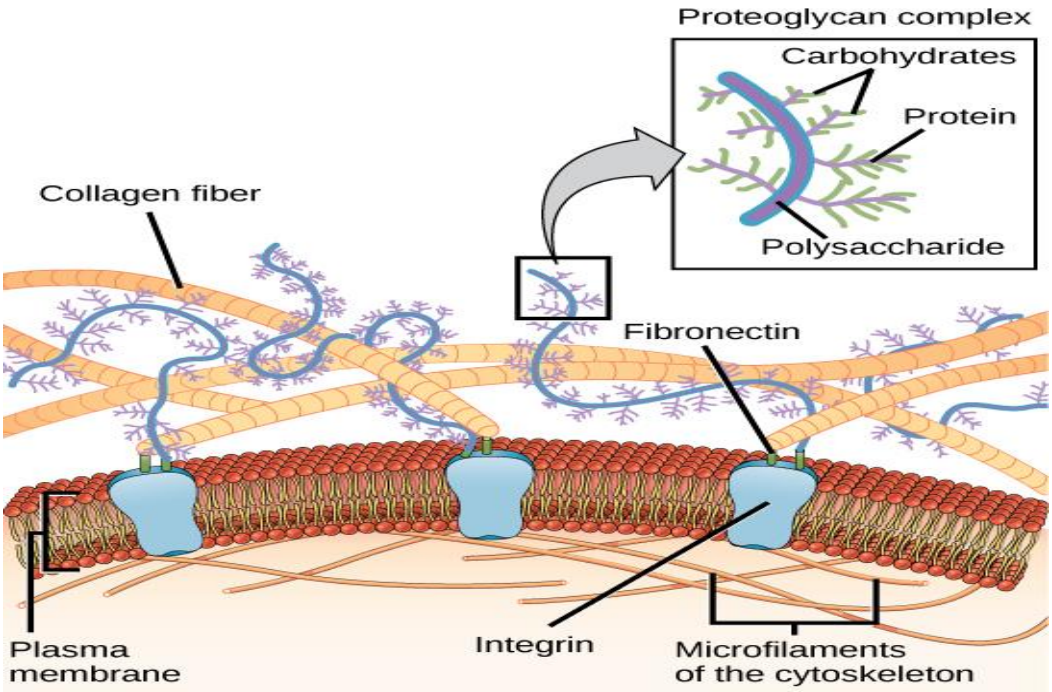

Figure 12: The extracellular matrix. The two main domains that make up the ECM are the basement membrane and the condensed stromal matrix layer. Laminins form an essential component of the basement membrane, whilst MMPs and several glycoproteins such as fibronectins, laminins, collagens, elastin, and integrins contribute to the composition of the stromal matrix (http://www.boundless.com/biology/)

1.2.6.1 The family of laminins

Laminins are glycoproteins that play a major role in the formation of the ECM[106]. Essentially, laminins are composed of three types of chains namely the α-, β- and γ-chains (Fig.13), which are joined together by disulphide bonds and are differentially assembled in order to form 17 heterotrimeric laminin isoforms[107]. These heterotrimers comprise several sites for binding of ECM components such as proteoglycans, integrins, collagen and entactin[107]. Additionally, there is also a binding site for other molecules of laminin, to allow for the formation of basal lamina sheets[107]. Functionally, laminin has been identified to play key roles in processes such as cell migration[108], adhesion[109], neurite outgrowth[110], cell differentiation and proliferation[111], as well as tumourigenic processes such as metastasis and angiogenesis[112]. Moreover, laminin also contains sites that allow for binding to cell surface receptors[112]. One such receptor – and of particular importance to the present study – is the 37kDa/67kDa non-integrin laminin receptor precursor/ high-affinity laminin receptor (LRP/LR)[113].

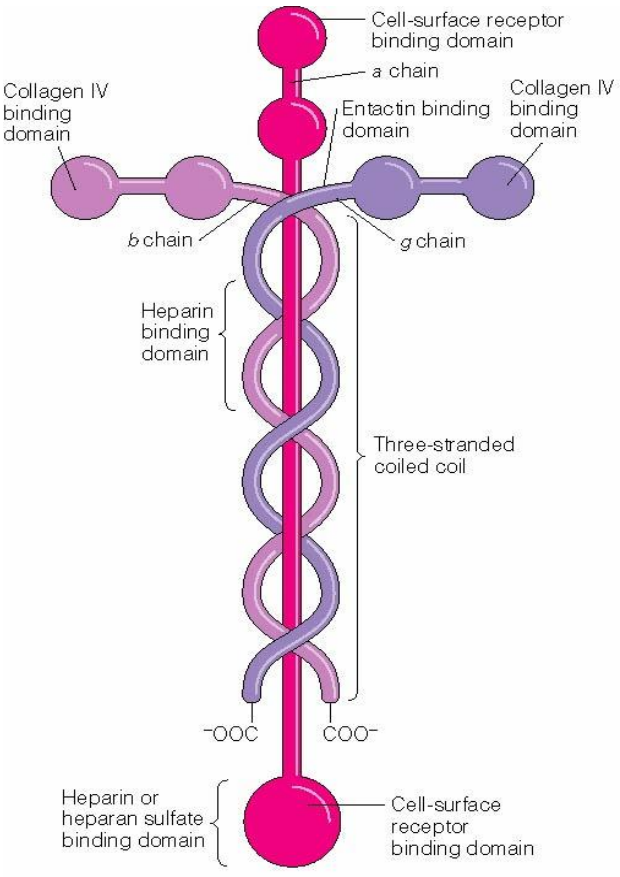

Figure 13: The structure of laminin. The α-, β- and γ-chains of laminin are stabilized by interchain disulphide bonds. Laminin contains binding sites for several ECM molecules such as collagen IV, entactin and heparin, and also binds an array of cell surface receptors in order to carry out its many functions (http://themeekgroup.com/Christian/laminin.htm.)

1.2.7 The 37kDa/67kDa laminin receptor precursor/laminin receptor (LRP/LR)

LRP/LR is a major extracellular matrix receptor that was initially isolated and purified from normal muscle cells[114], murine melanoma[115] and human breast carcinoma cells[116] in the year 1983. This non-integrin laminin receptor was shown to have an approximate molecular mass of 67kDa and exhibited high specificity and affinity for binding to laminins, in particular laminin-1[117]. It was also discovered that the mRNA that encodes for this 67kDa laminin receptor (LR) results in the formation of a protein with a length of 295 amino acids and an approximate molecular mass of 37kDa[118, 119]. This protein was then deemed as the precursor of the mature 67kDa laminin receptor, and consequently named the 37kDa laminin receptor precursor (LRP)[118]. To date, the precise mechanism by which the 37kDa LRP forms the 67kDa LR has not been elucidated. Early studies speculated that this mechanism may entail a process of homodimerization that occurs through certain post-translational modifications[118], however this theory was later disproved when a yeast two hybrid study showed failure of LRP to interact with itself and dimerize[120]. Other studies suggest that fatty acylation by fatty acids such as oleate, stearate and palmitate may be the key modification that results in the maturation of the 37kDa LRP into the 67kDa LR form[119]. More recent evidence has proposed that LRP is a target for small ubiquitin-like modifier (SUMO) proteins, which are 8-14kDa in size and have the potential to form oligomeric chains[121]. Thus, it is suggested that SUMOylation of LRP may be the modification that results in the maturation of 37kDa LRP into 67kDa LR[122].

It is known that LRP/LR (Fig.14) is predominantly found as a transmembrane receptor, particularly a type II transmembrane receptor[113]. In this location, it plays physiological roles in processes such as cellular adhesion, migration, proliferation, and the maintenance of cellular viability[113]. LRP/LR knockdown studies have also suggested physiological functions of this receptor in the cell cycle[123], protein synthesis[124] and processing of ribosomal RNA[125]. Additionally, this receptor localises in the cytosol[126] and the nucleus[127] – facilitating translational processes and the maintenance of nuclear structures, respectively. LRP/LR has also been identified in the perinuclear region[127]. As previously mentioned, laminin-1 binds to LRP/LR and this binding occurs at three distinct sites within the C-terminus of the receptor: 1) amino acid residues 161-180[128], 2) amino acid residues 205-229[128], and 3) most C-terminal 53 residues[129]. The receptor also has binding sites for viruses[130], elastin[131], carbohydrates[131], infectious as well as non-infectious prion proteins[131, 132], heparin[131], and immunoglobulin G antibodies[131].

Numerous studies have implicated LRP/LR as a key contributor to the pathogenesis of certain viral and bacterial infections[130], prion-protein related diseases such as Transmissable Spongiform Encephalopathies[133], neurodegenerative diseases such as Alzheimer's disease[134, 135], and several cancer types – the latter being of importance to the present study. Importantly, an array of cancer cells have exhibited an over-expression of LRP/LR in comparison to their normal counterparts, thereby suggesting that this receptor plays a role in the processes leading to tumour formation and progression.

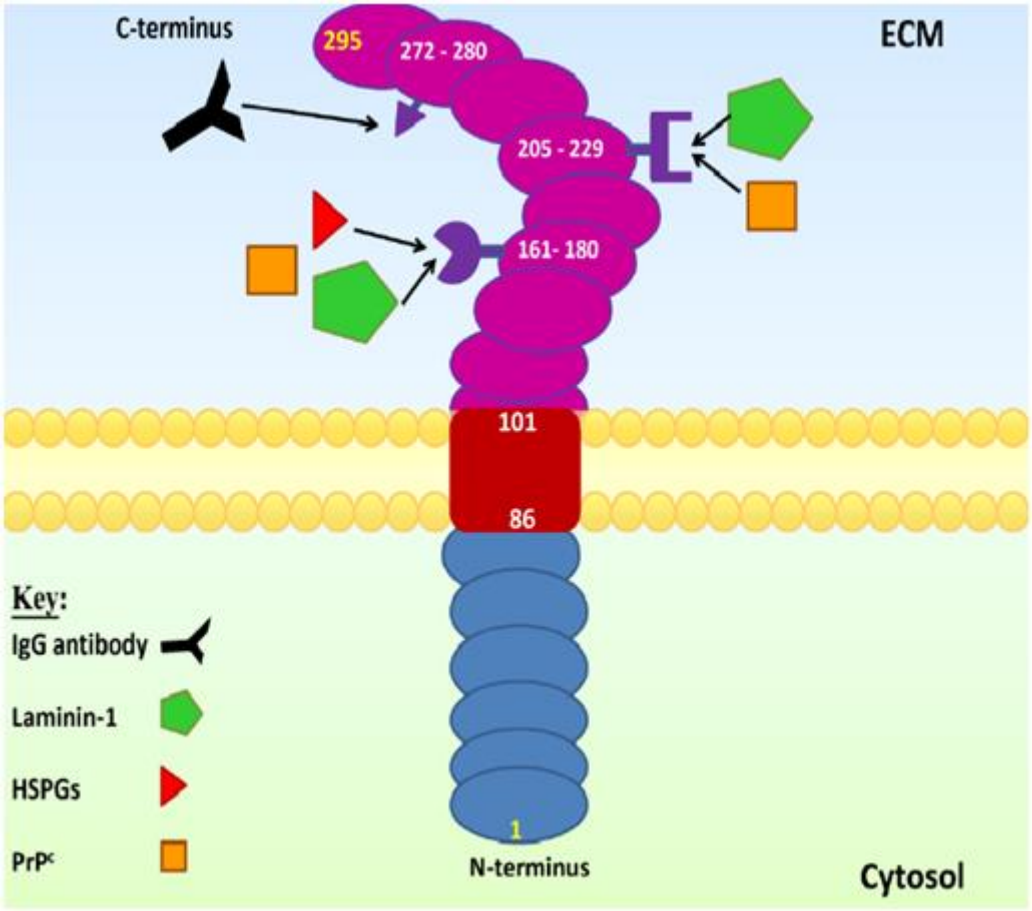

Figure 14: The 37kDa/67kDa laminin receptor precursor/laminin receptor (LRP/LR). This transmembrane receptor is 295 amino acids in length and comprises three main domains: 1) The N-terminal intracellular cytosolic domain (blue), 2) The transmembrane domain (red) and 3) The C-terminal extracellular domain (purple). The C-terminal domain contains sites for binding of substrates such as laminin-1, elastin, heparin, carbohydrates, prion proteins and IgG antibodies[136].

1.2.8 Involvement of LRP/LR in tumour progression

In contrast to normal cells, several tumourigenic cell lines arising from lung[137], colon[138], liver[139], gastric[140], prostate[141], cervical[142], ovarian[143], uterine[144], leukaemia[145] and breast[146] cancers have all exhibited up-regulated LRP/LR expression (Fig.15A). The exact

mechanism that contributes to the over-expression of LRP/LR in cancer cells when compared to normal cells is not fully understood. However, numerous studies have analysed the effects of this over-expression and have suggested that tumour progression is promoted via the action of LRP/LR-mediated enhancement of metastasis, angiogenic induction, and apoptotic evasion[112].

1.2.8.1 LRP/LR and metastasis

As previously described, LRP/LR has a high affinity for binding to laminin-1[117, 118]. This LRP/LR-laminin-1 interaction has been found to play a crucial role in promoting tumour metastasis[146,147] (Fig.15B). Particularly, tumourigenic cells possess increased levels of LRP/LR which – upon tumour cell adhesion - interacts with laminin-1 found in the basement membrane[146]. This interaction triggers subsequent activation of type IV collagenase enzymes that facilitate the degradation of type IV collagen within the basement membrane[148]. Tumour cells are then permitted to invade the stroma, migrate to distant organs via the bloodstream, and initiate the formation of secondary tumours at these distal sites[148]. Two key events involved in metastasis are adhesion and invasion, and the LRP/LR-laminin-1 interaction has been implicated in the enhancement of both of these events[148].

Recent studies have shown that fibrosarcoma[149], lung[150], cervical[150], colon[150], prostate[150], breast[151], oesophageal[151] and liver[139] cancers all exhibit increased levels of LRP/LR and thus increased occurrence of the LRP/LR-laminin-1 interaction. Moreover, blockage of this interaction by use of anti-LRP/LR specific antibody IgG1-iS18 resulted in diminished levels of adhesion and invasion (Fig.15B)[139,150,151]. Therefore, it is evident that LRP/LR is crucial for metastatic processes and is a promising target for metastatic cancer therapeutics.

1.2.8.2 LRP/LR and angiogenic induction

Angiogenesis can be described as a process that entails new blood vessel formation from pre-existing capillaries[122]. This process is critical in embryonic development, growth of tissues, wound healing, vascular remodelling, and the menstrual cycle[153]. Angiogenesis is able to assist with the afore-mentioned processes by providing nutrients and oxygen to the cells that require them[153]. Normal, healthy individuals display tightly regulated angiogenesis. However, dysregulation of angiogenesis has been observed in several conditions such as limb disease, rheumatoid arthritis and importantly, growth and metastasis of tumour cells[154]. In

the case of tumour development, angiogenesis involves the formation of tubular structures that permit tumour cells to migrate to distant sites and initiate metastasis at those sites[153]. Moreover, tumour angiogenesis provides a means by which tumour cells can gain oxygen and essential nutrients to facilitate and sustain their growth[153].

As previously described, the binding of LRP/LR on the cell surface to laminin-1 in the basement membrane leads to proteolytic activation that subsequently results in degradation of the basement membrane[148]. This process of cell-ECM interactions and the resultant membrane degradation has been identified as a crucial step in the formation of angiogenic tubular structures[152], thus probing research into the involvement of LRP/LR in tumour angiogenesis. A recent study showed that anti-LRP/LR specific antibody W3 significantly impeded tube formation in human umbilical vein endothelial cells (HUVEC's), thus confirming that LRP/LR does indeed play an important role in the process of angiogenesis[155] (Fig.15C).

1.2.8.3 LRP/LR and evasion of apoptosis

As previously mentioned, LRP/LR plays a role in the maintenance of cellular viability. Specifically, studies have shown that LRP/LR maintains the viability of yeast cells through the ribosomal processing and maturation[156]. Additionally, studies have proposed that LRP/LR maintains the viability of mammalian cells through the up-regulation of certain signalling cascades and also through interactions with Midkine – a growth factor that enhances cell migration and promotes cellular proliferation and survival[127]. Essentially, Midkine has been suggested to assist LRP/LR with binding to the nuclear envelope and chromatin in order to stabilize chromosomes, thereby aiding in the maintenance of cellular viability[127].

The up-regulated expression of LRP/LR in tumourigenic cells contributes to the maintenance of the viability of such cells. Additionally, it is suggested that LRP/LR facilitates tumour enhancement and progression by promoting apoptotic evasion[157]. The role played by LRP/LR in the inhibition of apoptosis has been confirmed by a study involving the use of small-interfering RNAs (siRNAs) directed towards the mRNA of the 37kDa LRP in liver (Hep3b) cancer cells, which led to apoptotic induction as a result of siRNA-mediated knockdown of LRP expression[157]. More recent studies made use of siRNA-LAMR1 to down-regulate LRP expression in cervical (HeLa)[158], lung (A549)[158], breast (MCF-7 and MDA-MB 231)[159] and oesophageal (WHCO1)[159] cancer cells, and it was observed that

LRP down-regulation induced apoptosis in these two cancerous cell lines (Fig.15D). These findings affirm the critical role played by LRP/LR in the maintenance of cellular viability and therefore deems this receptor as a potential therapeutic target for cancer treatment.

The mechanism of siRNA action is discussed in section 1.2.9 below.

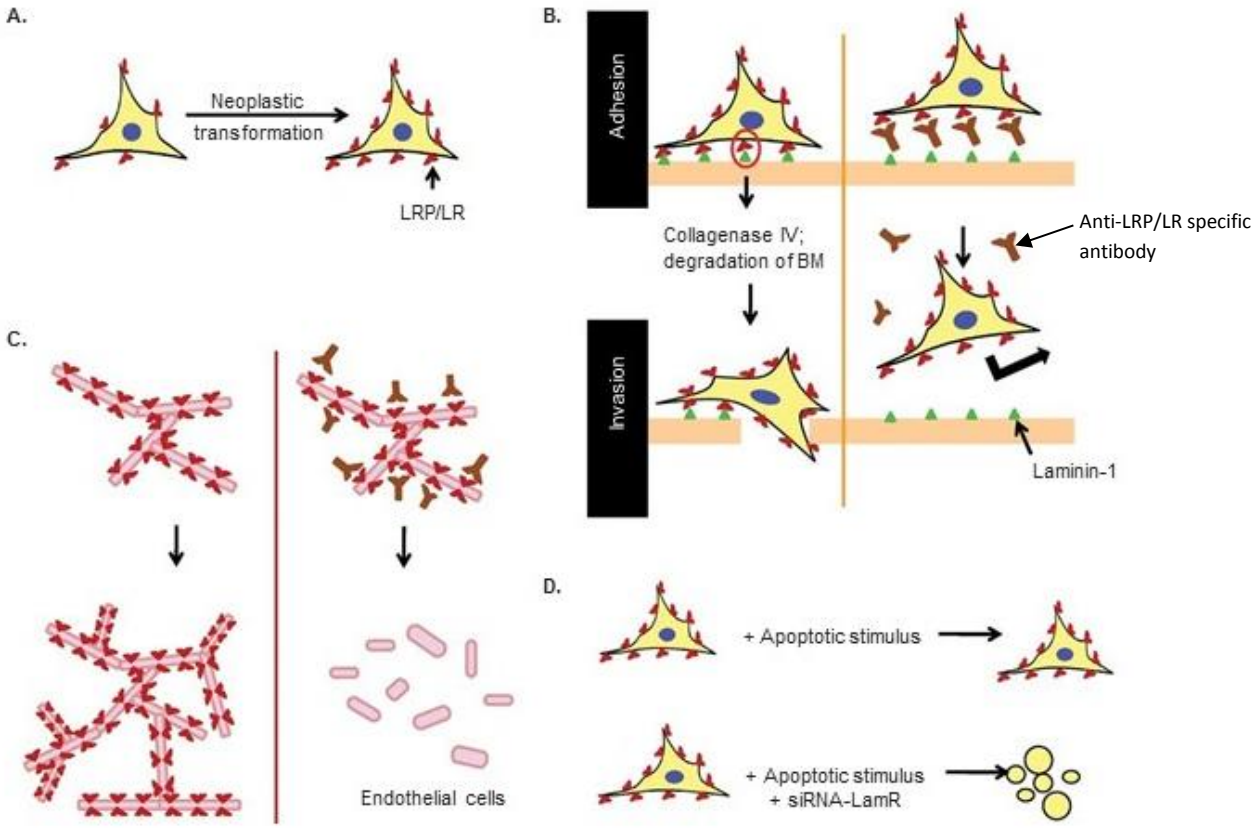

Figure 15: The role of the 37kDa/67kDa laminin receptor (LRP/LR) in tumourigenesis. A) Several types of cancerous cells exhibit higher levels of LRP/LR in comparison to their normal counterparts. B) Up-regulated LRP/LR expression in tumour cells facilitates more binding of the receptor to laminin-1 in the basement membrane (adhesion), leading to activation of type IV collagenase which triggers the degradation of type IV collagen in the basement membrane – thereby allowing tumour cells to invade organs (invasion). Anti-LRP/LR specific antibody IgG1-iS18 blocks the LRP/LR-laminin-1 interaction, thus impeding adhesion and invasion – the key events in metastasis. C) Increased levels of LRP expression have been implicated in the formation of endothelial tubes, thus suggesting that the receptor plays a pivotal role in tumour angiogenesis. Use of anti-LRP/LR specific antibody W3 blocked the formation of these tubular structures. D) Tumour cells survive by evading apoptosis. However, use of siRNAs directed towards LRP/LR successfully induced apoptosis in certain cancer types via LRP down-regulation (Adapted from [136]).

1.2.9 RNA interference

RNA interference refers to a conserved, specific mechanism of gene silencing used by a vast majority of eukaryotes[160]. This silencing functions to either suppress transcription (a process referred to as transcriptional gene silencing/ [TGS]) or activate a process of degradation of RNA (a sequence-specific process that is referred to as RNA interference/ [RNAi] or post-transcriptional gene silencing/ [PTGS])[160]. This regulatory silencing mechanism is employed by siRNAs, which are double-stranded RNA (dsRNA) molecules that facilitate the degradation of sequences that are present in and complimentary to the target mRNA[161]. The key players in the [PTGS]/[RNAi] process are dsRNA molecules that serve to activate and induce this process due to their involvement in inducer-molecule cleavage and degrading of target mRNA[161].

Two enzyme complexes, namely Dicer and RNA-induced signalling complex (RISC), have been identified to play pivotal roles in the [PTGS]/[RNAi] processes[161] (Figure 16). Specifically, Dicer facilitates initiation of this process[162], whilst RISC is responsible for degradation of the target mRNA[163].

1.2.9.1 Dicer

Dicer is an enzyme complex that is highly specific for dsRNA and forms part of the RNase III nuclease family[164]. Structurally, Dicer is composed of four domains: a dsRNA-binding domain, dual RNase III motifs, a PAZ domain, and an amino-terminal helicase domain[164]. Functionally, Dicer acts by binding to long dsRNA molecules and cleaving them in order to yield short nucleotide duplexes (approximately 21-23 bp in length) consisting of 2-overhanged nucleotides present at the 3' ends[165]. These duplexes are known as small interfering ribonucleic acids (siRNAs)[165]. Further dsRNA digestion by RNase III nucleases yields fragments of about 12-15 bp in length, and this occurs as a result of the action of two centres of catalysis found within the dimeric RNase III enzyme[165]. Moreover, it is stipulated that four catalytic sites are formed as a consequence of Dicer folding onto the dsRNA substrate – thereby facilitating dsRNA digestion to form siRNA[166]. Essentially, it is suggested that two catalytic sites remain active and share maximum homology with the catalytic sequence of RNase III[166]. The other two sites experience loss-of-function due to the sharing of only partial homology[166]. Resultantly, Dicer-processed dsRNA exhibit a doubling in size in comparison to fragments processed by alternative RNase III nuclease enzymes[166].

1.2.9.2 RNA-induced silencing complex (RISC)

According to a study conducted on *Drosophila* cells, it was established that dsRNA molecules assist with the sequence-specific targeting of exogenous RNA transcripts[162]. Moreover, this study identified RNA as an essential component of the nuclease activity that leads to degradation of the target mRNA[162]. This nuclease activity that results in mRNA degradation is called the RNA-induced silencing complex or RISC[162]. Domain proteins that contribute to the formation of RISC include fragile X-related (FXR), Argonaute (Ago) and Tudor-*Stapylococcus* (Tudor SN) proteins[167].

1.2.9.3 RNA interference mechanism

Although not fully elucidated, a proposed mechanism exists for the [PTGS]/[RNAi] process[167]. The mechanism incorporates three essential steps:

Step 1 (Initiation) entails the binding of Dicer to a dsRNA molecule, with subsequent cleavage of this dsRNA into fragments that are 21-25 nucleotides in length[168]. These fragments are termed siRNA[168](Fig.16A).

Step 2 (Effector) involves the joining of siRNAs to the RISC[169]. siRNAs are organized into double-stranded, duplexed structures consisting of 3' two-nucleotide overhangs and 5' phosphate termini[167]. This organization permits siRNAs to be incorporated into RISC[167]. ATP addition, as well as siRNA unwinding, are implicated in the enzymatic activation of RISC zymogens, with the outcome of only the most stable siRNA strand staying in attachment with RISC[166] (Fig.16B).

Step 3 (Gene silencing) is characterized by cleavage of target mRNA by the activated RISC multinuclease complex accompanied by the bound, stable siRNA strand[168] (Fig.16C). It is understood that target mRNA is endonucleolytically cleaved at a point that is central to the complementary siRNA region, and this point is located ten nucleotides upstream from the paired nucleotide at the guide siRNA's 5' end[169]. Cleavage of target mRNA may occur independently of ATP, but the presence of ATP increases the efficiency at which cleaved products are released[168].

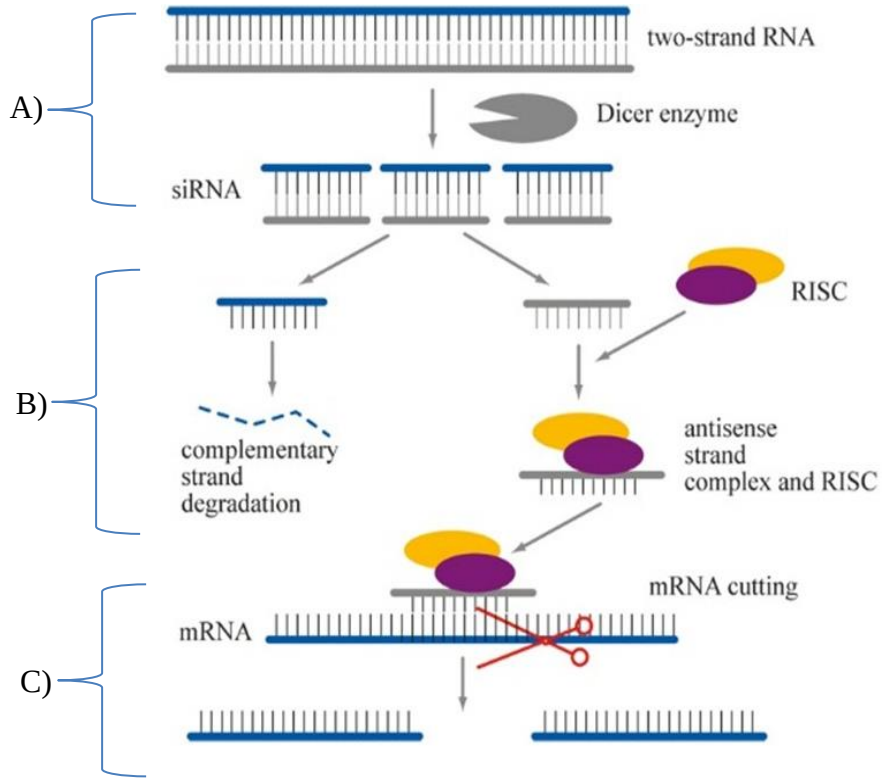

Figure 16: The mechanism of RNA interference. A) Enzyme complex dicer binds to and cleaves dsRNA into siRNA fragments. B) RISC incorporates siRNA and binds to complementary sequences on target mRNA. C) After binding, the activated RISC facilitates cleavage and degradation of the target mRNA (http://eng.thesaurus.rusnano.com/upload/iblock/775/RNA-interference_1.jpg)

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1.2.9.4 RNA interference applications

The use of RNA interference as a means of therapeutics has gained much attention with regards to the treatment of several gene-related ailments[170] including genetic and autoimmune diseases, viral-based infections, and cancer – the latter being integral to the present study. Essentially, RNA interference may be applied to one gene or several genes to correct their disease-causing activity[170]. *In vitro* introduction of siRNAs and subsequent gene silencing is easily achievable, but due to off-target effects that may negatively impact healthy cells, siRNA-mediated gene silencing *in vivo* proves to be an ongoing challenge.

Although challenging, several lines of research have permitted the development of RNA interference-based therapeutics to treat an array of viral infections, commonly HIV and hepatitis viruses[171]. Moreover, studies have provided insight into the use of RNAi-based therapeutics to treat neurodegenerative disorders and cancer[172].

CHAPTER 2: RATIONALE, AIM AND OBJECTIVES

2.1 Rationale and research question

It has been observed that several types of tumour cells exhibit a characteristic over-expression of the 37kDa/67kDa laminin receptor (LRP/LR) in comparison to their normal cell counterparts. This over-expression has been identified as a key factor in the enhancement of tumour progression. Importantly, LRP/LR has been implicated as a critical component in the maintenance of tumour cell viability – evidenced by reductions in cellular viability upon down-regulation of this receptor. Studies have proven that siRNA-mediated LRP/LR knockdown led to reductions in the viability of liver (Hep3B), lung (A549) and cervical (HeLa) cancer cells via apoptotic induction. Another study suggested that siRNA-mediated down-regulation of LRP/LR reduced the viability of breast (MCF-7 and MDA-MB231) and oesophageal (WHC01) cancer cells. These findings prompted the question whether siRNA-mediated knockdown of LRP/LR will reduce the viability of pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells, and to establish whether apoptosis is the form of cell death responsible for the possible reduction in cellular viability.

2.2 Aim

To determine how siRNA-mediated down-regulation of the 37-kDa/67-kDa laminin receptor (LRP/LR) impacts on the cellular viability of pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells.

2.3 Objectives

- To assess cell surface LRP/LR levels on the afore-mentioned cell lines (Section 2.1) by employing flow cytometry
- To successfully down-regulate the expression of LRP/LR in the cell lines mentioned above (Section 2.1) via cell transfection with siRNAs targeted to LRP mRNA.
- To determine endogenous levels of LRP/LR in the above-mentioned cell lines (Section 2.1) and also to detect siRNA-mediated LRP/LR down-regulation by use of Western blotting
- To analyse the effect of siRNA-mediated LRP/LR down-regulation on the viability of the above-mentioned cell lines (Section 2.1) using MTT assays
- To determine the effect of siRNA-mediated LRP/LR knockdown on the proliferation of the above-mentioned cell lines (Section 2.1) using BrdU assays

- To assess if any nuclear morphological changes occur upon siRNA treatment targeting LRP/LR in the afore-mentioned cell lines (Section 2.1) using immunofluorescence microscopy
- To detect possible apoptosis-inducing effects of siRNA-mediated LRP/LR down-regulation on the cell lines stated in Section 2.1 by use of Annexin-V-FITC assays as well as caspase-3 assays
- To analyse specific pathways used by the afore-mentioned cell lines (Section 2.1) to undergo siRNA-assisted apoptotic induction by performing caspase-8 and caspase-9 assays
- To analyse data for statistical significance

CHAPTER 3: MATERIALS AND METHODS

N.B. : A detailed list of suppliers/manufacturers of antibodies, reagents and equipment used to carry out the following experiments is given in APPENDIX A.

3.1 Cell culture

3.1.1 Cell lines

- AsPC-1 (ATCC® CRL-1862): Human pancreatic adenocarcinoma – isolated from ascites of a 62-year old Caucasian female diagnosed with pancreatic cancer and initiated in nude mouse xenografts.
- IMR-32 (ATTC® CCL-127): Human neuroblastoma – derived from an abdominal mass of a 13-month old Caucasian male.

3.1.2 Cell culture media

3.1.2.1 Specific requirements per cell line

- AsPC-1: RPMI 1640 supplemented with 2mM L-glutamine, 10mM HEPES and sodium pyruvate
- IMR-32: Eagle Minimum Essential Medium (EMEM) supplemented with 2mM L-glutamine and 1% non-essential amino acids

For complete growth media, the above-mentioned media was additionally supplemented with:

- 10% Fetal calf serum (FCS) which is heat inactivated
- 1% Penicillin-Streptomycin

3.1.3 Method of cell cultivation

Both cell lines were cultivated in their respective media as described in section *3.1.2.1* above. The cells were grown in a humidified 5% CO_2 incubator set at a temperature of 37°C in order to mimic *in vivo* conditions. Cells were seeded and sub-cultured at appropriate dilution factors and densities as often as was necessary. Seeding and sub-culturing of cells involved aspiration of the media followed by washing of the cells with 1-2ml of phosphate buffered

saline (PBS) to remove excess media. Thereafter, cells were detached using trypsin/EDTA and re-suspended in fresh media specific to the cell type as stated in section *3.1.2.1*.

3.1.4 Cryopreservation and storage of cells

Both of the mammalian cell lines employed in this study were cryopreserved to alleviate cell loss occurring as a result of contamination or reaching too high a passage number. Cryopreservation also allowed for cell storage over long periods of time or until needed.

The process of cryopreservation involved cell detachment by use of trypsin/EDTA post washing of the cells with PBS. Thereafter, detached cells were resuspended in their growth media and centrifuged at 1200 rpm for 10 minutes before discarding of the supernatant and re-suspension of the cell pellet in fresh warmed cell culture medium. Additional supplementation of the medium with 10% FCS and 10% DMSO served as the freezing medium. Following re-suspension, cells in 1ml aliquots were incubated for 1 hour at a temperature of -20°C prior to being incubated at -70°C overnight. Ultimately, cells were transferred to and stored in liquid nitrogen until required.

3.1.5 Thawing of cells

In the event that a cryopreserved stock of cells was needed, 1ml of fresh cell culture medium pre-warmed at 37°C was used to thaw the 1ml frozen cell stock and the resulting suspension was centrifuged at 1200 rpm for 10 minutes. After discarding the supernatant, the cell pellet was re-suspended in fresh media containing an additional 10% FCS and left to attach and grow at 37°C as previously described.

3.1.6 Cell counting

Cell counting was performed in order to ensure that a constant number of cells was used for experiments performed on both cell lines, thereby allowing for effective comparison between the cell lines involved in this study. Essentially, cell counting involved staining of cells with Trypan blue – in principle, viable cells appear clear whilst dead cells are stained blue. Post-staining, cells were then counted using a Neubauer haemocytometer. After washing of the cells with PBS and detaching them with trypsin/EDTA, cells were re-suspended in the appropriate media and an aliquot of this cell suspension was combined with an equal amount of Trypan- blue solution (0.4%). Thereafter, 10µl of this combination was placed on the haemocytometer and the mean cell average in 16 small squares was counted under the light

microscope. The following formula was employed in order to determine the total number of cells (for each of a total of four quadrants):

Number of cells/ml= Average count of unstained cells per 16 squares x 10^4 x dilution factor

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

3.2.1 Materials

- 24-well, 6-well, and 6cm plates
- siRNA-LAMR1 (targeted to LRP/LR)
- siRNA-scrambled (employed as a negative control)
- DharmaFect1 Transfection reagent
- Serum-free media (containing no FCS)
- Antibiotic-free media (containing no penicillin-streptomycin)
- esiRNA-RPSA (targeted to LRP/LR)*
- esiRNA-RLUC (employed as a negative control)*
- Lipofectamine transfection reagent*

***N.B.: These treatments were used following the same procedure described in section 3.2.2 below, but were used only in SDS-PAGE/Western blotting (sections 3.3 and 3.4) and MTT assays (section 3.6) as a means of confirmation of the results obtained using siRNA-LAMR1 and siRNA-scrambled.**

3.2.2 Transfection procedure

Prior to transfection, cells were seeded on appropriate plates at specific densities dependent on the experiment or assay to be performed. Cells were allowed to reach an approximate confluency of 50-70% before transfection was carried out. The target siRNA as well as the negative control siRNA were both diluted in specified amounts of serum-free media (as per manufacturers recommendations – Refer to Appendix A), followed by further dilution in suggested volumes of antibiotic-free media prior to being added to the cells. It is noteworthy that cells were also supplemented with recommended amounts of transfection reagent in order to facilitate the transfection procedure. Thereafter, the plates were incubated at 37°C for

a period of 72 hours to allow for transfection to occur prior to downstream experiments being performed.

3.3 Protein biochemistry

3.3.1 Preparation of cell lysates

3.3.1.1 Materials

- Cell scrapers
- Phosphate buffered saline (1X PBS)
- Lysis buffer [composed of: 10mM Tris/HCl pH 7.5, 10mM EDTA, 100mM NaCl, 0.05% (w/v) deoxycholic acid (DOC), 0.5% (v/v) Nonidet-P40]

3.3.1.2 Methodology

Attached cells were washed with PBS prior to incubation in 500µl of lysis buffer for 2 minutes. Thereafter, cell scrapers were used to facilitate cell detachment and membrane disruption. The resultant solution was placed in an Eppendorf tube and incubated at 4°C for 15 minutes. This was followed by centrifugation of the tube at 14000 rpm for 2 minutes, after which the supernatant containing the extracted proteins was retained and the pellet discarded. The supernatant was stored at -20°C and thawed at 4°C whenever required.

3.3.2 BCA™ assay for protein quantification

3.3.2.1 Principle underlying BCA™ assays

BCA™ assays are used for the determination of the total protein concentration in the cells. This particular assay exploits the reduction of Cu^{2+} to Cu^{+} by peptide bonds found in proteins. Cu^{+}, as well as a violet solution of bicinchoninic acid (BCA, 2,2'-Bichinolin-4,4'-dicarbonicacid) is produced. Hence, measurement of absorbance at 562nm is proportional to the amount of protein in the sample, thus allowing for protein concentration to be determined.

3.3.2.2 Materials

- 96-well plates
- Eppendorf tubes
- BCA assay kit

- ELISA reader

3.3.2.3 Methodology

Bovine serum albumin (BSA) standards of concentrations 0, 0.2, 0.4, 0.6, 0.8 and 1mg/ml were prepared and subsequently, 25µl of each standard was loaded into separate wells of a 96-well plate in triplicate. Similarly, 25µl of cell lysates obtained from section 3.3.1 and diluted (1:5) was added to the 96-well plate in duplicate. Thereafter, 200µl of BCA reagents (copper II sulphate and bicinchoninic acid) combined in a ratio of 1:50 was added to the wells containing the BSA standards as well as the lysates. The plate was then incubated for 30 min at room temperature and thereafter, the absorbance of the resultant solutions was measured at a wavelength of 562nm by use of an ELISA reader. Construction of a standard curve allowed for extrapolation of the concentration of proteins.

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

3.3.3.1 Materials

- Stacking Tris buffer [0.5M Tris, pH 6.8]
- Separating Tris buffer [3M Tris, pH 8.8]
- 10% ammonium persulfate (APS) in distilled water
- TEMED
- Protein molecular weight marker (pre-stained)
- 2X loading buffer [100mM Tris/HCl pH 6.8, 4% SDS, 2% β-mercaptoethanol, 20% glycerol, 0.02% bromophenol blue]
- 1X SDS running buffer [1% of 10X running buffer]
- 10X SDS running buffer [250mM Tris, 192mM glycine in distilled water, 10% SDS]
- Gel casting apparatus

3.3.3.2 Methodology

In order to separate proteins in a lysate sample, SDS-PAGE was employed. This technique was performed before the detection of total and down-regulated LRP/LR levels by use of Western blotting. SDS-PAGE exploits the molecular weight of proteins as the main criterion for separation. Heating of the lysate samples in the presence of SDS allowed for proteins to be denatured and additionally, lysate samples were subjected to β-mercaptoethanol to facilitate protein denaturation through the reduction of disulphide bridges within proteins.

Apart from denaturation of proteins, SDS also imparts a uniform negative charge on proteins thereby allowing molecular weight to be the principal determinant in the separation process. The negatively charged proteins migrate to the anode of the electrical field and separation of the proteins is subsequently achieved on the basis of molecular size using a polyacrylamide gel with a specific pore size. The pore size is dependent on the amount of acrylamide used, whereby larger amounts of acrylamide allows for smaller pore sizes to be achieved and smaller amounts of acrylamide will result in larger pore sizes. In line with the present study, a 12% polyacrylamide gel was used (Refer to Appendix A for gel composition).

Post heating of the lysate samples at 95°C for 5 min in loading buffer to allow for protein denaturation, 10μg of samples were loaded onto the gel. The gel was resolved at 200 V for approximately 30-45 min.

3.4 Western blotting and immunodetection of proteins

3.4.1 Materials

- Transfer buffer [20% methanol in 192mM glycine and 25mM Tris]
- Blocking buffer [3% BSA in 1X PBS-Tween]
- Wash buffer [0.1% Tween in 1X PBS]
- Polyvinyldifluoride (PVDF) membrane
- Whatman filter papers
- 100% methanol
- Primary antibody (anti-LRP/LR specific antibody IgG1-iS18) solution (Refer to: Zuber *et al, J.Gen.Virol.,* 2008)
- Secondary antibody (anti-human IgG-HRP conjugated antibody) solution
- Conjugated primary antibody (mouse monoclonal anti-β-actin peroxidase) for detection of loading control
- Chemiluminescent substrate
- Blotting device

3.4.2 Experimental procedure

Endogenous LRP/LR levels as well as siRNA-treated LRP/LR levels were determined by employing Western blotting that makes use of a primary and secondary antibody (Section

3.4.1). This technique entails the transfer of the proteins separated during SDS-PAGE (Section 3.3.3) onto a PVDF membrane followed by exposure to the afore-mentioned antibodies. The chemiluminescent substrate was used in order to detect the horseradish peroxidase (HRP) that is conjugated to the secondary antibody. Thereafter, the fluorescent light emission was captured onto an x-ray film and this step was performed in a dark room.

Prior to exposure of the PVDF membrane to relevant antibodies, electro-blotting was performed. Soaking of Whatman filter papers in transfer buffer was carried out for 5 min whilst PVDF membranes (cut to the dimensions of the SDS-PAGE gel) were soaked firstly in ethanol for 2 min and for 5 min in transfer buffer thereafter. PVDF membranes were then placed between soaked Whatman papers that were assembled onto the electro-blotting device and the gels were placed on top of the membranes. Electro-blotting took place for 45 min at 300V and 350A. Thereafter, the membranes were blocked for 1 hour using blocking buffer followed by a 1 hour incubation of the membranes in the primary antibody diluted in blocking buffer. Three washes in washing buffer (10 min each) were performed before incubation of the membranes in secondary antibody diluted in blocking buffer for 1 hour. Three washes were again performed as previously mentioned before the development of chemiluminescent detected proteins onto x-ray film. It is noteworthy that the afore-mentioned β-actin antibody, which was used to detect β-actin and thus serves as a loading control, is directly conjugated to HRP and therefore does not require a secondary antibody. Densitometry was performed to quantify protein levels and this was done using ImageJ™ software.

3.5 Flow cytometry (FACS analysis)

3.5.1 Materials

- Confluent cells
- Trypsin/EDTA
- 4% paraformaldehyde
- Primary antibody (anti-LRP/LR specific antibody IgG1-iS18)
- Secondary antibody (anti-human phycoerythrin (PE)- coupled)
- Anti-chloramphenicol acetyltransferase antibody (anti-CAT) produced in rabbit – to be used as a negative control primary antibody

- Allophycocyanin (APC) – produced in goat and directed towards rabbit IgG – to be used as a negative control secondary antibody
- PBS

3.5.2 Methodology

Determination of the cell surface levels of laminin receptor (LRP/LR) was achieved through the use of fluorescence activated cell scanning (FACS analysis). This technique employs the principle of immunodetection whereby primary antibodies that are recognised by specific fluorochrome-coupled secondary antibodies are used for the detection of a target protein. The flow cytometer is comprised of an argon laser beam that facilitates differentiation between antibody-labelled cells and unlabelled cells, thus allowing for the expression of target proteins on the cell surface to be quantified.

One of the essential requirements of flow cytometry is that cells need to be in suspension, hence trypsin/EDTA was used in order to assist with cell detachment. Thereafter, detached cells were centrifuged for 10 min at 1200 rpm followed by the resultant pellet being fixed with 4% paraformaldehyde for 10 min at 4°C. Post fixation, two cell suspensions were prepared by re-suspension of cell pellets in PBS and the halved – one half of the cell suspension was treated with primary antibody IgG1-iS18 (30 μg/ml) in PBS whilst the other half of the cell suspension was treated with only PBS (thus serving as a control). Following an overnight incubation at 4°C, cells were washed three times in PBS by centrifugation at 5000 rpm for 5 min and both cell pellets were re-suspended in PBS and the PE-coupled secondary antibody. After incubation of both cell suspensions for 1 hour in the dark, the resulting cell suspensions were washed 3 times in PBS as previously described and re-suspended in PBS before flow cytometric evaluation. The same procedure was followed in order to obtain results for the negative control bacterial protein, chloramphenicol acetyltransferase (CAT), using the negative control antibodies outlined in section 3.5.1 above. Additionally, unlabelled cells were also used as a control.

3.6 Assessment of cellular viability

3.6.1 MTT assay

3.6.1.1 Principle behind the assay

This assay is a colorimetric assay that relies on the ability of viable cells to cleave the mitochondrial tetrazolium salt (MTT), which is water-soluble and yellow in colour. Cleavage of MTT occurs via the mitochondrial succinate dehydrogenase (reductase) enzyme to produce water-insoluble purple formazan crystals (Fig.17). Therefore, by measurement of the absorbance of the resulting purple formazan crystal solution, a percentage of cell survival can be determined – with lower absorbance values corresponding to an enhanced level of cell death due to lower levels of MTT cleavage.

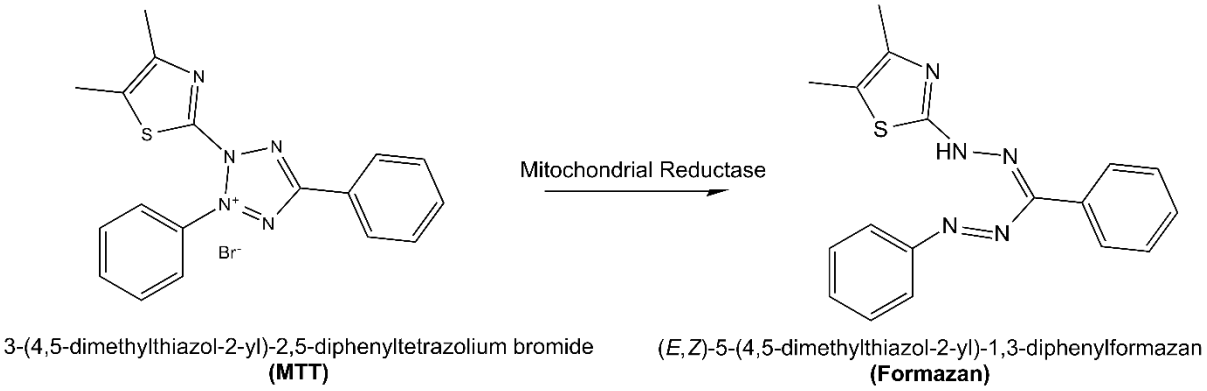

Figure 17: The MTT assay reaction. Yellow MTT salt is cleaved by the action of mitochondrial succinate dehydrogenase to form purple formazan crystals (https://upload.wikimedia.org/wikipedia/commons/d/de/MTT_reaction.png.)

3.6.1.2 Materials

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

3.6.1.3 Experimental procedure

Both AsPC-1 and IMR-32 cells were grown to reach a density of approximately 1 x 10^5 cells/ml and 100µl of cells at this density were seeded per well prior to siRNA transfection. Post transfection, cells were incubated for 72 hours at 37°C. Thereafter, 100µg of MTT dissolved in PBS was added to each well, followed by a 2 hour incubation period at 37°C. Post incubation, the MTT-containing medium was discarded from each well and the

remaining formazan crystals were dissolved in 500µl of DMSO. The absorbance of the resulting formazan solution was measured at 570nm using an ELISA plate reader. It is important to note that controls included untreated cells, negative control siRNA treated cells, and positive control protocatechuic acid (PCA) treated cells.

3.7 Analysis of cellular proliferation

3.7.1 Bromodeoxyuridine (BrdU) assay

3.7.1.1 Principle of the assay

This assay is a colorimetric immunoassay that quantifies bromodeoxyuridine (BrdU) that becomes incorporated into newly-formed DNA molecules of cells that are proliferating, as an indication of the proliferative state of cells. During the synthesis of nucleic acids, BrdU – which is an analogue of thymidine, becomes incorporated in place of thymidine (Fig.18). Use of a monoclonal detector antibody directed towards BrdU (anti-BrdU) and a horseradish peroxidase-conjugated antibody (directed towards the detector antibody) allows for the amount of actively proliferating cells to be determined. Essentially, horseradish peroxidase catalyzes the conversion of tetra-methylbenzidine (TMB), a chromogenic substrate, from a colourless solution to a blue solution – which can be quantified by measuring the absorbance using an ELISA plate reader.

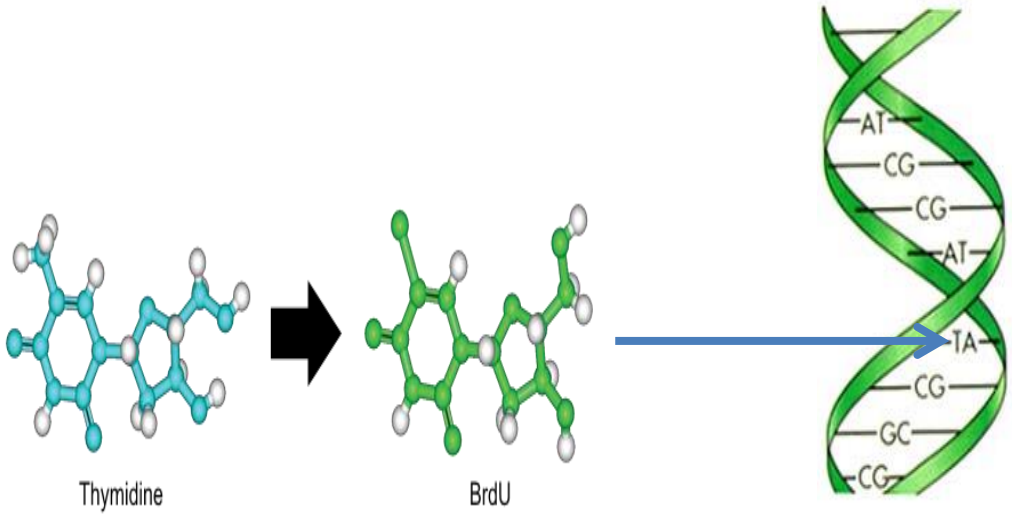

Figure 18: Principle of the bromodeoxyuridine (BrdU) assay. BrdU is an analogue that becomes incorporated into newly-formed DNA of actively proliferating cells in place of thymidine. Use of an antibody directed to BrdU facilitates detection and quantification of cell proliferation (Adapted from: https://www.abdserotec.com/brdu-bromodeoxyuridine.html).

3.7.1.2 Materials

- 24-well plate
- BrdU label
- Fixative/denaturing solution
- 100X anti-BrdU antibody
- BrdU antibody diluent
- Peroxidase goat anti-mouse IgG
- BrdU conjugate diluents
- TMB chromogenic substrate solution
- 20X plate wash concentrate
- Stop solution
- PBS
- Cell culture media
- De-ionized water

3.7.1.3 Experimental procedure

Post seeding and transfection of cells at a density of 1 x 10^5 cells/ml and 100µl of cells/well, 80µl of a working stock of BrdU (dilution of BrdU label in fresh culture medium (1:2000)) was added to each well followed by a 2-24 hour incubation period at 37°C. Following aspiration of contents, 800µl of fixative solution was loaded to each well and incubated for 30min at room temperature. After removal of the fixative by gentle blotting onto a paper towel, cells were subjected to an hour incubation in 100µl of anti-BrdU antibody (diluted to 1X by use of the antibody diluent). Prior to a 30min incubation of cells (at room temperature) with 400µl of the peroxidase anti-mouse IgG HRP-conjugate antibody (reconstituted in 1X PBS and further diluted 1:1000 with conjugate diluent), wells were washed thrice with wash buffer (diluted to 1X using deionized water). Thereafter, 400µl of substrate solution was loaded followed by a subsequent incubation period of 15min, after three washes with wash buffer. Finally, absorbance was measured within 30min of addition of stop solution (400µl) at dual wavelengths of 450-540 nm. It is noteworthy that in addition to untreated cells being used as a control, siRNA-scr and PCA were used as negative and positive controls, respectively.

3.8 Assessment of nuclear morphological changes

3.8.1 Confocal microscopy

3.8.1.1 Materials

- 4% paraformaldehyde
- PBS
- Hoescht 33342
- Slides
- Coverslips
- Mounting fluid

3.8.1.2 Methodology

This technique was employed in order to assess the nuclear morphological changes that occur after down-regulation of LRP/LR via the use of siRNA technology.

Firstly, cells were grown at an approximate density of $1x10^5$ cells/ml and 100µl of cells at this density were seeded onto coverslips prior to siRNA transfection. Thereafter, cells were fixed in 4% paraformaldehyde for 15 minutes followed by three washes with PBS. Excess PBS was blotted off between each PBS wash and post washing, cells were treated with Hoescht 33342 nuclear stain diluted in PBS (1:100) for 5 to 10 minutes in the dark. The coverslips containing the Hoescht-treated cells were then washed 2-3 times in PBS and thereafter, coverslips were mounted (cell side down) onto a clean microscope slide using mounting fluid. After setting for 45 minutes in the dark, slides were stored at 4ºC until ready to view using a confocal microscope. Importantly, controls included untreated cells, cells treated with siRNA-scr (negative control) and PCA (positive control).

3.9 Assessment of apoptotic induction

3.9.1 Annexin V-FITC/ 7-AAD assay

3.9.1.1 Principle of the assay

This assay is dependent on the ability of the calcium-dependent Annexin V protein to preferentially bind to the phosphatidylserine (PS) protein. Normally found to be located on

the intracellular membrane, PS translocates from the intracellular membrane to the outer surface during apoptosis and subsequent changes in the cellular surface (Fig.19). Translocation of PS to the outer surface enables Annexin V to bind to the protein in the presence of calcium, hence Annexin V staining allows for measurement of translocated PS and therefore allows for detection of apoptosis by means of flow cytometry. Additionally, 7-amino-actinomycin D (7-AAD), which is a red-fluorescent dye that binds specifically to guanine-cytosine DNA base pairs, is employed in this assay (Fig.19) - allow for distinction between early apoptotic and late apoptotic or necrotic cells. Thus, viable cells will show no fluorescence, whilst apoptotic cells will fluoresce green and dead cells green as well as red.

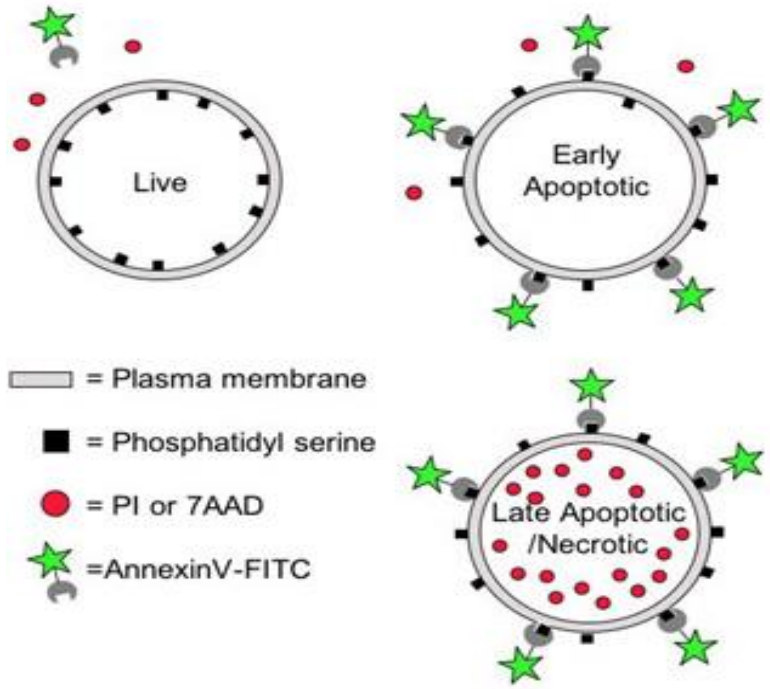

Figure 19: Principle of the Annexin V-FITC/ 7-AAD assay. Cells undergoing apoptosis experience a membrane-flip reaction that exposes phosphatidylserine (PS) on the outer cell membrane. Annexin V conjugated to FITC binds to this exposed PS, and FITC allows for detection of apoptotic cells. 7-AAD is a viability dye that is used to distinguish between early and late apoptotic cells (http://www.lifesci.dundee.ac.uk/sites/default/files/annexin.jpg).

3.9.1.2 Materials

- PBS
- Trypsin/EDTA
- Annexin V-FITC solution
- 1X Annexin-binding buffer
- 7-AAD viability dye

- Flow cytometer

3.9.1.3 Experimental procedure

Post seeding of cells to a density of 5 x 10^6 cells/ml and subsequent siRNA transfection, cells were washed with ice-cold PBS after harvesting with trypsin/EDTA and thereafter, cell suspensions were centrifuged at 500 x *g* for 5 minutes. This was followed by discarding of the supernatant and re-suspension of the cell pellet in 1X annexin-binding buffer. Subsequently, 10µl of Annexin V-FITC solution and 20µl of 7-AAD viability dye was added to 100µl of the cell suspensions. The cell suspensions were then incubated on ice for 15 minutes in the dark. Finally, samples were supplemented with 400µl of ice-cold 1X annexin binding buffer prior to flow cytometric analysis which was performed within 30 minutes. It must be noted that untreated cells, siRNA-scr treated cells, and PCA treat cells were used as controls, as with previously-described methods. The FL1 laser was used to detect Annexin V-FITC staining and FL3 was used for detection of 7-AAD staining. Compensation and quadrants were set using the following controls: unstained cells, Annexin V-FITC stained cells, 7-AAD stained cells, cells stained with both Annexin V-FITC and 7-AAD, and PCA-treated cells stained with both Annexin V-FITC and 7-AAD.

3.9.2 Caspase-3, -8 and -9 assays

3.9.2.1 Principle of the assays

Caspase-3, -8 and -9 assays are colorimetric assays that rely on caspase-mediated hydrolysis of a chromophore known as *p*-nitroaniline (*p*-NA) that is coupled to a peptide substrate. In the case of caspase-3 assays, the peptide substrate is Asp-Glu-Val-Asp *p*-nitroaniline (DEVD-*p*-NA). Caspase-8 and -9 assays make use of substrates known as Ile-Glu-Thr-Asp *p*-nitroaniline (IETD-*p*-NA) and Leu-Glu-His-Asp *p*-nitroaniline (LEHD-*p*-NA), respectively. In essence, each caspase recognizes a specific peptide sequence and subsequently cleaves that peptide, thereby releasing the *p*-NA chromophore – which possesses a high absorbance at 405nm. Therefore, the concentration of *p*-NA released can be used as a directly-proportional indicator of caspase activity and consequently the occurrence of apoptosis.

3.9.2.2 Materials

- Cell lysis buffer*
- 2X reaction buffer*

- *p*-NA-coupled peptide substrate (4mM)*
- DTT (1mM)
- Eppendorf tubes
- 96-well plates

***N.B.: Each of these components differ depending on the type of caspase assay being performed**

3.9.2.3 Experimental procedure

Cells were grown to reach a density of $1x10^6$ cells/ml and after siRNA transfection, $1x10^6$ cells were pelleted by centrifugation at 1200 rpm for 10 minutes. Thereafter, cells were re-suspended in 50µl of cell lysis buffer and incubated on ice for 10 minutes. This was followed by centrifugation of cells at 10000 x *g* for 1 minute. The supernatant was then transferred to a fresh Eppendorf tube which was immediately placed on ice and the pellet was discarded. Following this, the protein concentration of the supernatant was determined by use of a BCA™ assay (as described in section 3.3.2) and subsequently, 200µg of protein was diluted to 50µl of cell lysis buffer per sample and added to appropriate wells of a 96-well plate. The 2X reaction buffer was then supplemented with DTT (10µl DTT/1ml reaction buffer) and 50µl of this solution was added to each sample, followed by the addition of 5µl of peptide substrate. After a 2 hour incubation period at 37ºC, the plate was read at 405nm using an ELISA plate reader. In addition to untreated cells, siRNA-scr treated and PCA treated cells were used as negative and positive controls, respectively.

3.10 Statistical evaluation

The two-tailed Student's t-test with a confidence interval of 95% was used in order to analyse the data, with p-values of less than 0.05 being considered as significant. This statistical analysis was done using Graphpad Prism version 5.03. Pearson's correlation coefficient was also used to measure the correlation between LRP/LR levels and cell viability, cell proliferation and apoptotic induction. A positive coefficient was an indication of direct proportionality between the two variables, whereas a negative coefficient implied inverse proportionality. Values close to or rounding off to 1 signifies high positive correlation.

CHAPTER 4: RESULTS

4.1 High percentages of pancreatic cancer and neuroblastoma cells exhibit LRP/LR on their cell surface.

It is understood that LRP/LR plays a pivotal role in the maintenance of cellular viability. Therefore, in light of the present study, it was necessary to confirm the presence of LRP/LR on the cell surface and to quantify the percentage of cells displaying cell surface LRP/LR within a specific cell population. This was achieved by use of flow cytometry. Figure 20 depicts that both of the tumourigenic cell lines exhibited high percentages of cells within a given cell population that display LRP/LR on their cell surface. The distinct shift between the peaks is characteristic of changes in fluorescence intensity occurring as a result of treatment of cells with anti-LRP/LR specific antibody IgG1-iS18 and a relevant secondary antibody that is conjugated to a fluorochrome. It was observed that 93.48% of pancreatic cancer (AsPC-1) cells and 84.90% neuroblastoma (IMR-32) cells displayed LRP/LR on the cell surface, thus confirming the presence of the protein on the surface of both of the cell lines in question.

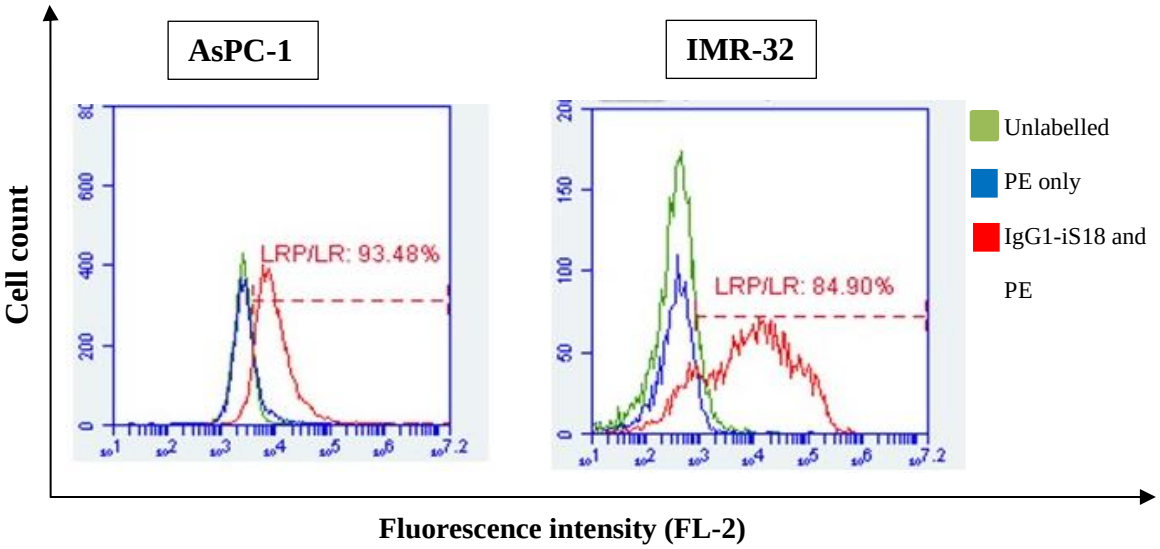

Figure 20: Detection of the 37kDa/67kDa laminin receptor LRP/LR levels on the surface of pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells. The green peak is representative of cells that have not been labelled with antibodies, whilst the blue peak represents cells that have been stained only with goat anti-human phycoerythin (PE)-coupled secondary antibody. The red peak is indicative of cells that have been stained with both anti-LRP/LR specific antibody IgG1-iS18 and the afore-mentioned secondary antibody. It is noteworthy that the inclusion of an unlabelled control confirms that the secondary antibody does not bind non-specifically, hence no shift in fluorescence intensity is observed between the green and blue peaks. The shifts observed between the green and red peaks indicate the presence of LRP/LR on the cell surface of both the tumourigenic cell lines under study. The above plots represent an average of experiments that were carried out in triplicate and repeated at least three times, with 20 000 cells being counted per sample and samples being analysed using filter 2 (FL-2) which is specific for the PE fluorochrome.

Similarly, anti-CAT specific antibody targeted to chloramphenicol acetyltransferase (CAT) was used as a negative control due to the absence of CAT on the surface of mammalian cells. As expected, both cell lines showed no shift in fluorescence intensity, thus indicating that CAT was indeed absent on the cell surface of the tumourigenic cell lines and therefore served as an effective negative control (Fig.21).

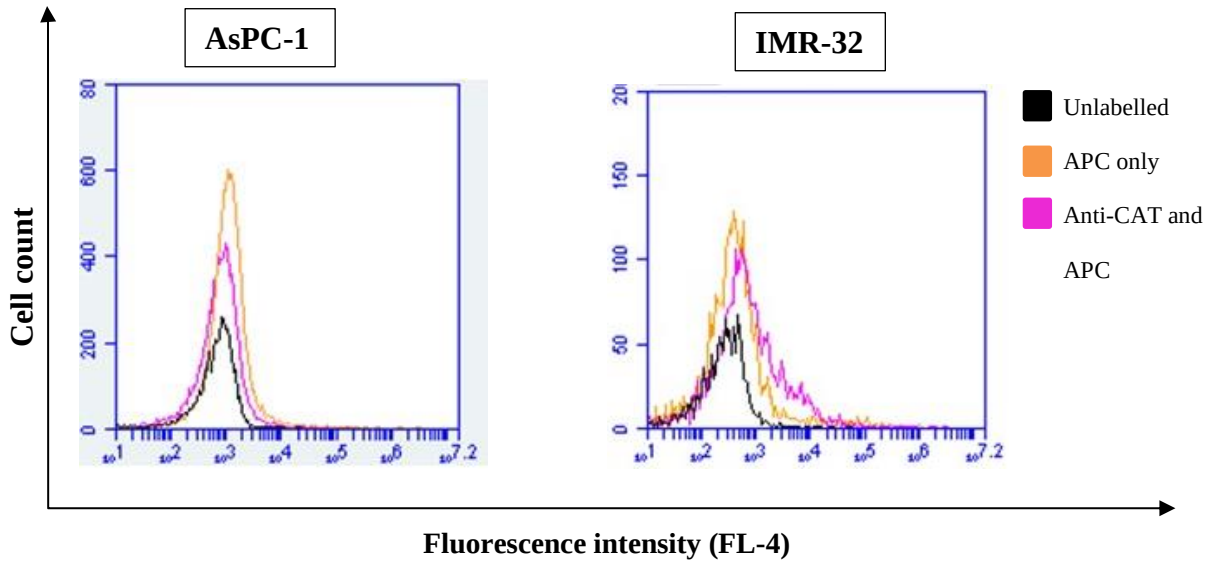

Figure 21: Determination of chloramphenicol acetyltransferase levels on the surface of pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells. The black peak is indicative of cells that are unlabelled, whilst the orange peak is representative of cells that have been stained with goat anti-rabbit allophycocyanin (APC)-coupled secondary antibody only. The pink peak represents cells that have been labelled with both anti-CAT primary antibody and the afore-mentioned secondary antibody which is specific for the primary antibody. No distinct shift was seen between the black and orange peaks, thus indicating that the secondary antibody did not bind non-specifically. Moreover, no shift was observed between the unlabelled peak and the peak representing cells stained with both primary and secondary antibodies – thereby indicating the absence of the CAT protein on the surface of both the tumourigenic cell lines in question. It is noteworthy that the above plots are representative of an average of experiments that were carried out in triplicate and repeated at least three times, with 20 000 cells being counted per sample. Samples were analysed using filter 4 (FL-4) which is specific for detection of the APC fluorochrome.

4.2 Neuroblastoma cells exhibit higher cell surface LRP/LR levels in comparison to pancreatic cancer cells.

In addition to determining the percentage of cells within a given population that exhibit cell surface LRP/LR, the actual levels of cell surface LRP/LR within that cell population was also analysed using flow cytometry. In essence, the same concentration of the primary antibody IgG1-iS18 and PE-coupled secondary antibody was used to label the same number of cells (20 000) within a specific population of both cell lines over the same period of time. It therefore follows that the more LRP/LR present on the surface of these cancer cells, the

higher the level of binding of the IgG1-iS18 primary antibody and subsequently the IgG-specific PE-coupled secondary antibody will be. Therefore, the median fluorescence intensity (MFI) can be used as an indicator of differential cell surface LRP/LR expression (Table 1). It was observed that neuroblastoma (IMR-32) cells display significantly higher cell surface LRP/LR levels (70% more) in comparison to pancreatic cancer (AsPC-1) cells, as depicted in figure 22.

Table 1: Median fluorescence intensity (MFI) values used to determine differential expression of LRP/LR on the surface of AsPC-1 and IMR-32 cells.

Cell line	MFI of unlabelled cells (A)	MFI of cells stained with IgG1-iS18 and PE-coupled secondary antibody (B)	Difference in MFI values i.e. (B) – (A)
AsPC-1	6857.19	10768.00	3910.83
IMR-32	996.17	13690.33	12694.17

***All MFI values are representative of an average of experiments carried out in triplicate and repeated at least three times.**

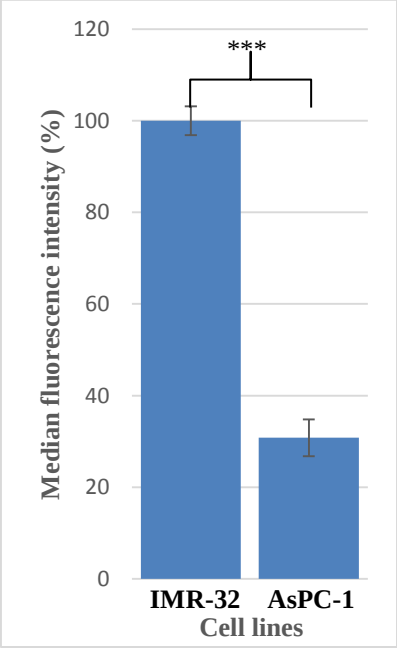

Figure 22: Quantification of LRP/LR levels on the surface of pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells by flow cytometry. For both tumourigenic cell lines, cells were labelled with primary antibody IgG1-iS18 and anti-human phycoerythrin (PE)-coupled secondary antibody. Analysis was performed on 20 000 cells per sample and the median fluorescence intensity (MFI) was used as an indicator of differential cell surface LRP/LR expression across both cell lines. It is noteworthy that the MFI values presented in the last column of table 1 was used in the construction of this graph and the MFI value representative of the IMR-32 cell line was set to 100%. IMR-32 cells were found to have 70% more cell surface LRP/LR than AsPC-1 cells. Experiments were carried out in triplicate and repeated at least three times. ***$p<0.0001$

A similar analysis was performed using the median fluorescence intensity values obtained for the CAT negative control protein (Table 2). As previously described, 20 000 cells within a specific population of each cell line was labelled with the same concentration of primary anti-CAT antibody and goat anti-rabbit allophycocyanin (APC)-coupled secondary antibody and incubated over the same time period. Thus, MFI values could be used as an indicator of differential surface expression of CAT protein levels across both the tumourigenic cell lines. As was expected, no significant difference was observed in cell surface CAT expression between AsPC-1 and IMR-32 cells (Fig.23).

Table 2: Median fluorescence intensity (MFI) values used as an indicator of differential expression of chloramphenicol acetyltransferase (CAT) on the surface of AsPC-1 and IMR-32 cells.

Cell line	MFI of unlabelled cells (A)	MFI of cells stained with anti-CAT primary and APC-coupled secondary antibody (B)	Difference in MFI values i.e. (B) – (A)
AsPC-1	427.33	819.00	391.67
IMR-32	99.33	446.33	347.00

***All MFI values are representative of an average of experiments carried out in triplicate and repeated at least three times.**

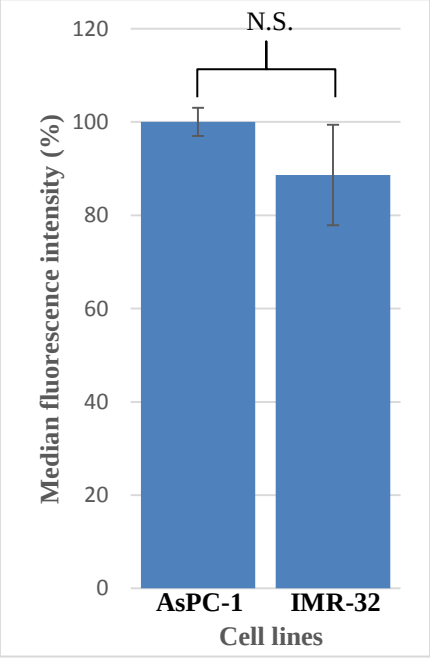

Figure 23: Quantification of cell surface chloramphenicol acetyltransferase (CAT) levels on pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells by flow cytometry. Both cell lines were labelled with anti-CAT primary antibody and allophycocyanin (APC)-coupled secondary antibody. Median fluorescence intensity (MFI) values were used to assess differential expression of CAT on the cell surface of both cell lines and 20 000 cells were analysed per sample. MFI values given in the last column of table 2 were used to construct this graph and the value corresponding to AsPC-1 cells was set to 100%. Experiments were performed in triplicate and repeated at least three times. N.S.:p= 0.2225, non-significant.

4.3 Neuroblastoma cells display significantly higher total LRP/LR levels in comparison to pancreatic cancer cells.

As previously stated, LRP/LR is not solely expressed on the cellular surface and is additionally found in the cytosol, nucleus and the perinuclear region. Therefore, Western blotting was carried out in order to determine total LRP/LR levels across both of the tumourigenic cell lines under study. Experiments were performed in triplicate and repeated three times, and a representative blot (Fig.24A) is given for both LRP/LR and β-actin – with the latter being used as a loading control. It is important to note that anti-LRP/LR specific antibody IgG1-iS18 was only successful in detection of the 37kDa laminin receptor precursor (LRP) form. The Western blots revealed that both the pancreatic cancer (AsPC-1) and the neuroblastoma (IMR-32) cells express LRP. However, densitometric quantification suggested that IMR-32 cells display 63% more total LRP when compared to AsPC-1 cells (Fig.24B).

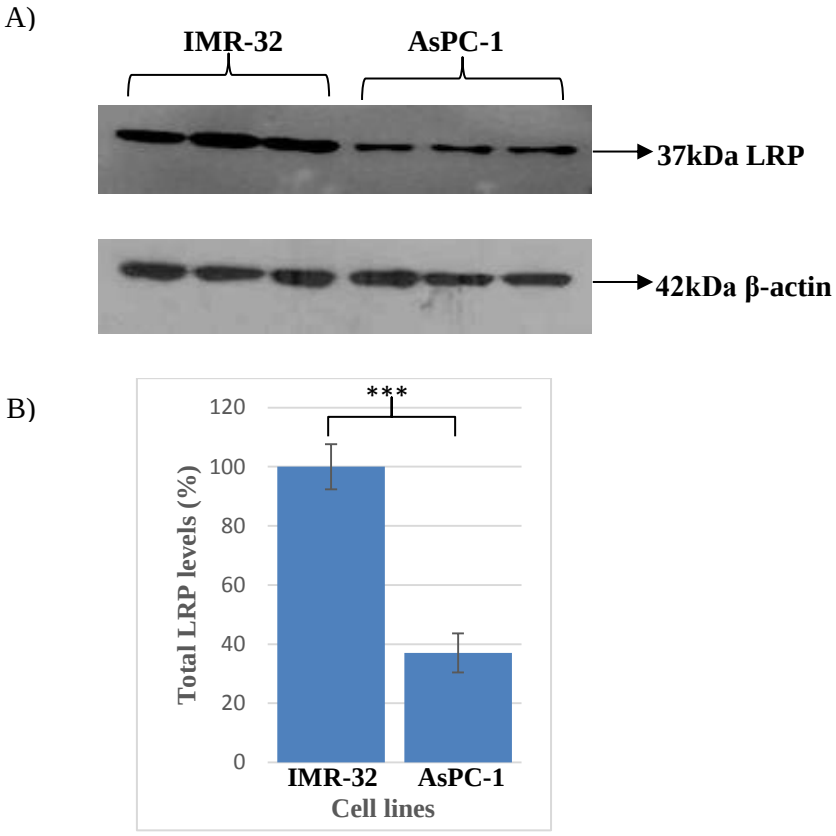

Figure 24: Assessment of relative expression of total 37kDa laminin receptor precursor (LRP) levels on pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells. A) Anti-LRP/LR specific primary antibody IgG1-iS18 and a HRP-coupled secondary antibody specific for the primary antibody was used to detect total 37kDa LRP levels in both of these tumourigenic cell lines. β-actin was used as a loading control. B) Densitometric analysis performed on these blots revealed that IMR-32 cells possess 63% more total LRP in comparison to AsPC-1 cells. It is noteworthy that the data is representative of an average of experiments performed in triplicate and repeated three times. Values obtained from quantification of LRP were divided by values obtained from β-actin quantification, and the resultant values were used in the construction of this graph. The resultant value for IMR-32 cells was set to 100%. ***p=0.0003

4.4 Use of siRNA technology leads to successful down-regulation of LRP expression in pancreatic cancer and neuroblastoma cells.

In order to gain insight into the effect of LRP/LR expression on cellular viability, siRNA-mediated knockdown of LRP/LR was performed. Post transfection of pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells with siRNA-LAMR1 (specifically targeting the mRNA of the 37kDa LRP), Western blot analysis and subsequent densitometry was performed as previously described. Densitometric analysis revealed that LRP was successfully down-regulated in both AsPC-1 (Fig.25A and 25B) and IMR-32 (Fig.26A and 26B) cells by transfection with the afore-mentioned siRNA. It was observed that siRNA-LAMR1 transfected AsPC-1 and IMR-32 cells exhibited a 90% and 71% reduction in LRP expression, respectively, when compared to non-transfected cells whose LRP/LR levels were set to 100%. Moreover, transfection of AsPC-1 cells with siRNA-scr (negative control scrambled siRNA) showed no significant LRP knockdown when compared to non-transfected cells (Fig.25C and 25D). Likewise, the same effect was observed when IMR-32 cells were transfected with siRNA-scr (Fig.26C and 26D).

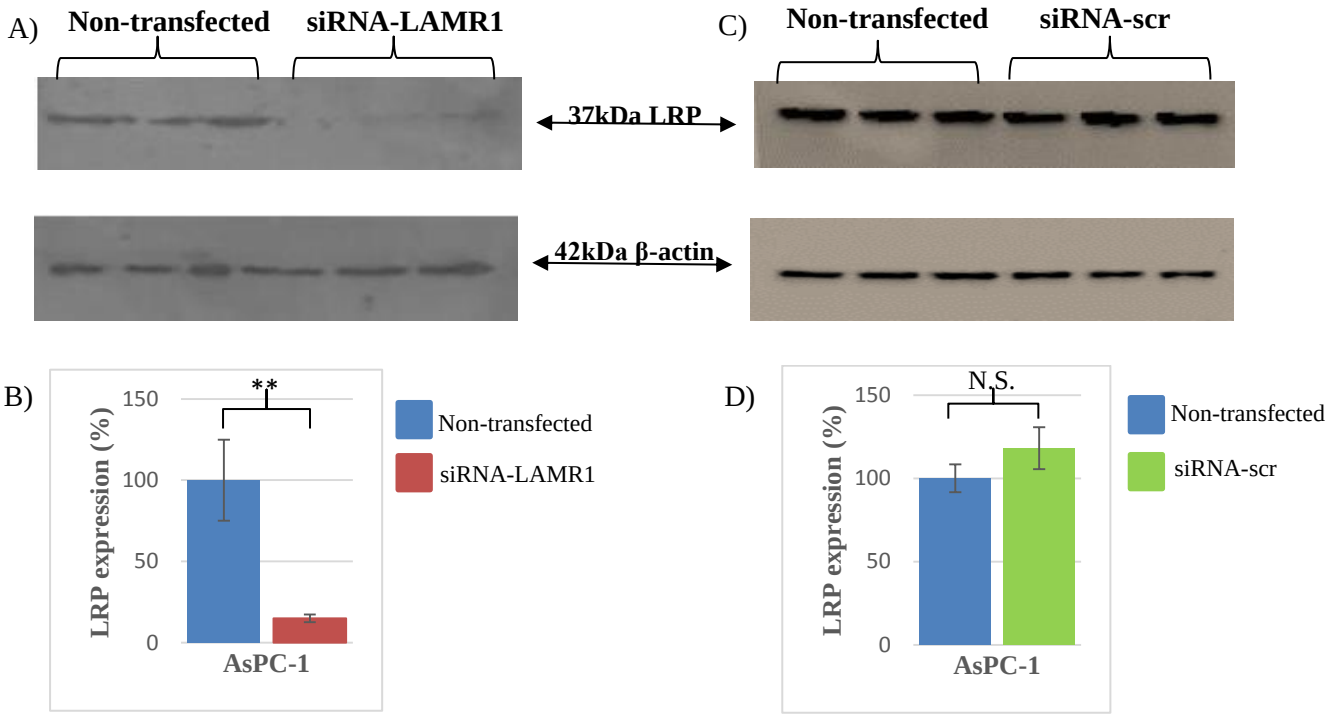

Figure 25: LRP expression in pancreatic cancer (AsPC-1) cells after siRNA transfection. A and B) Transfection of AsPC-1 cells with siRNA-LAMR1 resulted in significant LRP knockdown of 90% when compared to non-transfected cells. Densitometry values obtained for LRP were divided by those for the β-actin loading control and resultant values were used to construct this graph. The value for the non-transfected cells was set to 100%. C and D) Densitometry revealed no significant difference in LRP expression between non-transfected and siRNA-scr control transfected cells. **p=0.0086, N.S.:p=0.1665. Graphs are representative of an average of experiments performed in triplicate and repeated three times.

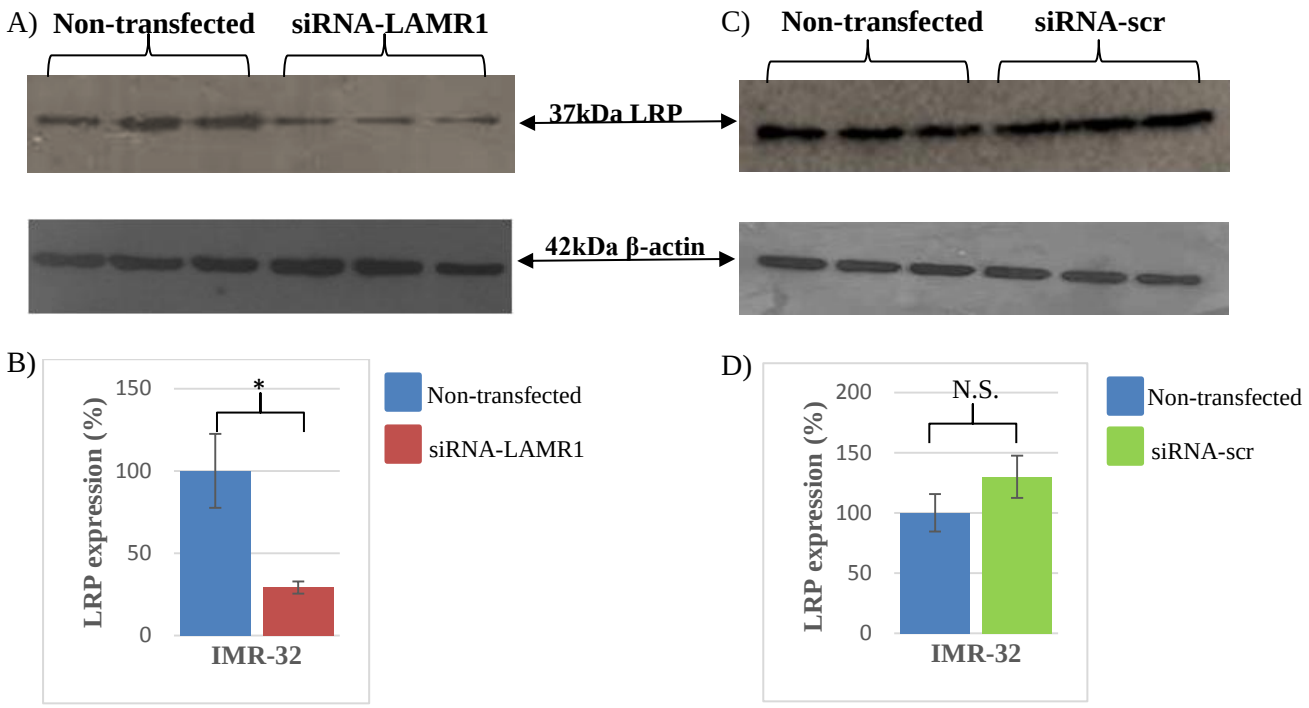

Figure 26: Detection of LRP expression in neuroblastoma (IMR-32) cells post siRNA transfection. A and B) Densitometry revealed that there was a significant reduction of 71% in LRP expression after transfection of IMR-32 cells with siRNA-LAMR1 when compared to non-transfected cells. Densitometry values obtained for LRP were divided by values for the β-actin loading control. Resultant values were used in the construction of this graph and the value for non-transfected cells was set to 100%. C and D) No significant difference in LRP expression was observed between non-transfected and siRNA-scr transfected cells. Graphs depict values that are an average of experiments done in triplicate and repeated three times. *p=0.0117, N.S.: p=0.1148 hence non-significant.

4.5 siRNA-mediated down-regulation of LRP expression results in significantly reduced viability of pancreatic cancer and neuroblastoma cells.

After siRNA-mediated knockdown of LRP was observed, the effect thereof on cellular viability was analysed. Following treatment of AsPC-1 and IMR-32 cells with siRNA-LAMR1, MTT assays revealed that cellular viability was significantly reduced in comparison to controls. This signifies that siRNA-mediated LRP knockdown leads to the observed decreases in cellular viability in both of the tumourigenic cell lines. It was seen that AsPC-1 and IMR-32 cells treated with siRNA-LAMR1 exhibited 82% and 75% reductions in cellular viability, respectively, when compared to non-transfected cells whose cell viability readings were set to 100% (Fig.27). Moreover, no significant reduction in cellular viability was observed for both cell lines when treated with the negative control siRNA-scr and compared to non-transfected cells (Fig.27). Protocatechuic acid (PCA) was used as a positive control treatment as it is a known apoptosis inducer which acts by impacting the activity of cell-cycle regulatory proteins and inflammatory cytokines – thereby hampering oncogenic cell growth[173].

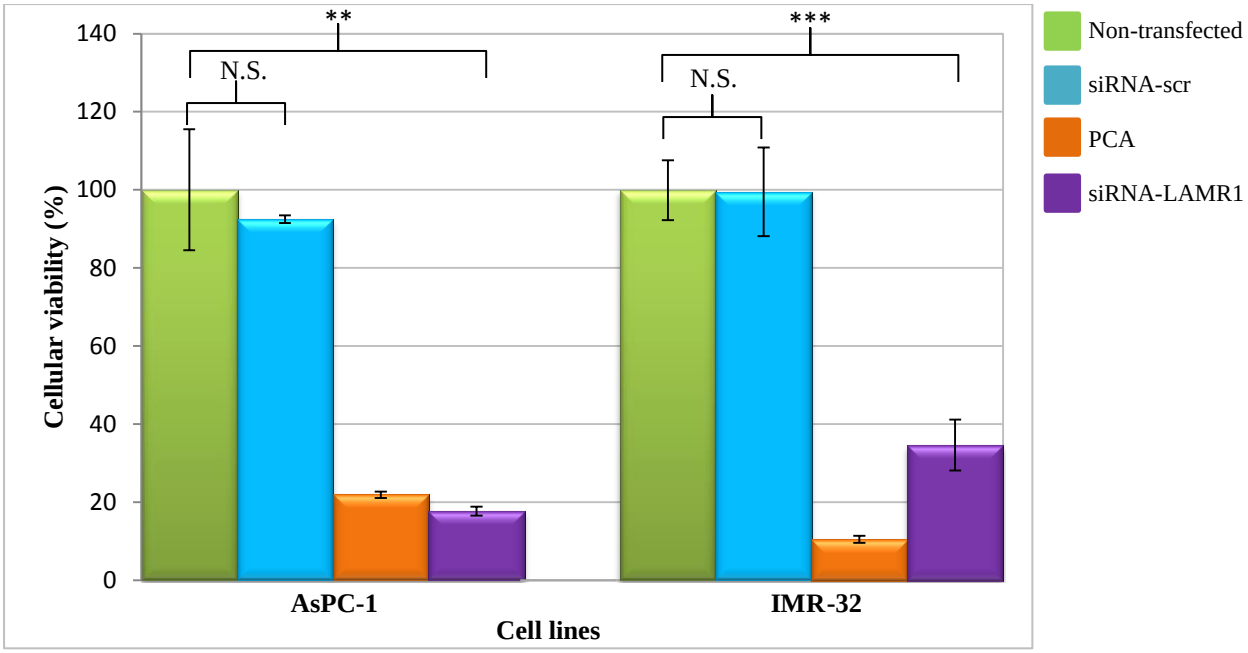

Figure 27: The effect of siRNA-mediated LRP knockdown on the viability of pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells. MTT assays were used to analyse the viability of AsPC-1 and IMR-32 cells after treatment with siRNA-LAMR1. The value for non-transfected cells was set to 100% for both cell lines and by comparison, AsPC-1 and IMR-32 cells treated with siRNA-LAMR1 displayed a significant reduction of 82% and 65% in cellular viability, respectively. Cells treated with the negative control siRNA-scr showed no significant differences in cellular viability when compared to non-transfected cells for both cell lines. Protocatechuic acid (PCA) was used as a positive control. Graph is representative of an average of experiments performed in triplicate and repeated three times. **p=0.0017, ***p=0.0008, N.S.: p>0.05 hence non-significant.

4.6 Use of an alternative siRNA confirms that LRP down-regulation is responsible for reductions in cellular viability in pancreatic cancer and neuroblastoma cells.

In order to ascertain whether the observed reduction in cellular viability occurred as a result of siRNA-LAMR1-mediated LRP down-regulation and not an off-target effect, another siRNA that targets a different region of LRP was used. Post transfection of cells with this alternative siRNA (esiRNA-RPSA), Western blot analysis and densitometry was performed. By comparison to non-transfected cells (which was set to 100%), pancreatic cancer (AsPC-1) cells showed a significant knockdown of 83% in LRP expression (Fig.28A and 28B). Similarly, neuroblastoma (IMR-32) cells exhibited a significant down-regulation of 64% in LRP expression when compared to non-transfected cells (Fig.29A and 29B). Additionally, treatment of AsPC-1 cells with esiRNA-RLUC (negative control siRNA) displayed no significant difference in LRP expression when compared to non-transfected cells (Fig.28C and 28D). Likewise, the same effect was observed for IMR-32 cells treated with esiRNA-RLUC (Fig.29C and 29D).

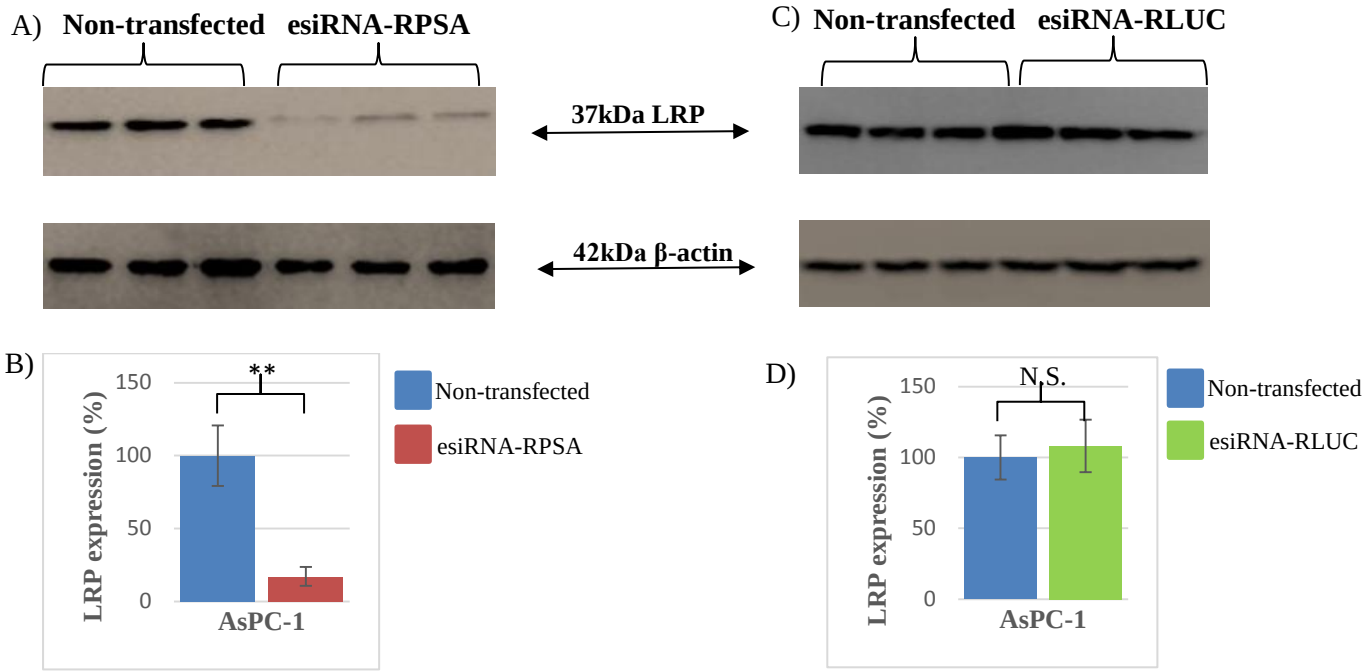

Figure 28: Detection of LRP expression in pancreatic cancer (AsPC-1) cells post transfection with an alternative siRNA. A and B) After transfection of cells with esiRNA-RPSA, densitometry showed that there was a significant reduction of 83% in LRP expression when compared to non-transfected cells, which was set to 100% as previously described. β-actin was used as a loading control. C and D) Densitometry revealed that there was no significant difference in LRP expression between cells treated with the negative control siRNA (esiRNA-RLUC) and non-transfected cells. Graphs are representative of an average of experiments performed in triplicate and repeated three times. **p=0.0057, N.S.:p=0.6613 hence non-significant.

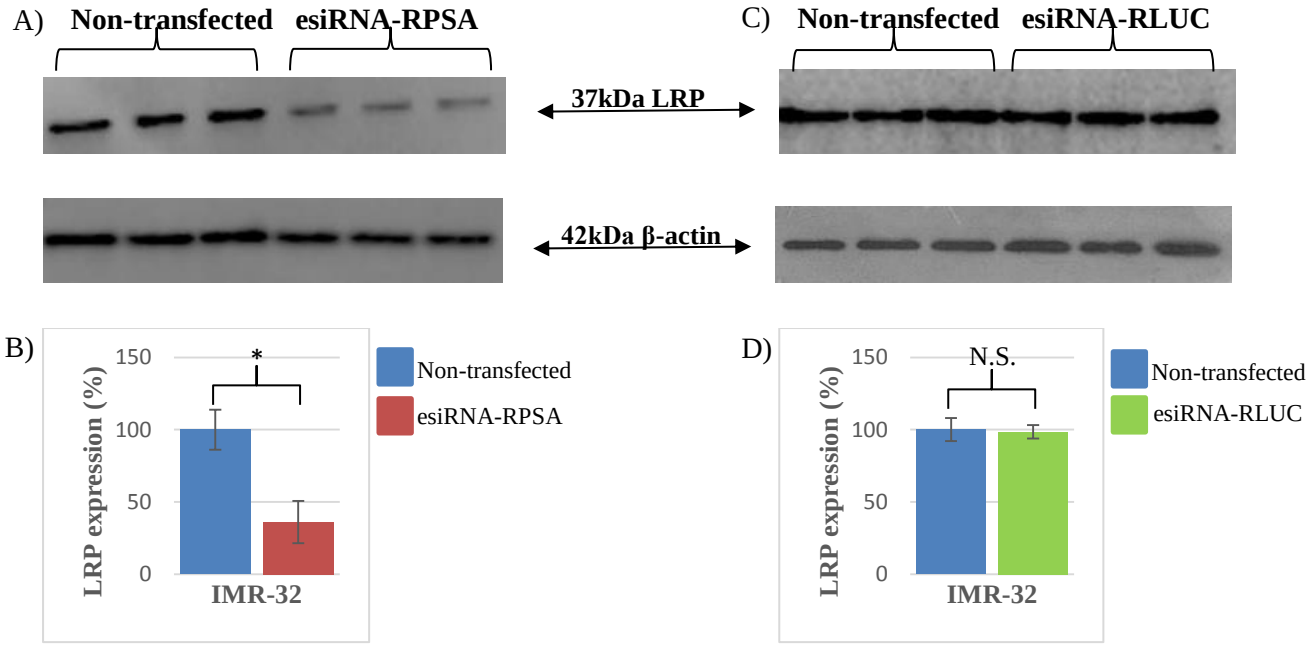

Figure 29: LRP expression in neuroblastoma (IMR-32) cells after transfection with an alternative siRNA. A and B) Densitometry revealed that after treatment of IMR-32 cells with esiRNA-RPSA, there was a significant reduction of 64% in LRP expression when compared to non-transfected cells. Non-transfected cells were set to 100% to allow for comparison and β-actin was used as a loading control. C and D) Densitometric quantification showed no significant difference in LRP expression between non-transfected cells and cells treated with esiRNA-RLUC (negative control siRNA). Graphs are indicative of an average of experiments carried out in triplicate and repeated three times. *p=0.0250, N.S.: p=0.8402.

To assess the effects of esiRNA-RPSA-mediated LRP knockdown on the viability of AsPC-1 and IMR-32 cells, MTT assays were performed. It was found that treatment of cells with this alternative siRNA resulted in significantly reduced cellular viability in both the tumourigenic cell lines under study. Specifically, AsPC-1 cells showed an 82% reduction in cellular viability post siRNA treatment whilst IMR-32 cells exhibited a 60% reduction when compared to non-transfected cells – which were set to 100% (Fig.30). Moreover, treatment of both cell lines with the negative control siRNA (esiRNA-RLUC) did not result in a significant change in cellular viability when compared to non-transfected cells (Fig.30). Only Western blotting and MTT assays were repeated using the alternative siRNA and control siRNA. The same trend is seen with both siRNA-LAMR1 and esiRNA-RPSA with regards to cell viability studies, confirming that it is the specific targeting and knock-down of LRP/LR that reduces the cell viability in the tumourigenic cells being investigated.

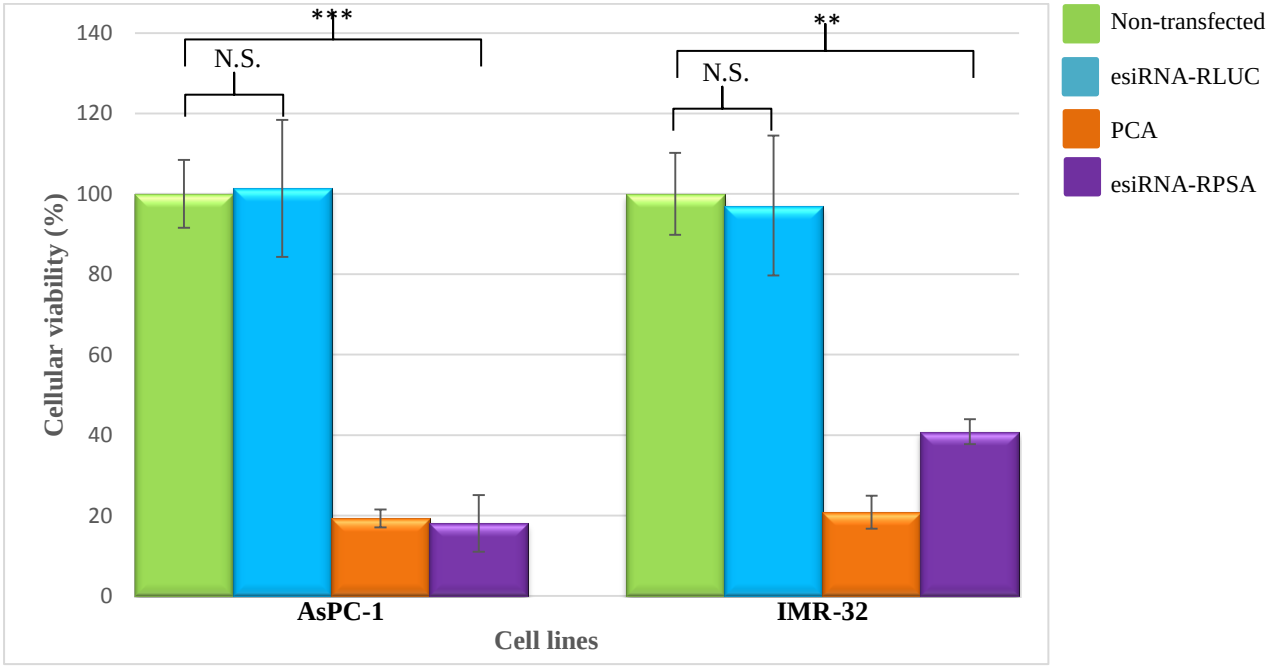

Figure 30: The effect of an esiRNA-RPSA-mediated LRP down-regulation on the viability of pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells. Post treatment of cells with this alternative siRNA, MTT assays were performed to assess cellular viability. Compared to non-transfected cells (set to 100%), AsPC-1 and IMR-32 cells showed reductions of 82% and 60% in cellular viability, respectively, after being treated with esiRNA-RPSA. No significant differences were observed in the viability of cells treated with the negative control siRNA (esiRNA-RLUC) when compared to non-transfected cells for both cell lines. PCA was used as a positive control and the graph is representative of an average of experiments performed in triplicate and repeated three times. **p=0.0014, ***p=0.0005, N.S.: p>0.05 therefore non-significant.

All of the results that follow are representative of experiments performed using only siRNA-LAMR1 and siRNA-scr.

4.7 Knockdown of LRP expression by siRNA significantly reduces proliferation of pancreatic cancer and neuroblastoma cells.

Due to the observed reduction in cellular viability of pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells upon treatment with siRNA, the effect of siRNA-mediated down-regulation of LRP expression on cellular proliferation was also investigated – since a loss in cellular viability could be a result of reduced proliferation rates. Cellular proliferation was analysed by use of BrdU assays.

It was observed that AsPC-1 and IMR-32 cells showed significant reductions of 76% and 44% in cellular proliferation, respectively, after being treated with siRNA-LAMR1 and compared to non-transfected cells which were set to 100% (Fig.31). Furthermore, treatment of both cell lines with the negative control siRNA (siRNA-scr) showed no significant differences in cellular proliferation when compared to non-transfected cells (Fig.31).

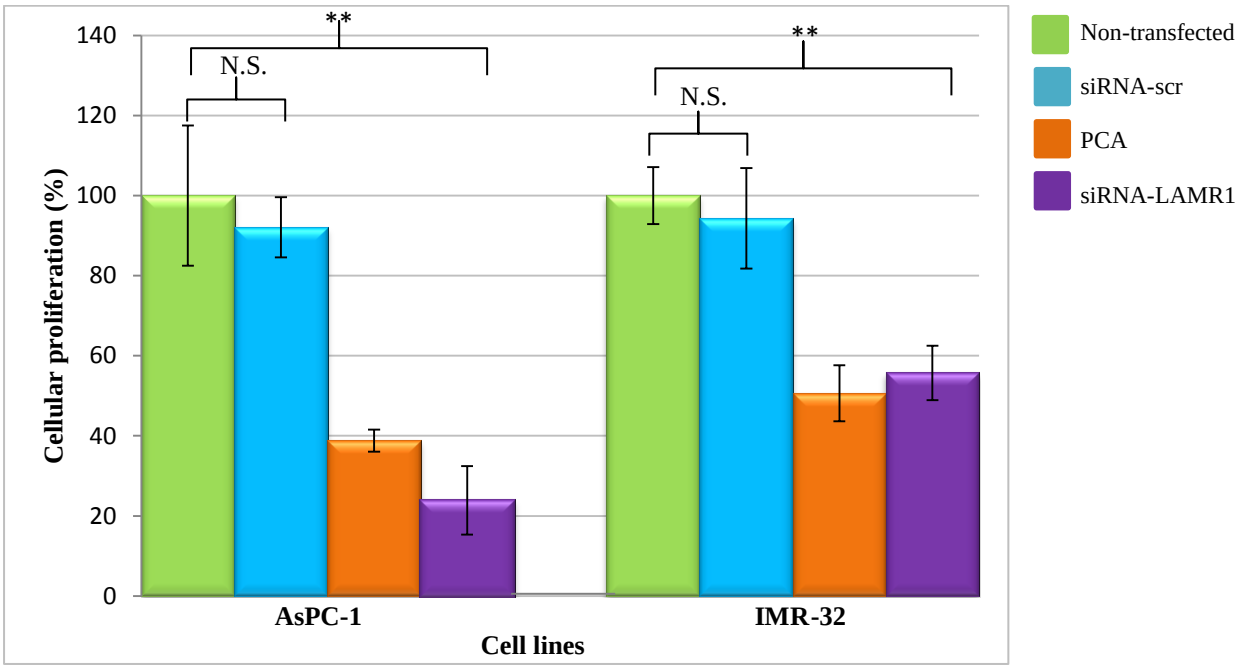

Figure 31: The effect of siRNA-mediated LRP knockdown on the proliferation of pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells. Post treatment of cells with siRNA-LAMR1, AsPC-1 cells showed a 76% reduction in cellular proliferation when compared to non-transfected cells (set to 100%). IMR-32 cells showed a decrease of 44% in proliferation after siRNA-LAMR1 treatment by comparison to non-transfected cells. Treatment of both cell lines with the negative control siRNA-scr revealed no significant changes in cellular proliferation when compared to non-transfected cells. Protocatechuic acid was used as a positive control and the graph is representative of an average of experiments carried out in triplicate and repeated three times. **p=0.0053 and 0.0031 for AsPC-1 and IMR32 cells, respectively. N.S.: p>0.05 thus non-significant.

4.8 siRNA-mediated knockdown of LRP expression results in nuclear morphological changes suggestive of apoptotic induction in pancreatic cancer and neuroblastoma cells.

To examine if apoptotic induction led to the reductions in cellular viability post siRNA-LAMR1 treatment, possible nuclear morphological changes were detected by confocal microscopy. Compared to the nuclei of non-transfected cells (Fig.32A), siRNA-LAMR1 treated pancreatic cancer (AsPC-1) cells displayed nuclear morphological changes such as the formation of apoptotic bodies (Fig.32D). Similarly, comparison of nuclei of siRNA-LAMR1 treated neuroblastoma (IMR-32) cells (Fig.33D) to the nuclei of non-transfected cells (Fig.33A) revealed nuclear morphological changes such as nuclear shrinkage, membrane blebbing and the formation of apoptotic bodies. Moreover, treatment of both cell lines with the negative control siRNA-scr did not result in nuclear morphological changes when compared to non-transfected cells (Fig.32B and 33B). PCA was used as a positive control for studying nuclear morphological changes under apoptotic conditions (Fig.32C and 33C).

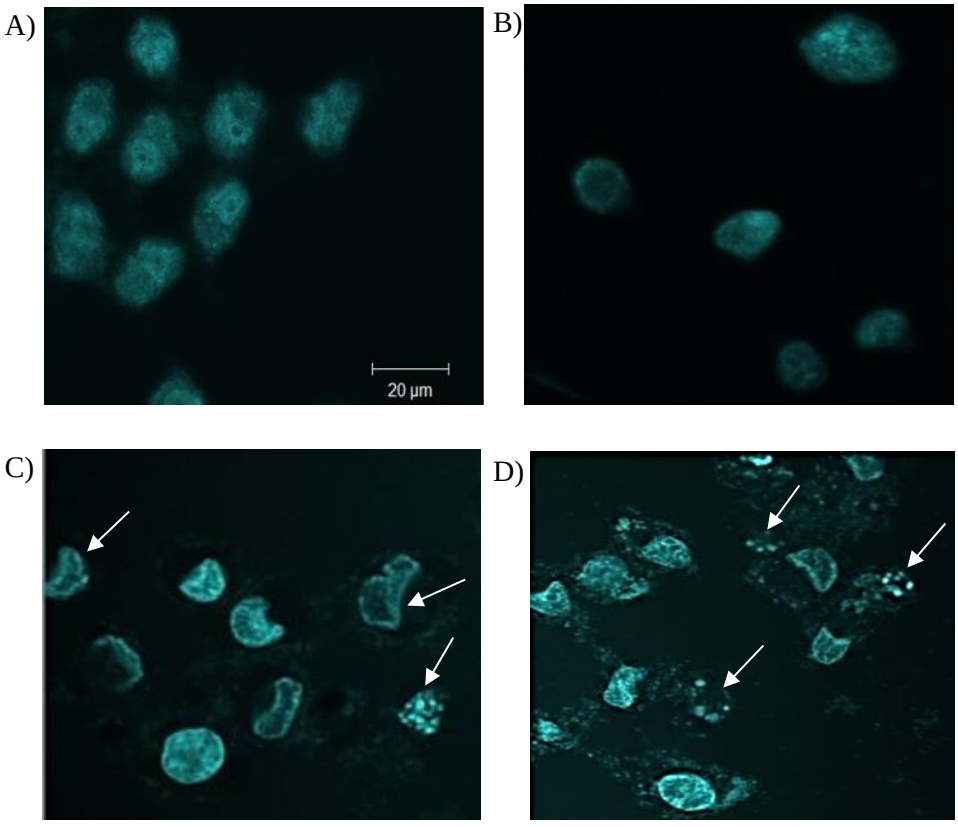

Figure 32: The effect of siRNA-mediated LRP knockdown on nuclear morphology of pancreatic cancer (AsPC-1) cells. A) Non-transfected cells showed normal rounded nuclei with uncompromised integrity. B) Cells treated with the negative control siRNA-scr displayed nuclei similar to that of the non-transfected cells. C) PCA treated cells showed nuclei that have undergone membrane blebbing and apoptotic body formation (indicated by white arrows). D) siRNA-LAMR1 transfected cells exhibited diminished nuclear integrity and the formation of apoptotic bodies (shown by white arrows), suggesting apoptotic induction**.** All images were obtained at a magnification of 63X. Scale bar applies to all images.

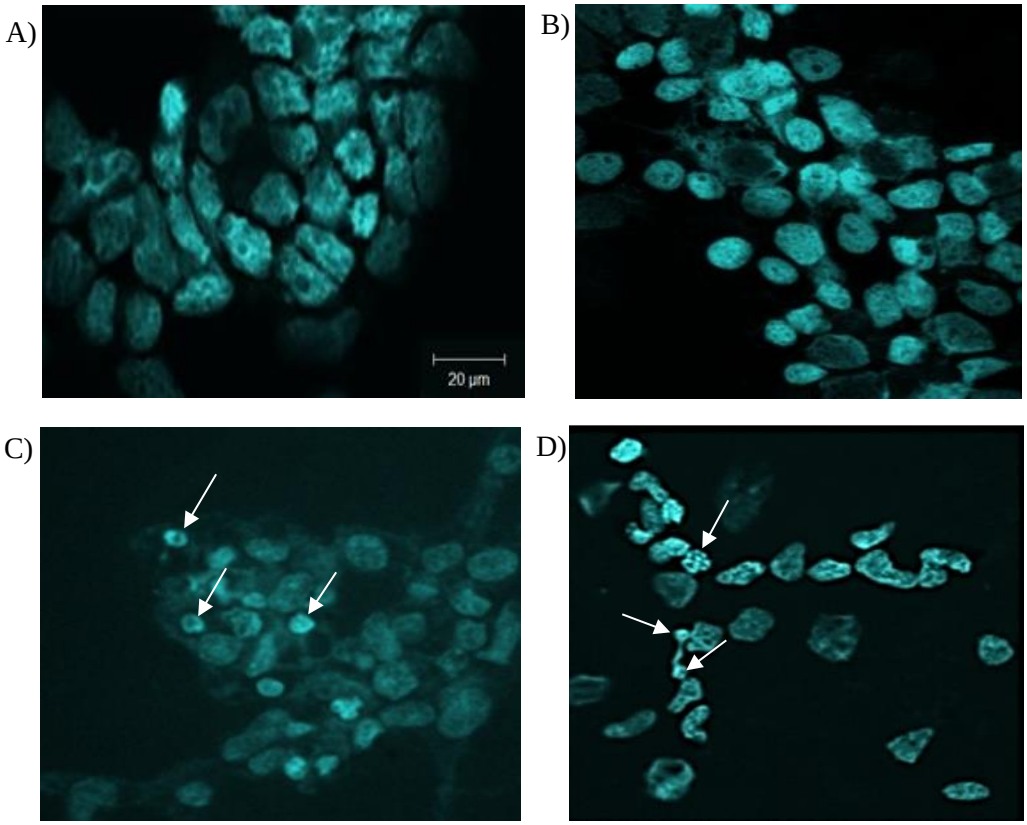

Figure 33: Changes in nuclear morphology in neuroblastoma (IMR-32) cells after siRNA-mediated LRP down-regulation. A) Nuclear integrity is maintained in non-transfected cells, evident by the normal rounded shape of nuclei. B) Negative control siRNA-scr transfected cells showed no interruptions in nuclear morphology, as expected. C) Positive control PCA treated cells showed nuclear shrinkage (indicated by white arrows). D) siRNA-LAMR1 transfected cells showed compromised nuclear integrity, made clear by the observation of nuclear shrinkage and apoptotic body formation (shown by white arrows). All images were obtained at 63X magnification and the scale bar applies to all images.

4.9 siRNA-mediated down-regulation of LRP expression leads to apoptotic induction in pancreatic cancer and neuroblastoma cells.

Confocal microscopy suggested that knockdown of LRP expression in pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells causes nuclear morphological changes that are characteristic of cells undergoing apoptotic induction. In order to confirm that this observation of apoptotic induction holds true for both of the tumourigenic cell lines in question, Annexin V-FITC/ 7-AAD assays were performed.

It was observed that transfection of AsPC-1 cells with siRNA-LAMR1 resulted in 76% of cells undergoing apoptotic induction (Fig.34D) when compared to non-transfected cells (Fig34A). Treatment of IMR-32 cells with siRNA-LAMR1 led to 42.7% of cells experiencing apoptotic induction (Fig.35D) in comparison to non-transfected cells (Fig.35A). Furthermore, treatment of both cell lines with the negative control siRNA-scr did not result in

apoptotic induction (Fig.34B and 35B). Protocatechuic acid (PCA) was used as a positive control and led to high percentages of both cell lines that underwent apoptotic induction (Fig.34C and 35C).

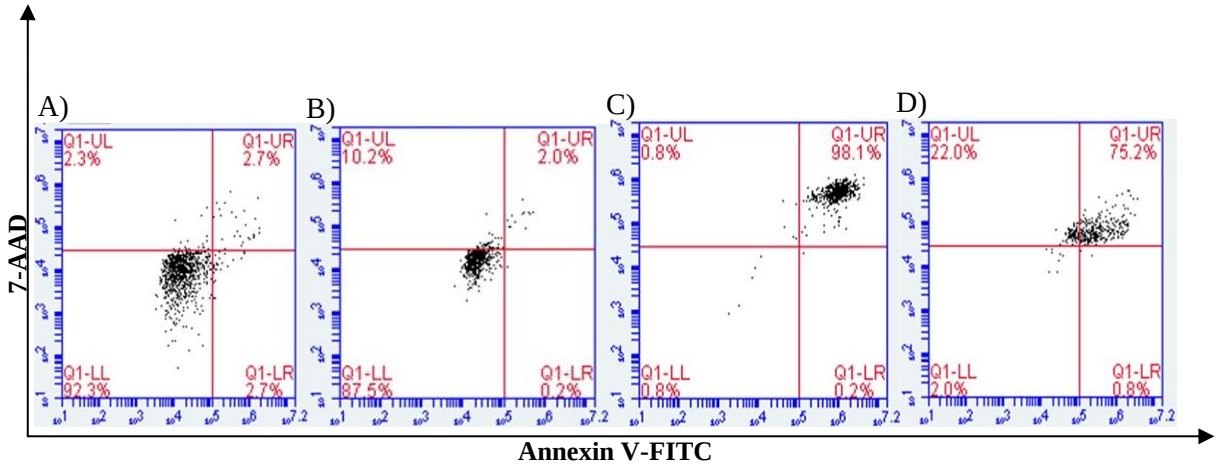

Figure 34: Apoptotic induction in pancreatic cancer (AsPC-1) cells after siRNA transfection. A) Non-transfected cells show that majority of cells lie in the lower left quadrant (Q1-LL) and this quadrant is indicative of normal living cells. B) Treatment of cells with the negative control siRNA-scr also resulted in majority of the cells appearing in the lower left quadrant. C) Protocatechuic acid (PCA) was used as a positive control and resulted in most of the cells appearing in the upper right quadrant (Q1-UR), which is representative of cells undergoing late apoptosis. D) Transfection of cells with siRNA-LAMR1 led to 0.8% of cells undergoing early apoptosis, as shown in the lower right quadrant (Q1-LR), and 75.2% of cells undergoing late apoptosis – therefore a total of 76% of cells underwent apoptosis after siRNA-LAMR1 treatment. The upper left quadrant (Q1-UL) is indicative of cells undergoing necrosis. 20 000 cells were counted per sample and plots are representative of an average of experiments done in triplicate and repeated three times.

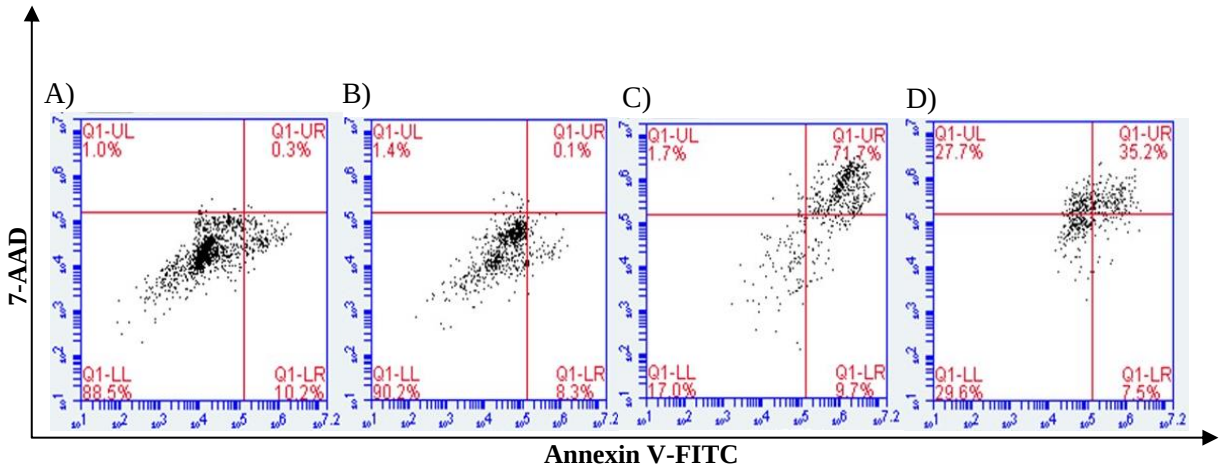

Figure 35: Induction of apoptosis in neuroblastoma (IMR-32) cells post siRNA transfection. A) Majority of non-transfected cells appeared in the lower left quadrant, indicative of living cells. B) siRNA-scr transfected cells were mostly also found in the lower left quadrant. C) PCA treated cells mostly appeared in the upper right quadrant, indicative of cells undergoing late apoptosis. D) 7.5% of cells transfected with siRNA-LAMR1 were found to be undergoing early apoptosis (lower right quadrant), whilst 35.2% of siRNA-LAMR1 transfected cells were observed as undergoing late apoptosis (upper right quadrant) – thus a total of 42.7% of siRNA-LAMR1 treated cells underwent apoptosis. 20 000 cells were counted per sample and plots are indicative of an average of experiments performed in triplicate and repeated three times.

4.10 The level of apoptotic induction after siRNA-mediated LRP knockdown is significantly higher in pancreatic cancer cells than neuroblastoma cells.

In order to quantify the levels of apoptotic induction occurring after treatment of pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells with siRNA-LAMR1, the combined percentage values obtained for early and late apoptosis for each cell line (Fig.34D and 35D) were used as an indication of total apoptosis levels. It was observed that AsPC-1 cells underwent 44% more total apoptosis than IMR-32 cells after siRNA-LAMR1 transfection (Fig.36).

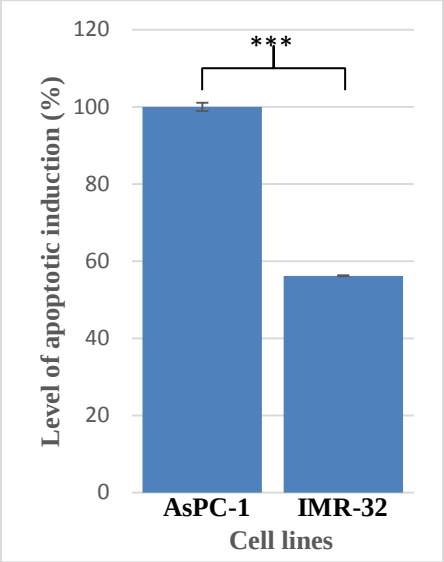

Figure 36: Total levels of apoptotic induction in pancreatic cancer (AsPC1) and neuroblastoma (IMR-32) cells after siRNA-mediated LRP knockdown. After transfection of cells with siRNA-LAMR1, it was observed that AsPC-1 cells underwent 44% more apoptosis than IMR-32 cells. Total apoptosis values post siRNA-LAMR1 transfection were obtained from the annexin V-FITC/ 7-AAD assays and the total apoptosis values obtained for the corresponding non-transfected cells were subtracted. Resultant values were used in the construction of this graph and AsPC-1 cells were set to 100%. Graph represents an average of experiments performed in triplicate and repeated three times. ***$p<0.0001$

4.11 siRNA-mediated knockdown of LRP expression leads to significantly increased caspase-3 activity in pancreatic cancer and neuroblastoma cells.

To further confirm that down-regulation of LRP as a result of treatment of cells with siRNA-LAMR1 leads to apoptotic induction in pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells, caspase-3 activity assays were performed. It was found that after transfection with siRNA-LAMR1, AsPC-1 cells displayed a significant 10-fold increase in caspase-3 activity when compared to non-transfected cells which were set to 100% (Fig.37). IMR-32

cells showed a 6-fold increase in caspase-3 activity after treatment of cells with siRNA-LAMR1 and compared to non-transfected cells (Fig.37). Additionally, treatment of both cell lines with the negative control siRNA-scr showed no significant differences in caspase-3 activity when compared to non-transfected cells (Fig.37).

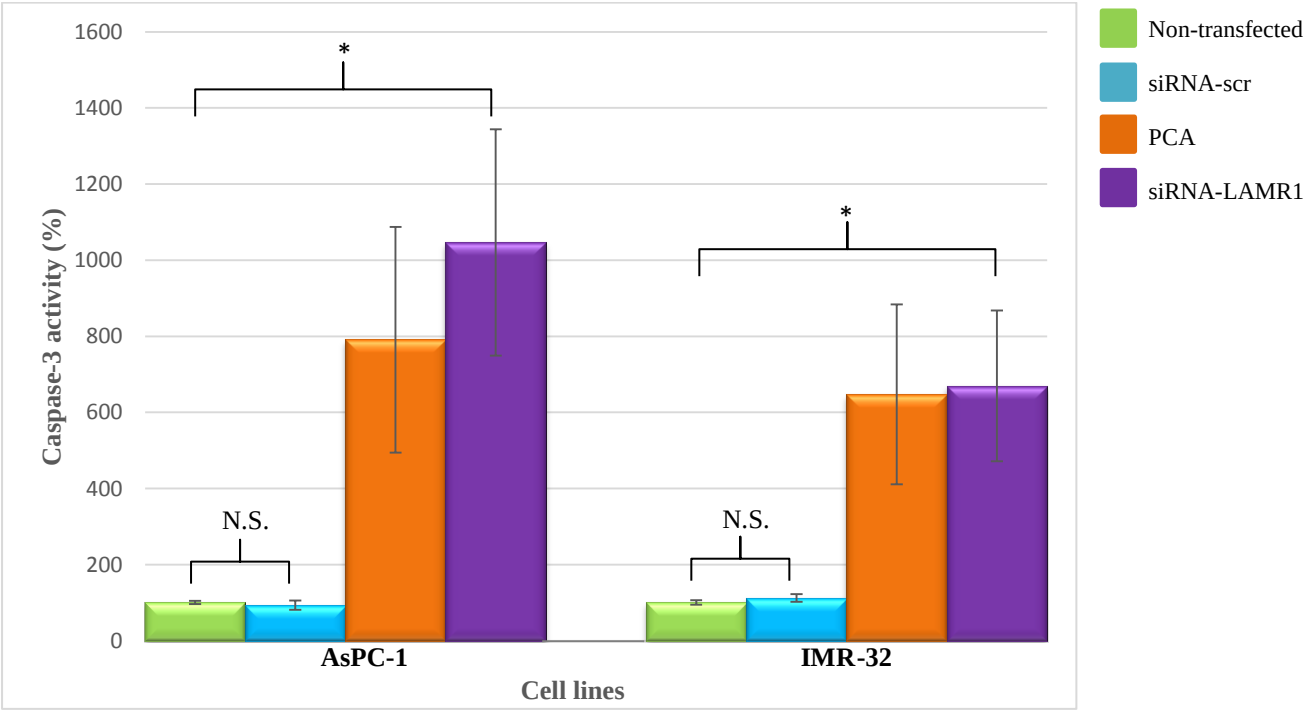

Figure 37: The effect of siRNA-mediated LRP down-regulation on caspase-3 activity in pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells. Caspase-3 activity assays showed that after treatment of cells with siRNA-LAMR1, AsPC-1 and IMR-32 cells showed a significant 10-fold and 6-fold increase in caspase-3 activity, respectively, when compared to non-transfected cells (which was set to 100%). No significant difference was observed when both cell lines were treated with the negative control siRNA-scr and compared to non-transfected cells. Prototechuic acid (PCA) was used as a positive control. Graph is indicative of an average of experiments performed in triplicate and repeated three times. *p=0.0109 and 0.0154 for AsPC-1 and IMR-32, respectively. N.S: p>0.05 hence non-significant.

4.12 Pancreatic cancer and neuroblastoma cells exhibit significantly increased caspase-8 activity after siRNA-mediated down-regulation of LRP expression.

To further ascertain that siRNA-mediated LRP knockdown induces apoptosis in pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells, caspase-8 activity assays were performed. Additionally, this assay was used to gain insight into whether or not the caspase-8-mediated extrinsic pathway is used by each of the two cell lines to induce apoptosis post transfection with siRNA-LAMR1. It was observed that AsPC-1 and IMR-32 cells treated with siRNA-LAMR1 experienced a significant 3-fold and 4-fold increase in caspase-8

activity, respectively, when compared to non-transfected cells which were set to 100% (Fig.38). Moreover, transfection of both cell lines with the negative control siRNA-scr revealed no significant differences in caspase-8 activity when compared to non-transfected cells (Fig.38).

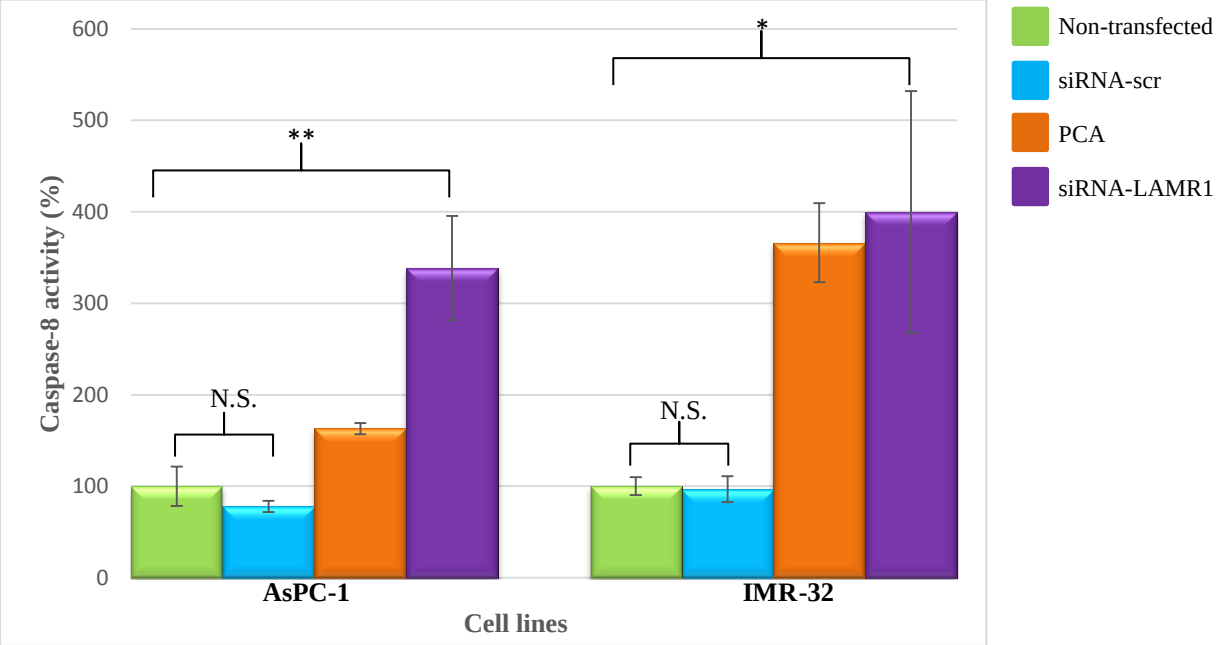

Figure 38: The effect of siRNA-mediated knockdown of LRP expression on caspase-8 activity in pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells. siRNA-LAMR1 transfected AsPC-1 and IMR-32 cells showed a significant 3-fold and 4-fold increase in caspase-8 activity, respectively, when compared to non-transfected cells (set to 100%). Treatment of both cell lines with the negative control siRNA-scr resulted in no significant difference in caspase-8 activity when compared to non-transfected cells. PCA was used as a positive control. The graph is representative of an average of experiments carried out in triplicate and repeated three times. *p=0.0357, **p=0.0053, N.S: p>0.05 thus non-significant.

4.13 Pancreatic cancer cells show a significant increase in caspase-9 activity after siRNA-mediated LRP knockdown.

Caspase-9 activity assays were used in order to establish whether or not pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells use the caspase-9-mediated intrinsic pathway to induce apoptosis after siRNA-mediated LRP knockdown. It was found that AsPC-1 cells exhibit a significant 3-fold increase in caspase-9 activity after transfection of cells with siRNA-LAMR1, when compared to non-transfected cells which were set to 100% (Fig.39). No significant difference was observed in caspase-9 activity in IMR-32 cells treated with siRNA-LAMR1 by comparison to non-transfected cells (Fig.39). Furthermore, treatment of

both cell lines with the negative control siRNA-scr resulted in no significant differences in caspase-9 activity when compared to non-transfected cells, as expected (Fig.39).

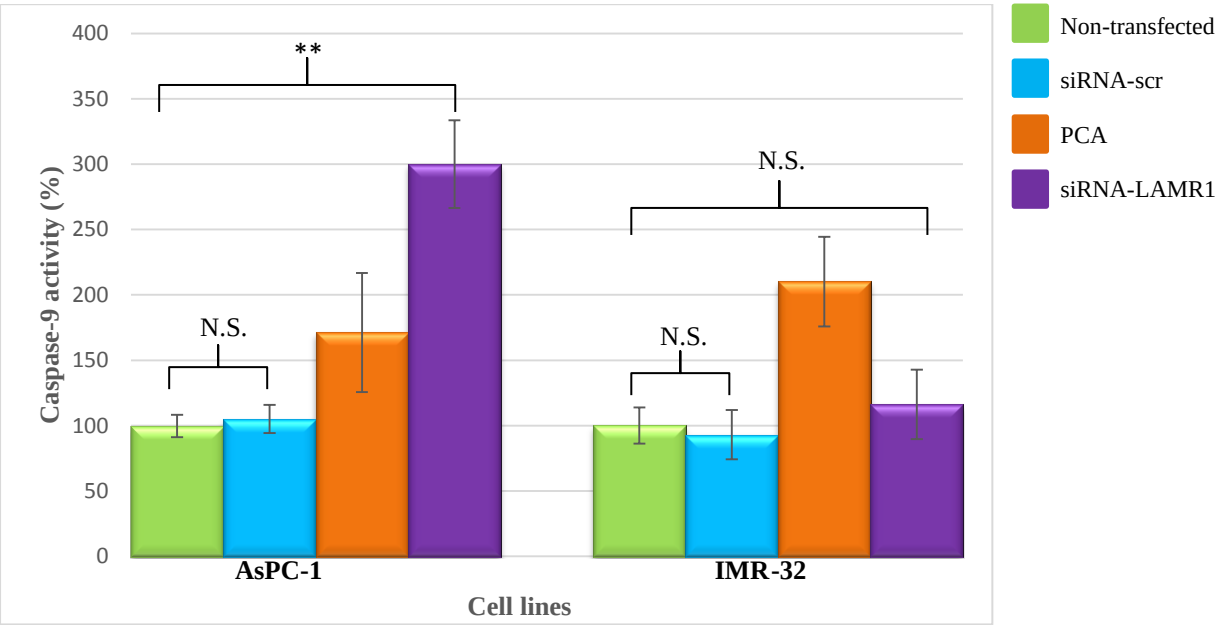

Figure 39: The effect of siRNA-mediated down-regulation of LRP expression on caspase-9 activity in pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells. siRNA-LAMR1 transfected AsPC-1 cells showed a significant 3-fold increase in caspase-9 activity when compared to non-transfected cells (set to 100%). Treatment of IMR-32 cells with siRNA-LAMR1 showed no difference in caspase-9 activity by comparison to non-transfected cells. Both tumourigenic cell lines displayed no significant difference in caspase-9 activity after treatment of cells with the negative control siRNA-scr, and by comparison to non-transfected cells. PCA was used as a positive control. Graph represents an average of experiments carried out in triplicate and repeated three times. **p=0.0016, N.S.: p>0.05 thus non-significant.

4.14 Pearson's correlation co-efficient

Table 3: Pearson's correlation co-efficients (R) between cell surface and total LRP levels prior to siRNA transfection.

Cell lines	Correlation between cell surface and total LRP levels prior to siRNA transfection (R-value)
AsPC-1	0.72
IMR-32	0.97

Table 4: Pearson's correlation co-efficients (R) between total LRP levels prior to and post siRNA-LAMR1 transfection.

Cell lines	Correlation between total LRP levels prior to and post siRNA-LAMR1 transfection (R-value)
AsPC-1	0.90
IMR-32	0.92

Table 5: Pearson's correlation co-efficients (R) between total LRP levels prior to and post transfection with esiRNA-RPSA.

Cell lines	Correlation between total LRP levels prior to and post esiRNA-RPSA transfection (R-value)
AsPC-1	0.80
IMR-32	0.94

Table 6: Assessment of correlation between levels of siRNA-mediated LRP knockdown and reductions in the viability of pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells, using Pearson's correlation co-efficients (R).

Cell lines	Correlation between siRNA-LAMR1-mediated LRP knockdown and reduction in cellular viability (R-value)	Correlation between esiRNA-RPSA-mediated LRP knockdown and reduction in cellular viability (R-value)
AsPC-1	0.99	0.92
IMR-32	0.96	0.99

Table 7: Correlation between siRNA-mediated LRP down-regulation and reductions in cellular proliferation and viability in AsPC-1 and IMR-32 cells by use of Pearson's correlation co-efficients (R).

Cell lines	Correlation between siRNA-LAMR1-mediated LRP knockdown and reduction in cellular proliferation (R-value)	Correlation between siRNA-LAMR1-mediated reductions in both cellular proliferation and cellular viability (R-value)
AsPC-1	0.99	0.99
IMR-32	0.85	0.96

Table 8: Pearson's correlation co-efficients (R) between total LRP levels post siRNA-LAMR1 transfection and total levels of apoptosis.

Cell lines	Correlation between total LRP levels post siRNA-LAMR1 transfection and total levels of apoptosis (R-value)
AsPC-1	0.92
IMR-32	0.99

Table 9: Pearson's correlation co-efficients (R) between total LRP levels post siRNA-LAMR1 transfection and increases in caspase-3 activity after siRNA-LAMR1 treatment.

Cell lines	Correlation between total LRP levels post siRNA-LAMR1 transfection and increases in caspase-3 activity after siRNA-LAMR1 treatment (R-value)
AsPC-1	0.99
IMR-32	0.91

CHAPTER 5: DISCUSSION

Over time, the involvement of LRP/LR in tumourigenesis has largely gained the interest of the scientific community due to evidence that this receptor exhibits a characteristic over-expression in several cancerous cell types in comparison to normal cells. Specifically, tumour progression is enhanced by the LRP/LR-assisted promotion of processes such as tumour angiogenesis, metastasis and apoptotic evasion. As previously stated, LRP/LR is not confined only to the cell surface but is additionally localized in the cytosol, nucleus and the perinuclear region. The vast localization of LRP/LR implicates it in many protein interactions, allowing the receptor to serve several physiological functions such as cell adhesion, cell migration and proliferation, cellular viability maintenance, regulation of cell cycle functioning, protein synthesis and ribosomal RNA processing. Due to LRP/LR over-expression, these functions may be exploited in tumour cells, leading to the occurrence of the afore-mentioned tumour-promoting processes. Thus, this receptor may be considered as a promising target for cancer prognosis and therapeutics.

5.1 Analysis of the difference in cell surface LRP/LR levels between pancreatic cancer and neuroblastoma cells

Flow cytometry revealed significantly high percentages of both pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells displaying LRP/LR on the cell surface. However, these percentages are only indicative of the percentage of cells within a given population that display an unknown amount of cell surface LRP/LR. To further investigate and quantify the actual levels of LRP/LR expression on the cell surface, median fluorescence intensities were used and revealed that IMR-32 cells exhibited significantly higher (70% more) cell surface LRP/LR levels in comparison to AsPC-1 cells. This difference might be due to the possibility that neuroblastoma cells are more dependent on LRP/LR to carry out processes such as cell adhesion and invasion, however this assumption will require further investigation involving comparative studies of the invasive potential of IMR-32 and AsPC-1 cells and the involvement of LRP/LR therein. Additionally, it may be speculated that IMR-32 cells could be more reliant on LRP/LR to facilitate cellular proliferation and the maintenance of cellular viability – two essential functions served by LRP/LR as a transmembrane cell-surface receptor. Another possible explanation as to why IMR-32 cells exhibit increased LRP/LR levels may be due to interactions of this receptor with the cellular prion protein (PrP). PrP is a ligand for LRP/LR[174] and, although ubiquitously expressed, it has been found to be

expressed in higher quantities in neuronal cells[175]. Thus, it may be possible that increased LRP/LR expression observed in the neuroblastoma cell line under investigation is a means of compensation used by neuronal cells to allow for adequate binding of PrP molecules.

Moreover, the bacterial protein chloramphenicol acetyltransferase (CAT) was used as an IgG isotypic negative control due to its expected absence in eukaryotic cells. CAT proved to be a highly efficient control, showing no significant differences in cell surface expression between the two tumourigenic cell lines and confirming that it is the specificity of IgG1-iS18 that results in LRP/LR recognition and not simply due to non-specific IgG binding.

5.2 Assessment of the difference between total LRP levels in pancreatic cancer and neuroblastoma cells

As previously described, LRP/LR is predominantly a cell-surface transmembrane receptor but is also found in other cellular locations. Therefore, even though differences were observed in cell-surface LRP/LR levels between AsPC-1 and IMR-32 cells, total LRP/LR levels (which incorporates LRP/LR found in the nucleus, cytosol, perinuclear region, and also on the cell surface) were also determined. Western blotting and densitometry revealed that IMR-32 cells contain 63% more total LRP compared to AsPC-1 cells. This increased total LRP level in IMR-32 cells may suggest that, in comparison to AsPC-1 cells, this cell line could be more dependent on LRP/LR to maintain nuclear structures and execute translational processes – in line with the functions served by LRP/LR in the nucleus and cytosol, respectively. Importantly, it must be noted that majority of the observed total LRP levels can be attributed to cell-surface LRP due to the high correlation observed between these two parameters. This is relevant due to cell-surface LRP being predominantly implicated in the maintenance of cellular viability.

5.3 Silencing of LRP

RNA interference was used to down-regulate LRP/LR in order to assess the role of this receptor in cellular viability. A specific siRNA called siRNA-LAMR1 was employed to facilitate this down-regulation. siRNA-LAMR1 targets the mRNA of the 37kDa laminin receptor precursor (LRP) form, particularly targeting the open reading frame region of the human LRP gene. Western blotting was employed to detect LRP knockdown using anti-LRP/LR specific antibody IgG1-iS18, which only successfully detects the 37kDa LRP – possibly due to cytoplasmic extractions containing the 67kDa LR form in significantly low

concentrations. Treatment of AsPC-1 and IMR-32 cells with siRNA-LAMR1 revealed a 90% and a 71% reduction in LRP expression, respectively, when compared to non-transfected cells – thereby proving the efficacy of siRNA-LAMR1.

Moreover, the fact that AsPC-1 cells showed a greater reduction in LRP expression after siRNA-LAMR1 supplementation in comparison to IMR-32 cells may possibly be attributed to IMR-32 cells having higher total LRP levels than AsPC-1 cells before siRNA treatment. Essentially, the same density of cells from both cell lines were treated with the same volume and concentration of siRNA-LAMR1 with regards to preparation of lysates. Hence, it can be suggested that this volume of siRNA-LAMR1 was more effective in targeting majority of the LRP in AsPC-1 cells due to the lower total LRP level in AsPC-1 cells prior to siRNA transfection. Similarly, it may be possible that the volume of siRNA-LAMR1 used was less effective in targeting LRP in IMR-32 cells due to the higher total LRP level before siRNA transfection. Furthermore, a high correlation was observed between total levels of LRP prior to and post siRNA-LAMR1 transfection, thus suggesting that higher levels of LRP expression prior to siRNA treatment leads to less LRP knockdown after siRNA treatment, and vice versa.

As previously mentioned, siRNA-LAMR1 targets the 37kDa LRP and not the 67kDa LR form of the receptor. However, owing to the fact that LRP is a precursor of LR, reduced expression of LRP would consequently result in reduced maturation to the LR form. Moreover, the post-translation modification known as fatty acylation is responsible for the maturation of the 37kDa LRP into the 67kDa LR form and since this maturation does not occur through direct transcription or translation, the 67kDa LR is unable to be directly targeted.

It is noteworthy that a negative control scrambled siRNA, called siRNA-scr, was used to control for the effects of siRNA-LAMR1. The control siRNA was designed to not target any known gene within the cell and is necessary to distinguish between silencing that is sequence-specific and non-specific effects. Additionally, this control siRNA has been designed to exert minimal or no effect on cellular viability and phenotype. It is evident that siRNA-scr did not significantly affect LRP expression in both AsPC-1and IMR-32 cells.

Even though siRNA-LAMR1 is specific for LRP in relation to the control siRNA-scr, which had no effect on LRP expression, it cannot be disregarded that the effects of siRNA-LAMR1 on cell viability might have been due to off-target effects and not specifically the down-

regulation of LRP/LR. Therefore, another siRNA called esiRNA-RPSA which is specific for LRP was used. This siRNA also specifically targets the mRNA of the 37kDa LRP, similarly to siRNA-LAMR1. However, BLAST analysis revealed that siRNA-LAMR1 targets the 37kDa LRP mRNA at nucleotides 250-268, whilst esiRNA-RPSA targets nucleotides 521-929 of the mRNA sequence. Treatment of AsPC-1 and IMR-32 cells with esiRNA-RPSA yielded significant reductions of 83% and 64%, respectively, in LRP expression. This result is similar to that of the outcome observed when using siRNA-LAMR. Furthermore, a high correlation was observed between total LRP levels prior to and post esiRNA-RPSA treatment. This reiterates the suggestion that higher levels of total LRP before siRNA treatment results in less LRP knockdown post siRNA treatment.

Due to the fact that both of the afore-mentioned LRP-specific siRNAs target completely different regions of the LRP mRNA but yield similar effects, it can be said that the results observed upon using siRNA-LAMR1 are a direct outcome of down-regulation of LRP specifically. Furthermore, esiRNA-RLUC served as the negative control siRNA that controls for the action of esiRNA-RPSA. This control siRNA targets Renilla Luciferase (RLUC), which is absent in the cell lines under study. No significant difference was observed in LRP expression when AsPC-1and IMR-32 cells were treated with this negative control siRNA, thus proposing high specificity of esiRNA-RPSA for LRP.

5.4 Association between LRP expression and cellular viability maintenance

In order to gain insight into the role played by LRP/LR in the maintenance of cellular viability, siRNAs were used to down-regulate LRP expression and the effect thereof on the cellular viability of AsPC-1 and IMR-32 cells was assessed using MTT assays. Treatment of AsPC-1 and IMR-32 cells with siRNA-LAMR1 resulted in an 82% and 65% reduction in cellular viability, respectively, when compared to non-transfected cells. This observation reveals a crucial role for LRP/LR in the maintenance of cellular viability, as the knockdown of this receptor results in significantly lowered cell viability. Moreover, high correlation was observed between the extent of siRNA-LAMR1-mediated LRP knockdown in AsPC-1 and IMR-32 cells, and the siRNA-LAMR1-induced reduction in cellular viability in these two cell lines. This further justifies that LRP/LR is essential to the maintenance of cellular viability. Similarly, transfection of AsPC-1 and IMR-32 cells with esiRNA-RPSA resulted in reductions of 82% and 60% in cellular viability, respectively, when compared to non-transfected cells. This observation is in line with that of cells treated with siRNA-LAMR1,

hence providing further evidence of the critical role of LRP/LR in cellular viability maintenance. Furthermore, high correlation exists between esiRNA-RPSA-mediated LRP down-regulation in AsPC-1 and IMR-32 cells and reductions in cellular viability induced by esiRNA-RPSA treatment.

Additionally, treatment of AsPC-1 and IMR-32 cells with siRNA-scr and esiRNA-RLUC - the negative control siRNAs for siRNA-LAMR1 and esiRNA-RPSA, respectively - showed no effect on cellular viability, thus proving their high efficacy as negative controls and affirming the specificity of their respective target-siRNA counterparts. Treatment of cells with 8mM protocatechuic acid (PCA) - an apoptotic inducer employed as a positive control in this study - showed reductions in cellular viability paralleled to that of cells treated with siRNA-LAMR1 and esiRNA-RPSA, as expected.

It is clear that LRP/LR plays an important role in the maintenance of cellular viability, and moreover, tumour cells characteristically evade apoptosis to facilitate tumour progression. As previously stated, a proposed mechanism by which LRP/LR maintains cellular viability is through interactions with a growth factor known as Midkine. This growth factor exhibits high affinity for binding to heparin and is critically involved in the retention of the stability of chromosomes that affords LRP/LR the ability to maintain cellular viability[127]. Essentially, Midkine has been associated with cellular processes such as angiogenesis, cellular proliferation and migration[127]. Furthermore, several cancer types have displayed up-regulated Midkine expression in comparison to their normal cell counterparts. Recent studies have revealed an over-expression of Midkine in neuroblastoma[176] and pancreatic[177] tumour cells - the two cell types central to the present study. It is suggested that this over-expression results in the up-regulation of cell survival promoting factors (such as Bad and PKB) and assists in apoptotic inhibition via caspase-3 suppression[178]. Ultimately, siRNA-mediated LRP knockdown may possibly result in subsequently reduced LRP/LR-Midkine interactions, thus causing the observed reductions in cellular viability.

5.5 Involvement of LRP expression in cellular proliferation

In order to establish whether impaired cellular division is responsible for the reductions in cellular viability of AsPC-1 and IMR-32 cells, cellular proliferation was analysed. BrdU assays revealed that after transfection of both these cell lines with siRNA-LAMR1, AsPC-1 and IMR-32 cells experience reductions of 76% and 44% in cellular proliferation, respectively, in comparison to non-transfected cells. This suggests that LRP/LR plays a role

in cellular division that consequently facilitates cellular viability maintenance. Treatment of both cell lines with the negative control siRNA-scr showed no significant impact on cellular proliferation when compared to non-transfected cells. On the other hand, treatment of cells with the positive control PCA showed reductions in cellular proliferation that are similar to those observed for siRNA-LAMR1 treated cells.

Furthermore, a high correlation was observed between total levels of siRNA-LAMR1-mediated LRP knockdown in AsPC-1 and IMR-32 cells, and siRNA-LAMR1-induced reductions in cellular proliferation. This further confirms the crucial involvement of LRP/LR in cellular proliferation. Additionally, a high correlation was seen between siRNA-LAMR1-mediated reductions in cellular viability and cellular proliferation, thus confirming that the processes of cellular proliferation and cellular viability maintenance are both affected by LRP/LR RNA interference.

It is evident that LRP/LR is involved in the process of cellular proliferation and additionally, Midkine is a growth factor that also plays a role in this process - as previously highlighted. The use of siRNA-LAMR1 to knock down LRP resulted in decreased proliferation of AsPC-1 and IMR-32 cells, and this may be attributed to decreased LRP expression being inadequate in interactions with Midkine. The reduction in the LRP/LR-Midkine interaction possibly impeded cellular proliferation and hence the maintenance of cellular viability was compromised. LRP/LR has also been identified in interactions with certain proteins that govern cell cycle progression, such as cyclins A2 and B1, CDK's 1 and 2, and p21[123], thus knockdown of this receptor may result in hampered cellular growth and proliferation.

5.6 Influence of LRP expression on apoptotic induction

To identify whether apoptosis was the form of cell death responsible for the observed reduction in the viability of AsPC-1 and IMR-32 cells after LRP down-regulation, confocal microscopy was used. After treatment of these two cell lines with siRNA-LAMR1, evident alterations in nuclear morphology were observed – such as nuclear shrinkage, nuclear membrane blebbing, and the formation of apoptotic bodies – when compared to the nuclear morphology of non-transfected cells. Due to the maintenance of nuclear structures by the binding of nuclear and perinuclear LRP/LR to histones[113], it is clear that knockdown of this receptor leads to diminished nuclear integrity and altered nuclear morphology. These siRNA-LAMR1-induced changes to the nuclear morphology of AsPC-1 and IMR-32 are indicative of apoptotic induction in these cell lines. Moreover, apoptotic induction exhibits characteristic

disruptions in DNA structure[99], thereby deeming Hoescht staining as an effective method of detection of apoptosis due to its capacity to bind to double stranded DNA. Additionally, treatment of both cell lines with the negative control siRNA-scr showed no disruptions in nuclear morphology, evidenced by the similarity to non-transfected cells. Positive control PCA-treated cells showed alterations in nuclear morphology that are suggestive of apoptotic induction, and this was expected due to PCA being an apoptotic inducer.

Although confocal microscopy indicated the occurrence of apoptosis in AsPC-1 and IMR-32 cells, further confirmation and quantification of apoptotic induction was required. This was achieved by use of Annexin-V-FITC/ 7-AAD assays. Essentially, apoptosis involves loss of integrity of cell membranes that results in a membrane-flip reaction which exposes intracellularly-located phosphatidylserine (PS) on the outer cell membrane – thereby permitting Annexin-V to bind to PS when calcium is present[179]. Importantly, the Annexin-V protein is conjugated to a fluorochrome known as FITC, which allows for the binding of Annexin-V to PS to be detected without impacting on the binding capacity of Annexin-V. However, it is impossible to differentiate between early and late apoptosis solely by Annexin-V binding, thus a viability dye known as 7-AAD is additionally used to achieve this. Specifically, 7-AAD is only incorporated into late apoptotic cells which have experienced membrane alterations (allowing the dye to stain DNA within the nucleus), and is excluded from early apoptotic cells which lack this feature[180].

It was observed that non-transfected and negative control siRNA-scr transfected cells for AsPC-1 and IMR-32 cells exhibited negative staining for Annexin-V, therefore appearing in the lower left quadrant which is representative of living cells. On the other hand, 8mM PCA-treated and siRNA-LAMR1-treated AsPC-1 and IMR-32 cells displayed positive staining for Annexin-V – appearing in the lower right and upper right quadrants. The shift from negative to positive Annexin-V staining signifies that siRNA-LAMR1-mediated LRP down-regulation in AsPC-1 and IMR-32 cells triggers blebbing of the cell membrane, which is characteristic of cells undergoing apoptosis. This blebbing exposes PS on the outer cell membrane and allows Annexin-V to bind, as previously described. Both cell lines showed majority of cells experiencing late apoptosis after siRNA-LAMR1 transfection, specifically 75.2% of AsPC-1 and 35.2% of IMR-32 cells. Further quantification suggested that AsPC-1 cells underwent 44% more total apoptosis than IMR-32 cells, thus proposing that siRNA-LAMR1 is more successful in knocking down LRP in AsPC-1 cells than it is in IMR-32 cells and subsequently causing more cell membrane blebbing in AsPC-1 cells. This was further

justified by the high correlation observed between total levels of LRP after siRNA-LAMR1 treatment and total levels of apoptosis for both cell lines.

Although majority of AsPC-1 and IMR-32 cells underwent late apoptosis after transfection with siRNA-LAMR1, relatively high percentages of these cell types also experienced necrosis after this treatment. Necrotic cells appear in the upper left quadrant and it was observed that 22% of AsPC-1 cells and 27.7% of IMR-32 cells underwent necrosis post siRNA-LAMR1 transfection. One possible explanation for this observation may be that the 72-hour transfection period was too long, thus causing necrotic cell death instead of late apoptosis. However, this speculation requires further time-dependent studies to assess whether the same effect is observed when the transfection period is shortened.

Nuclear morphological studies as well as Annexin V assays proved that siRNA-mediated knockdown of LRP expression leads to apoptotic induction in AsPC- 1 and IMR-32 cells. Additionally, caspase-3 assays were performed in order to further ascertain the occurrence of apoptotic induction after treatment of these cell lines with siRNA-LAMR1. Caspase-3 is an effector caspase that exhibits increased activity in cells that are actively undergoing apoptosis[181]. After treatment of AsPC-1 and IMR-32 cells with siRNA-LAMR1, there was a marked increase in caspase-3 activity in both cell lines when compared to non-transfected cells. These findings clearly indicate that siRNA-mediated LRP down-regulation induces apoptosis in these two tumourigenic cell lines, possibly due to the reduction in or inhibition of the afore-mentioned LRP/LR-Midkine interaction. This observation of apoptotic induction after siRNA-mediated LRP knockdown may also be attributed to the interaction of LRP/LR with focal adhesion kinase (FAK). A recent study showed that after LRP/LR binds to laminin, interactions can occur between LRP/LR and FAK[182]. These interactions were implicated in the activation of PI3-kinase/AKT and MEK/ERK 1/2 cell survival pathways and also the up-regulation of Bcl-2 - an anti-apoptotic protein[182]. Thus, siRNA-mediated knockdown of LRP in the present study may perhaps be preventing the LRP/LR-FAK interaction and thereby inducing apoptosis. Alternatively, down-regulation of LRP by siRNAs in the present study may have led to apoptotic induction due to the ribosomal functions of LRP/LR. It is known that LRP/LR plays a role in the biogenesis of ribosomes by facilitating 21S pre-rRNA processing into mature 18S rRNA[125], and also associates with and acts as a component of the 40S ribosomal small subunit where it assists with protein translation and synthesis[183,184,185]. Therefore, knockdown of LRP in this study could have interfered with ribosome formation and consequent translation of proteins that are critically

involved in proper cell functioning and survival, thereby leading to cell death. Additionally, it is suggested that ribosomes carry out translational processes in association with cytoskeletal components – in particular, tubulin[186]. Importantly, LRP/LR has been identified as a protein that tethers tubulin to the ribosome[126,186] and thus, knockdown of this receptor in the present study may possibly be hampering this interaction – causing attenuated translation and resulting in cell death. Moreover, AsPC-1 cells exhibited a greater increase than IMR-32 cells in caspase-3 activity after siRNA-LAMR1 transfection. This observation strongly reiterates that LRP/LR is crucial in the maintenance of cellular viability, thus lower levels of LRP expression lead to increased levels of apoptotic induction and vice versa. The high correlation observed between the total levels of siRNA-LAMR1-mediated LRP knockdown and increases in caspase-3 activity in both of the cell lines under study firmly supports this suggestion.

As previously stated, caspase-3 is an effector caspase meaning that it cleaves substrates in the cell in order to trigger the induction of apoptosis. Furthermore, caspase-3 is involved in both the intrinsic and extrinsic apoptotic pathways. Therefore, the observed increases in caspase-3 activity after siRNA-LAMR1-mediated down-regulation of LRP can only be used as an indication of the occurrence of apoptosis. Caspase-8 and caspase-9 assays had to be additionally performed in order to assess which specific pathway is used by AsPC-1 and IMR-32 cells to undergo apoptosis after siRNA-LAMR1-mediated knockdown of LRP expression. It was observed that both of the tumourigenic cell lines exhibited increased caspase-8 activity after treatment with siRNA-LAMRI, when compared to non-transfected cells. Due to caspase-8 being critically involved in the death receptor (extrinsic) pathway as previously described, it can be said that siRNA-mediated LRP down-regulation induces apoptosis in AsPC-1 and IMR-32 cells via the extrinsic pathway.

However, it was also observed that AsPC-1 cells treated with siRNA-LAMR1 exhibited significantly increased caspase-9 activity in comparison to non-transfected cells. Caspase-9 is intricately involved in the mitochondrial (intrinsic) pathway, thus suggesting that siRNA-mediated LRP knockdown also induces apoptosis in AsPC-1 cells via the intrinsic pathway. One possible explanation of why siRNA-LAMR1 treated AsPC-1 cells undergo apoptosis via both the intrinsic and extrinsic pathways may be that these cells experience what is known as retaliatory caspase activation[187,188] (Fig.40). This mechanism of caspase activation proposes that the two apoptotic pathways are able to activate each other using a feedback amplification loop[187,188]. Within this feedback loop, active caspase 9 is responsible for the cleavage and

activation of caspase-3, which in turn results in caspase-8 activation[187,188]. Therefore, this may possibly be the reason why increases in both caspase-8 and caspase-9 were observed in AsPC-1 cells after siRNA-mediated knockdown of LRP expression.

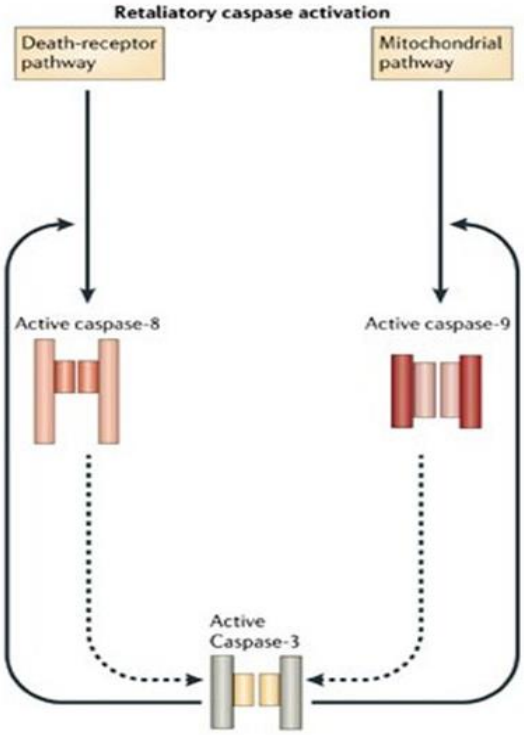

Figure 40: The feedback amplification loop between the intrinsic and extrinsic apoptotic pathways. Active caspase-9 leads to the cleavage and subsequent activation of caspase-3, which then results in the activation of caspase-8. In this manner, certain cells to undergo apoptosis via both apoptotic pathways[188].

Apart from the cancerous cell lines pertaining to the present study, the occurrence of retaliatory activation of caspases has also been implicated in studies involving other cell lines and diseases. One such study found that active caspase-8 effectively cleaved procaspase-3 molecules in murine cells in response to TNFα crosslinking, subsequently forming active caspase-3 molecules, which in turn facilitated the activation of procaspase-9 molecules to form active caspase-9[189]. Another study suggested the involvement of the feedback amplification loop in the cells of the substantia nigra of mice infected with Parkinson's disease – where a toxic component called MPTP induced caspase-9 activation in the substantia nigra cells, subsequently causing activation of caspase-3 which activated downstream caspase-8[190]. Additionally, research has suggested that activated caspase-8 (formed by the action of Etoposide) interacts with the mitochondrial membrane in breast cancer (MDA-MB231) cells – leading to the generation of truncated Bid (t-Bid) which

promotes the release of cytochrome-c, ultimately resulting in caspase-9 and downstream caspase-3 activation within the afore-mentioned feedback loop[191].

Taken together, the results of the present study suggest that AsPC-1 cells undergo apoptosis via both the intrinsic and extrinsic pathways after siRNA-mediated LRP knockdown – possibly using the mechanism of retaliatory caspase activation. On the other hand, the findings propose that IMR-32 cells undergo apoptosis solely via the death receptor (extrinsic) pathway post siRNA-mediated LRP down-regulation, further evidenced by the absence of a significant increase in caspase-9 activity when compared to non-transfected cells. It could be speculated that since IMR-32 cells exhibit higher LRP/LR levels, there might be higher levels of interactions between LRP/LR and ECM components, particularly laminin. It may be possible that there is up-regulated expression and activation of collagenase due to the increased LRP/LR-laminin interaction[192], and it may be worthwhile to examine whether collagenase somehow stimulates the death receptor pathway in neuroblastoma cells.

5.7 LRP/LR as a target for cancer therapeutics

Extensive research has implicated LRP/LR as a key contributor to the pathogenesis of viral diseases (such as Sindbis and Dengue), neurodegenerative diseases (such as Alzheimer's disease), and of importance to the present study – cancer. Specifically, LRP/LR has been found to promote the tumourigenic phenotype and enhance tumour progression through its involvement in processes such as metastasis, tumour cell proliferation and angiogenesis, apoptotic evasion by tumour cells, and the maintenance of tumour cell viability. Due to the critical function of LRP/LR in tumourigenesis, the receptor can be deemed as a promising therapeutic target for the treatment of several cancer types.

A vast array of *in vitro* studies targeting LRP/LR using anti-LRP/LR specific antibodies has been performed and have proved successful in the impediment of tumour- enhancing processes such as metastasis and angiogenesis. Additionally, the use of RNA interference technology to knockdown LRP expression has been observed as an effective means of hampering the maintenance of tumour cell viability – where siRNAs directed to LRP/LR of lung (A549), cervical (HeLa), liver (Hep3b), breast (MCF-7 and MDA-MB 231) and oesophageal (WHCO1) cancer cells all resulted in reduced viability of these tumourigenic cells as a consequence of siRNA-mediated reductions in total LRP/LR levels in all these cell lines. Similarly, the results of the present study suggest that siRNA-mediated down-regulation of LRP expression significantly reduces the viability of pancreatic cancer (AsPC-

1) and neuroblastoma (IMR-32) cells. Although past and present research suggests the high efficacy of siRNAs in reducing the viability of the afore-mentioned cell lines, it must be noted that each cancer type exhibits its own characteristic behaviour and therefore what holds true for one cancer type may not necessarily for other types of cancer. Hence, it is pivotal to test the use of LRP-specific siRNAs to a broad range of cancer types.

Due to the integral physiological functions of LRP/LR in normal cells, such as cell migration, growth and differentiation, one prominent challenge that remains is the administration of siRNAs *in vivo*. Solely targeting LRP/LR in tumourigenic cells without compromising the function of normal cells is pivotal if therapeutic tools targeting LRP/LR are to be used. Nevertheless, *in vitro* studies have shown promising results that suggest siRNAs are effective as a therapeutic tool for certain cancers, making LRP/LR an attractive therapeutic target for cancer treatment.

CHAPTER 6: CONCLUSIONS AND FUTURE WORK

6.1 Conclusions

The present study demonstrated that the siRNA-mediated knockdown of LRP expression significantly reduced the viability of pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells through hampering of cell proliferation and through the induction of apoptosis. Specifically, apoptosis occurred via both the intrinsic and extrinsic pathways in AsPC-1 cells likely due to feedback amplification loop entailing retaliatory caspase activation. Moreover, IMR-32 cells experienced apoptosis solely through the death receptor (extrinsic) pathway.

These findings exemplify the critical role played by LRP/LR in the maintenance of cellular viability in both of these tumourigenic cell lines. Furthermore, specific siRNAs targeting LRP expression may be deemed as potential therapeutic tools for the treatment of pancreatic cancer and neuroblastoma.

6.2 Future work

As previously mentioned, all cancer types behave characteristically and differently from each other, and due to the fact that the present study only focused on pancreatic cancer and neuroblastoma, several other cancer types remain to be investigated with regards to siRNA-mediated LRP down-regulation and cellular viability. Additionally, different cell lines within the same cancer type should be used to ensure that the observed effects are cancer-type specific and not just cell-line specific. Future studies could also consider making use of cell lines within a specific cancer type that are at different stages of their life cycle i.e early, middle and late stage cell lines in order to establish at which stage siRNA-mediated LRP knockdown is most effective at reducing cellular viability. Furthermore, time and dose-dependent studies could also be carried out in order to determine optimal transfection periods and siRNA dosages.

Due to LRP/LR playing several physiological roles in normal cells, it may be worthwhile to investigate the effect of siRNA-mediated LRP down-regulation on normal cells. In the event that this knockdown of LRP exerts little or no effect on the functioning of normal cells, further studies could entail the use of animal models such as nude mice to gain insight into *in vivo* siRNA administration. Nude mice are favourable animal models since they possess the ability to receive several types of tumour tissue grafts without exhibiting a rejection response or immune defence mechanisms towards these grafts[193]. Animal trials may possibly lead to

clinical trials, thereby allowing the effect of siRNA-mediated LRP down-regulation to be assessed in the human body. Furthermore, future studies could explore the use of siRNAs in conjunction with anti-LRP/LR specific antibodies to treat several tumour-promoting processes at the same time.

CHAPTER 7: REFERENCES

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APPENDIX A

General – Suppliers list:

- Media (RPMI and EMEM), BSA, de-ionized water, molecular weight marker – Thermo Scientific
- L-glutamine, HEPES, sodium pyruvate, non-essential amino acids, penicillin/streptomycin, FCS, trypsin, BCA reagents , APS, acrylamide, TEMED, paraformaldehyde, PCA, MTT, fluoromount mounting fluid, SDS– Sigma Aldrich
- PBS – Gibco by Life Technologies
- DMSO, lysis buffer, Tween – Merck Millipore
- Methanol – VWR chemicals
- PVDF membrane – Pall corporation
- Chemiluminescent substrate - Biorad
- Microscopy slides and coverslips – Labocare
- 24-well, 6-well and 6cm plates – Corning Inc.

Antibodies and siRNAs – Suppliers list:

- IgG1-iS18 – Affimed Therapeutics
- Anti-human IgG-HRP, anti-human PE, anti-rabbit APC– Abcam
- Anti-β actin peroxidase, anti-CAT, Hoescht 33342, esiRNA-RPSA, esiRNA-RLUC – Sigma
- Lipofectamine transfection reagent – Invitrogen by Life Technologies
- siRNA-LAMR1 (2nmol), siRNA-scr (5nmol), DharmaFect1 transfection reagent – Dharmacon
- 5x siRNA reconstitution buffer – Thermo Scientific

Kits – suppliers list:

- BrdU assay kit- Calbiochem
- Annexin V-FITC/ 7-AAD kit – Beckman Coulter
- Caspase 3,-8 and -9 kits – Abcam

Equipment list:

- Flow cytometer – BD Accuri C6
- Confocal microscope – Zeiss LSM 710 3-channel (images were captured using the blue laser, 63X magnification, and using Zen 2011 software).
- Laminar flow – Labotec
- ELISA reader – Tecan (using Magellan software)
- Centrifuge – Eppendorf 5417C
- Pipettes and micropipettes – Eppendorf research
- Gel casting and running apparatus – Biorad
- pH meter – Eutech instruments

Transfection procedure using siRNA-LAMR1 and siRNA-scr:

Pellets of siRNA-LAMR1 (2nmol) were reconstituted in 200µl of 1X siRNA buffer, and siRNA-scr (5nmol) was reconstituted in 500µl of 1X siRNA buffer before use. The table below shows amounts of siRNA and corresponding components used for transfections per well of a 6-well, 24-well and 96-well plate.

	6-well plate	24-well plate	96-well plate
Serum-free media (µl) – for addition of siRNA	195	48.74	12.19
Reconstituted siRNA-LAMR1 or siRNA-scr (µl)	5	1.25	0.31
Serum-free media (µl) – for addition of DharmaFect1 transfection reagent	190	47.5	11.89
DharmaFect1 (µl)	10	2.5	0.63
Antibiotic free media	1600	400	100

Basic procedure:

Add reconstituted siRNA-LAMR1 or siRNA-scr to the corresponding volume of serum-free media in an Eppendorf tube. In a second Eppendorf tube, add transfection reagent to the corresponding volume of serum-free media. Incubate both tubes for 5 minutes at room temperature and then mix the contents of both tubes together. Incubate for a further 20 minutes at room temperature before adding to suggested volumes of antibiotic-free media. Thereafter, the resultant siRNA solution may be added to the cells.

Transfection procedure using esiRNA-RPSA and esiRNA-RLUC:

esiRNA-RPSA and esiRNA-RLUC are purchased reconstituted. The table below shows volumes of esiRNA and corresponding components used for transfections per well of a 6-well, 24-well and 96-well plate.

	6-well plate	24-well plate	96-well plate
Opti-MEM media – for addition of esiRNA (µl)	125	25	6.25
esiRNA-RPSA or esiRNA-RLUC (µl)	5	1.25	0.31
Opti-MEM media – for addition of Lipofectamine transfection reagent (µl)	125	25	6.25
Lipofectamine (µl)	5	1.25	0.31

Basic procedure:

Add esiRNA-RPSA or esiRNA-RLUC to the corresponding volume of Opti-MEM media in an Eppendorf tube. In a second Eppendorf tube, add transfection reagent to the corresponding volume of Opti-MEM media. Incubate both tubes for 5 minutes at room temperature and then mix the contents of both tubes together. Thereafter, the resultant siRNA solution may be added to the cells.

Composition of 12% polyacrylamide gels (per gel):

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