PROBING THE ROLE OF THE 37-kDa/67-kDa LAMININ RECEPTOR PRECURSOR/ LAMININ RECEPTOR (LRP/LR) ON THE CELLULAR VIABILITY OF BREAST AND OESOPHAGEAL CANCER CELLS BY siRNA-MEDIATED DOWNREGULATION OF LRP/LR

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

Johannesburg, 2014
DECLARATION

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

Signature

Date

12-05-2015
ABSTRACT

Cancer is a global burden due to high incidence and mortality rates and is ranked the second most diagnosed disease amongst non-communicable diseases in South Africa. A high expression level of the 37kDa/67kDa laminin receptor (LRP/LR) is one characteristic of cancer cells. This receptor is implicated in the pathogenesis of cancer cells by supporting tumor angiogenesis, metastasis and especially for this study, the evasion of apoptosis. In the current study, the role of LRP/LR on cellular viability of breast MCF-7, MDA-MB 231 and WHCO1 oesophageal cancer cells was investigated. Western blot analysis revealed that total LRP expression levels of MCF-7, MDA-MB 231 and WHCO1 were significantly downregulated by targeting the mRNA of LRP using siRNA-LAMR1 by 100, 44% and 73%, respectively. This knockdown of LRP expression resulted in a significant decrease of viability in the breast and oesophageal cancer cells as determined by an MTT assay by 72%, 52% and 45% in WHCO, MCF-7 and MDA-MB 231 cells, respectively. Moreover, the reduction in cellular viability was accompanied by a significant decrease in cell proliferation by 26%, 43% and 59% in MCF-7, WHCO1 and MDA-MB 231 cells, respectively. The exposure of the phosphatidylserine protein on the surface of breast MCF-7, MDA-MB 231 and oesophageal WHCO1 cancer cells as detected by the Annexin-V/7-AAD assay indicates that the reduction in cellular proliferation and viability is due to the induction of apoptosis which is elaborated by the loss of membrane symmetry as well as observations of nuclear morphological changes in all cell lines post transfection with siRNA-LAMR1. This data indicates that LRP/LR is crucial for the maintenance of cellular viability of cancerous cells and our findings recommend siRNA technology as a novel alternative therapeutic approach for the treatment of metastatic cancer.
In loving memory of my grandparents

Jetro Fakezwe Makhubu

1931 – 2007

and

Ellen Makhubu

1937 – 2014
ACKNOWLEDGEMENTS

I would like to thank Prof. S.F.T. Weiss for affording me this wonderful opportunity, for his time, guidance, support, scientific input as well as making sure my project is a success.

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Thanks a million times!
RESEARCH OUTPUT

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Other publications


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Conference presentations during MSc dissertation


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<td>rpm</td>
<td>Revolutions per minute</td>
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<td>Second</td>
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<td>amino acid</td>
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<td>acquired immune deficiency syndrome</td>
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<td>APS</td>
<td>ammonium persulfate</td>
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<td>base pair</td>
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<td>bromodeoxyuridine</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>C-terminus</td>
<td>carboxyl-terminus</td>
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<td>CaCl₂</td>
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<td>CAD</td>
<td>caspase-activated DNase</td>
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<td>CAM</td>
<td>cell-cell adhesion molecule</td>
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<td>carbon dioxide</td>
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<td>DD</td>
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<td>DED</td>
<td>death effector domain</td>
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<td>dH₂O</td>
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<td>DISC</td>
<td>death inducing signalling complex</td>
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<td>dulbecco's modified eagle medium</td>
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<td>DNA</td>
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<td>deoxyribonucleotide triphosphate</td>
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<td>dsRNA</td>
<td>double stranded RNA</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>FADD</td>
<td>fas-associated protein with death domain</td>
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<td>FCS</td>
<td>fetal calf serum</td>
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<td>human hepatoma cell line</td>
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<td>human immunodeficiency virus</td>
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<td>human papillomavirus</td>
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<td>HRP</td>
<td>horse-radish peroxidase</td>
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<td>ICAD</td>
<td>inhibitor of caspase-activated DNase</td>
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<td>IOD</td>
<td>integrated optical density</td>
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<td>kDa</td>
<td>kilo Dalton</td>
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<td>laminin receptor precursor</td>
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<td>messenger ribonucleic acid</td>
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<td>polyvinylidene fluoride</td>
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<td>RNA-induced silencing complex</td>
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<td>RPSA</td>
<td>ribosomal protein SA</td>
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<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>small interfering RNA</td>
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<td>tetramethylethylenediamine</td>
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<td>transcriptional gene silencing</td>
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<td>tumour necrosis factor receptor</td>
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<td>(TNF)-related apoptosis-inducing ligand</td>
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1. CHAPTER 1 – INTRODUCTION

1.1. Cancer

Cancer is a generic term referring to the growth of abnormal cells due to uncontrolled proliferation and lack of apoptosis that is caused by the accumulation of mutations that in turn compromise proper regulation of homeostasis[1]. Cancer arises when mutations observed in a single cell allow the cell to deregulate certain physiological processes i.e DNA repair checkpoints during the cell cycle; thus leading to transformation to a tumorigenic cell[1].

This disease has gained huge interest from both the public and scientific community due to high prevalence and mortality rates worldwide (Figure 1, 2 and 3), affecting individuals of all races, ages, gender and class [2](GLOBOCAN 2012 and http://www.cancerresearchuk.org ).

To date, cancer is the major cause of death worldwide, claiming about 8.2 million lives in 2012 with 70% of these deaths observed in low- and middle-income countries (GLOBOCAN 2012). Furthermore, current research studies propose that annual cancer cases will ascend from the 14 million observed in 2012 to 22 million by the year 2034(GLOBOCAN 2012). Of the 14.1 million new cases observed in 2012, about 50% of these new cases are accounted for by the five most common cancer types namely, lung, breast, liver, stomach and colorectal cancer (Figure 2) (Available from http://www.cancerresearchuk.org ).

Of particular interest to this study, breast and oesophageal cancers were ranked the second and eighth most commonly diagnosed cancers in the world, respectively (Figure 2). Moreover, breast and oesophageal cancers were observed to be the fifth and sixth common causes of death amongst cancers worldwide, respectively [2] (Figure 3).

The above statistics exemplify the urgency to discover alternative cancer treatments that will assist in eradicating this enduring cancer dilemma.
Figure 1: Worldwide cancer statistics in the year 2012. A chart indicating the new cancer and death cases worldwide accompanied by most commonly diagnosed and death-causing cancers. (Image adopted from GLOBOCAN 2012)
Figure 2: The 10 most commonly diagnosed cancers in the year 2012, worldwide. Image adopted from http://www.cancerresearchuk.org

Figure 3: The 10 most common causes of cancer deaths in the year 2012 worldwide. Image adopted from http://www.cancerresearchuk.org
1.1.1. Cancer causes and prevention

The transformation from a normal to a cancerous cell is not an isolated process; however, it is as a complex process governed by a series of evolutionary steps that may be due to internal (genetic instability, immune condition and epigenetic modifications) and external (radiation, infectious agents and tobacco) factors [3]. Numerous cancers have been identified as both preventable and non-preventable, with approximately 30% of cancers being preventable by incorporating lifestyle changes in order to minimize obesity, physical inactivity, smoking and exposure to certain external causative factors [4].

Moreover, certain cancers are initiated or enhanced by infectious agents such as viruses including the Human Papilloma Virus (HPV) that causes cervical cancer [5, 6], Epstein-Barr Virus (EBV) that increases a person’s risk of being infected with nasopharyngeal cancer[7]. Kaposi sarcoma (KS) on the other hand is caused by infection with the Human herpesvirus (HHV-8) which also co-infects with the Human Immunodeficiency Virus (HIV) thus increasing a persons’ risk of developing KS when diagnosed with HIV, however the tumor may also develop in the presence of the HHV-8 or the Kaposi’s sarcoma-associated herpesvirus (KSHV), solely[8].

1.1.2. Cancer diagnosis and treatment

Early diagnosis is crucial for the patients’ health as this allows for detection and eradication of the cancer whilst it is diminutive and has not metastasized to distant organs because treatment at this stage is limited and highly ineffective. Over the years, numerous diagnostic tools have been developed including, genetic tests, X-ray machines, magnetic resonance imaging (MRI) scans, ultrasound scans and nucleic markers[9].

Furthermore, once diagnosed with this disease, there are numerous treatment options to assist in controlling or eradicating the tumor e.g. radiation, surgery, chemotherapy, immunotherapy and transplantation[9]. However, some of these techniques such as radiation tend to be non-specific and enhance cancer growth, thus studies focusing on understanding the mechanisms of cancer have paved way for the invention of advanced and more specific novel approaches including gene therapy, angiogenesis inhibitors, telomerase inhibitors and monoclonal antibody therapy[10].

A majority of these recent treatment options target the alterations fundamental to tumor formation which have been described by Hanahan and Weinberg as the hallmarks of cancer (Figure 4).
**Figure 4: The main hallmarks of cancer.** The six main processes that facilitate transition from a normal to a cancerous cell. (Image adopted from[11])
1.2. Cancer hallmarks

1.2.1. Sustaining proliferative signalling

Normal cells cautiously regulate the release and production of growth signals that control entry into the cell cycle thus resulting in proper tissue homeostasis; however, cancerous cells deregulate these signals and are able to proliferate uncontrollably\cite{1}.

Proliferation is well controlled in normal cells, with a distinct balance between growth-promoting and anti-growth signals that control cellular division. Cancer cells have however acquired the ability to deregulate these signal by autocrine or paracrine signalling thus resulting in continuous cellular proliferation\cite{12, 13}. They continue to proliferate even in the absence of nutrients or growth factors such the epidermal growth factor receptor (EGF-R)\cite{14}. These growth factors can be supplied by the cancer cells themselves or by normal cells that are stimulated by other cancer cells. Alternatively, somatic mutations such as PI3K, MAP-kinase, Ras or Myc may result in persistent activation of downstream proliferative pathways\cite{15}.

1.2.2. Evading growth suppressors

In addition to overexpression of growth-promoting signals, cancerous cells have also acquired the ability to evade anti-growth signals, mainly the tumor suppressor genes – retinoblastoma (RB) and P53\cite{1}. Tumor suppressors are crucial for normal growth as they regulate cell growth and proliferation in order to maintain proper tissue homeostasis and prevent tumor formation\cite{1}. However, cancer cells must be able to inhibit the effects of these tumor suppressors in order to progress. The two main tumor suppressors are encoded by the retinoblastoma (Rb) and TP53 proteins, which either induce cellular proliferation, cell death or senescence depending on the cellular environment\cite{11}. TP53 is a pro-apoptotic member that triggers apoptosis in unfavourable cellular conditions or excessive genome damage\cite{16}. RB on the other hand decides whether a cell progresses through the cell cycle or remains in the G\textsubscript{0} phase until the conditions are favourable\cite{17, 18}. However, cancer cells with mutations in these tumor suppressor genes lack the services of these key regulators of cell-cycle progression and result in persistent abnormal cell proliferation and lack of apoptosis, which are the basis of tumor progression\cite{1, 11}.

1.2.3. Inducing angiogenesis

Similar to normal cells, cancerous cells need nutrients and oxygen in order to proliferate. Tumors aberrantly express vital growth factors and receptors – specifically, the angiogenic inducing factors such as upregulating vascular endothelial growth factor (VEGF) which facilitates blood vessel formation, providing conditions sufficient for rapid turnover due to a constant supply of blood, nutrients and oxygen\cite{1, 19}. Moreover, tumors utilize the vasculature to eliminate metabolic waste and also as a route to migrate and metastasize to other organs\cite{1, 19}.
1.2.4. Activating invasion and metastasis

Invasion and metastasis of epithelial cells is principally regulated by the epithelial-mesenchymal transition (EMT). Tumorigenic cells selectively reduce their expression of cell-cell adhesion molecules (CAMs), such as E-cadherin [20], alter integrin expression so as to express integrins suited to the composition of the extracellular matrix (ECM) at the distal secondary tumour site. Moreover, cancer cells may upregulate their expression of matrix degrading proteases (i.e. collagenases) vital for the disruption of the basal lamina that stimulates extravasation and initiation of migratory events[21].

1.2.5. Enabling replicative immortality

Numerous studies have illustrated that the presence of telomeres support proliferation due to the prevention of DNA damage by protecting the ends of chromosomes [22, 23]. In order to proliferate continuously, cancer cells have upregulated the expression of telomerase[24]. Limitless replicative potential is due to the enhanced telomerase activity observed in a vast number of cancerous cells[24]. Normal cells have low levels of telomerase, the enzyme required for the addition of telomere repeat segments that are crucial for protecting the ends of chromosomal DNA which prevents DNA damage[22]. The length of telomeric DNA tends to control the number of cell divisions that a cell can undergo before the ends of chromosomal DNA are damaged and compromise cell viability[22]. In turn, the enhanced telomerase activity allows cancer cells to divide uncontrollably as this corresponds to resistance to the occurrence of senescence and apoptosis by preventing progressive telomere shortening and resulting DNA damage[1]. The subunit of the telomerase enzyme, TERT plays a role in enhancing proliferation by resisting apoptosis due to its involvement in repairing DNA damage, however, further studies need to be carried out in order to elucidate the particular mechanism that this protein exerts this function[25].

1.2.6. Resisting cell death

Induction of cell death due to physiological stress or DNA damage is vital for the maintenance of a healthy cellular microenvironment and homeostasis [26, 27]. However, tumors have evolved to acquire a selection of tactics to circumvent apoptosis and, in turn, progressively grow and produce highly aggressive malignant tumors[1]. As mentioned previously, one common strategy is the loss or aberrant expression of P53, which allows the tumorigenic cells to by-pass the apoptosis-inducing circuitry and proliferate aberrantly to form enormous malignant tumor types[1, 28]. Tumors also avoid cell death by altering the expression of pro- and anti-apoptotic regulators of the death pathways to be dealt with later on, i.e. cancerous cells upregulate and downregulate the expression of anti- and pro-apoptotic Bcl-2 family members, respectively[1, 29]. The evasion of apoptosis has been observed to be a major hallmark of most if not all cancer types, thus targeting of mechanisms surrounding this hallmark is crucial for elucidation of possible therapeutic options of this disease[1].

In addition to the above discussed hallmarks, there are two emerging cancer characteristics of tumors namely, reprogramming energy metabolism and avoiding immune destruction. The first entails the modification of the cellular energy metabolism inorder to sustain the
chronically proliferating tumor which requires supplementary nutrients and oxygen supply[11].

1.2.7. *Reprogramming energy metabolism*

Apart from maintaining chronic proliferation and evading apoptosis cancer cells alter their environment by upregulating their energy metabolism in order to provide conditions that are suitable for cellular growth and division thus promote tumor progression[11]. One example is glycolytic fuelling which is the increased uptake of glucose which is involved in the activation of mutated tumor suppressor genes as well as oncogenes which feed into the basic hallmarks of tumorigenecity[11].

1.2.8. *Evading immune destruction*

The immune system has mechanisms of inhibiting the formation or progression of tumors such as DNA repair systems and cell cycle checkpoints. However, tumors have acquired the ability to bypass or avoid detection by the immune system[11]. Not much is currently known about these two hallmarks but the enabling characteristics governing these processes are tumor-promoting inflammation and the genome instability and mutation[11].
1.3. Apoptosis

This phenomenon was first described by Carl Vogt in 1842 and 100 years later termed “apoptosis” by John Kerr[30]. Apoptosis is a physiological well-ordered type of programmed cell death, in contrast to the catastrophic type of unprogrammed cell death – necrosis[31]. It is a crucial process from embryonic development all through to adulthood for immune regulation and achieving proper tissue homeostasis by regulating cell numbers and tissue size[32]. It is well-orchestrated and important process throughout an persons’ lifespan, however, inappropriate execution of this process compromises physiological homeostasis and leads serious illnesses including AIDS[33], autoimmune diseases[34], liver diseases[34], neurodegenerative disorders[35] such as Alzheimer’s, Parkinson’s’ disease and crucial to this study cancer, which is as a result of minute apoptosis. Needless to say due to implications in such aggressive diseases, apoptosis has gained major interest from the scientific community aiming at formulating alternative therapeutic options regulating cell death mechanisms.

The induction of apoptosis is stimulated by external (oxidative stress, growth factor withdrawal) and internal (DNA damage) stimuli resulting in physical changes such as chromatin condensation, cell shrinkage as well as biochemical alterations including DNA cleavage [30, 36, 37] (Figure 5). These features and cell membrane blebbing which triggers phosphotidylserine exposure are referred to as the hallmarks of apoptosis [36, 37]. As the cell progresses from early to late apoptosis the membrane integrity is lost and leads to packaging of the cellular contents in spheres known as apoptotic bodies, which are digested by macrophages; thus not posing harmful consequences to other cells or the cellular environment[37] (Figure 5).

**Figure 5: The main hallmarks of apoptosis.** Apoptotic cells are characterized by physical and chemical changes. Once apoptosis is initiated, the cell shrinks and DNA fragmentation occurs followed by membrane blebbing that becomes convoluted forming apoptotic bodies. The apoptotic bodies package cellular contents which are digested by macrophages. (Image adopted from [http://www.buzzle.com/articles/how-do-cells-commit-suicide.html](http://www.buzzle.com/articles/how-do-cells-commit-suicide.html))
This process is executed by specific proteases known as caspases, both initiator i.e. caspase-8, -10 and effector caspase-3, -6 and -7[38]. These enzyme precursors are synthesized inactive and are activated by proteolysis or transactivation upon contact with other caspases[38]. Although caspases are the executers of apoptosis the events preceding caspase activation differ and are dependent on the pathway activated between the two major pathways, the death receptor-mediated and mitochondrion-mediated cell death pathway [31, 32, 36]. These processes differ in certain respects including the stimuli required for initiation, and it is noteworthy to add that although the receptor-mediated pathway is rarely targeted for the study of oncogenic mutations a brief discussion on this pathway will also follow.

1.3.1. **Extrinsic death receptor pathway**

This pathway is crucial for the regulation of the immune system and is triggered by the binding of the death receptors from the tumor necrosis factor (TNF) receptor superfamily composed of TNF-1, CD95 and the TNF-related apoptosis-inducing ligand (TRAIL)[39]. Binding of these receptors directs receptor trimerization where the adaptor Fas-associated death domain (FADD) proteins interact with Fas via the cytosolic domain [40-42]. This interaction induces the death effector domain (DED) of FADD to bind the DED of procaspase-8 thus forming the death inducing signalling complex (DISC) complex [43, 44]. In this complex, the procaspase-8 molecules are orientated in close proximity such that transactivation of these zymogens occurs and the active caspase-8 interacts with and activates one of the executioner caspases, caspase-3,-6 or -7 (Figure 6); resulting in the cleavage of key cellular substrates such as DNA molecules that are crucial for maintenance of the cell’s viability thus leading to cellular destruction [43, 44].

1.3.2. **Intrinsic mitochondrial pathway**

This is the most targeted pathway of cell death as most cell death proceeds via this pathway and is triggered by both internal and external stimuli. Detection of the apoptotic signal triggers the release of cytochrome c along with Smac/DIABLO and AIF (Apoptosis Inducing Factor) from the mitochondrial upon activation of the pro-apoptotic members of the Bcl-2 family (i.e. Bak, Bax, Bid & Bim) [45-47]. To date the precise mechanism regulating this event is still elusive, however, Ott et al. proposed that this release proceeds by a two-step mechanism initiated by the solubilisation of the cytochrome c molecules from the inner mitochondrial membrane by peroxidation of the anchoring-lipid, cardiolipin [48]. However, the solubilisation of these cytochrome c molecules is preceded by the permeabilization of the outer mitochondrial membrane by Bax thus initiating the release cytochrome c into the cytosol [48]. Subsequently, seven cytochrome c molecules interact with seven apoptotic protease activating factor-1 (Apaf-1) and seven procaspase-9 molecules, forming the apoptosome [49]. Within the apoptosome the oligomerization of Apaf-1 by cytochrome c induces dimerization of the procaspase-9 molecules resulting in the release of active caspase-9 molecules that in turn activate effector caspases such as caspase-3, leading to the cleavage of crucial cellular substrates to effect the occurrence of apoptosis[49] (Figure 6).
Indeed for apoptosis to occur the above mentioned events are crucial, however, induction of apoptosis is not solely controlled by pro-apoptotic signals but an integration between both pro- and anti-apoptotic signalling and the progression of this system is often more complex.

Figure 6: The two main pathways of apoptosis in mammalian cells. The death receptor pathway detects an external stimuli (i.e oxidative stress) leading to the interaction of FADD proteins with Fas by a process known as receptor trimerization. This complex induces the DED of FADD to bind to the DED of procaspase-8 molecules thus forming the DISC complex. This complex is crucial for the transactivation of procaspase-8 molecules to active caspase-8 molecules that activate the effector caspases thus inducing apoptosis. The intrinsic pathway is activated by both internal and external stimuli that trigger the release of cytochrome C that is required to interact with Apaf-1 and procaspase-9 molecules forming the apoptosome. Within the apoptosome, oligomerization of Apaf-1 by cytochrome C leads to dimerization of procaspase-9 molecules, releasing active caspase-9 molecules that activate effector caspases and induce apoptosis. (Image adopted from[50]).

Moreover, researchers have described as many as eleven cell death pathways in mammals; however, there are only two more types of cell death pathways that have been clearly defined, namely autophagic cell death and necrosis[51]. All cells can undergo either of the three cell death pathways and in order to produce appropriate potential therapeutics targeting cell death one has to be able to clearly identify the form of cell death between the three[51]. Therefore, a brief discussion on the characteristics to distinguish between the three cell death types and the distinct hallmarks of apoptosis follows.
1.4. Autophagic cell death

Autophagic cell death also referred to as type II cell death is stimulated by signals such as nutrient starvation, differentiation and environmental stress[52]. This form of cell death is crucial for the recycling of long-lived proteins and components of organelles by the lysosomes for the maintenance of energy and protein synthesis[52]. Upon detection of the signal, double-membrane bound structures assemble to form autophagosomes, vacuole-shaped structures that contain cytosolic material[52]. These structures fuse with lysosomes thus leading to the degradation of the encapsulated cytosolic material[52]. This is a physiologically critical process; however, excessive autophagy has pathological implications, causing neurodegenerative diseases, liver diseases and cancer [53, 54].

1.5. Necrosis

Necrosis is an energy-independent form of cell death that is considered to result in prominent energy depletion, activation of non-apoptotic proteases and formation of reaction oxygen species. [55]. This results in cytoplasmic swelling, dilation of prominent organelles (mitochondria, Golgi apparatus, and endoplasmic reticulum), rupture of the cell membrane as well as condensation of chromatin material[56]. Continuous swelling stimulates the cell to burst, resulting in the leakage of the cytoplasmic contents which cause an inflammatory response[56].

1.6. Hallmarks of apoptosis

1.6.1. Caspase activation

Caspases are a family of proteases that are produced in an inactive enzyme form known as zymogens with an N terminal prodomain, followed by a large and small subunit [38]. These enzymes undergo caspase maturation by cleavage on the carboxyl side of aspartate residues achieved by other caspases or the cytotoxic T lymphocyte serine protease granzyme B[38].

The prodomains of these zymogens differ in length, ranging from 23 amino acids to 219 amino acids. Initiator caspases such as caspase-8, -9 and -10 generally have long prodomains and effector caspases 3, 6 and 7 have short prodomains[38].

Upon activation, caspases cleave particular substrates thus resulting in their activation or inactivation. The cleavage of fundamental components of the cytoskeleton, nucleus and proteins of major signalling pathways results in numerous morphological changes associated with apoptosis as will be discussed shortly.
1.6.2. *Membrane alterations*

Cell membrane blebbing is one major characteristic of apoptosis induction, resulting in the exposure of phospholipid phosphatidylserine (PS) to the outer membrane[57]. This lipid bilayer-forming lipid is generally situated in the inner cell membrane in live cells; however, upon induction of apoptosis it is translocated to the outer cellular membrane [57, 58]. This caspase-dependent mechanism is crucial for recognition of apoptotic cells as well as serving as a signal to phagocytes that require binding to PS receptors, thus leading to prompt engulfment of apoptotic cells without provoking an inflammatory response[59] (Figure 5).

1.6.3. *DNA fragmentation*

DNA cleavage is another crucial feature as it assists in the elimination of DNA that codes for autoantibodies that can cause hyperactivity of the immune system thus resulting in the demolition of healthy tissue[60]. This process is initiated by the caspase-mediated cleavage of the inhibitor of caspase-activated DNase (ICAD), resulting in free, active CAD that produces multiple DNA double-stranded breaks of 180 base pairs in size [61, 62]. This is not a strictly specific process as the active CAD may degrade various parts of the entire genome leading to a loss of essential genes thus resulting in the induction of cell death[61].

1.6.4. *Mitochondrial changes*

In contrast to membrane alterations and DNA fragmentation, mitochondrial modifications precede apoptosis because it is crucial for the release of cytochrome c molecules that are required for the formation of the apoptosome in the intrinsic mitochondrial pathway[63]. It is proposed that the pro-apoptotic molecules of the Bcl-2 family such as Bax interacts with the mitochondrion and induce mitochondrial outer membrane permeabilization (MOMP) which is accompanied by the solubilisation of cytochrome c molecules from the anchoring lipid, cardiolipin thus leading to cytochrome c release and formation of the apoptosome in the intrinsic pathway[63].
1.7. The cell cycle

Proper functioning of an organism requires adequate completion of the cell cycle in all the cells in the body. This is a tightly regulated process that is crucial for cellular growth by mediating duplication[64], replication and transfer of genetic information in the form of chromosomes into daughter cells[65]. The coordination of these complex events is achieved by the cyclin dependent kinases (CDKs) by driving the cell through the five stages of the cell cycle, namely, gap 0 (G₀), gap 1 (G₁), gap 2 (G₂), mitosis (M phase) and the S phase that is responsible for DNA synthesis[65] (Figure 7).

During the G₀ phase the cell remains quiescent or senescent meaning it is currently inactive in the cycle, either temporarily or permanently but is metabolically active[65]. Extracellular signals such as growth factors, mitogens or nutrient availability stimulates quiescent cells to enter G₁ and proceed to the S phase for DNA synthesis[65]. Precise DNA replication allows for procession to the G₂ phase where cells expand in size and proteins required for the M phase are synthesized[65]. These proteins are required for the duplication of the mitotic spindles, separation and migration of sister chromatids to opposite poles of the cell prior to cytokinesis, resulting in two identical daughter cells[65].

There are cell cycle checkpoints (G₁-S and G₂-M) that are responsible for ensuring the order of events in the cell cycle and monitoring crucial events such as DNA replication[65]. Stringent regulation of these events is crucial as accumulation of mutations cause loss of genome integrity and leads to unstable genomes that could evolve normal cells to cancerous cells[65]. These mutations afford cells the ability to proliferate independently of growth signals and reduced sensitivity to death signals[65]. The checkpoints assist in the correct regulation of the cell cycle and should an error be encountered progression through the cell cycle is halted until it has been fixed, however, if it is a severe error cell death is immediately induced[66].

The above mentioned processes and the million events that occur in cells daily do not occur in an isolated system, however, there is an integration of communication amongst cells and their environment, the extra-cellular matrix (ECM).
Figure 7: The cell cycle. The cell cycle comprises of five stages; $G_0$ – the cell remains quiescent but metabolically active, $S$ – the cell’s DNA replicates, $G_1$ – cells enter this phase in response to nutrient availability, $G_2$ – the cell grows in size, $M$ – mitotic spindles are formed and cell division occurs following sister chromatid segregation. (Image adopted from://www.bdbiosciences.com/research/apoptosis/analysis/index.jsp)
1.8. Extracellular matrix (ECM)

The ECM is a complex matrix that functions to provide signals that regulate cell behaviour, navigational signals for migrating cells, contact with neighbouring cells and supply of nutrients and growth factors via specialized receptor molecules[67]. It is crucial for cell survival in both plants and animals, with the cell wall representative of the ECM in plants whereas in animals it appears in two forms, the basement membrane and the stromal matrix[67].

This matrix is made up of a mixture of diverse molecular components that are essential for regulation of these cellular processes and varies in composition and volume between tissues of an organism as well as with developmental age. It is mainly composed of proteoglycans and glycoproteins such as, collagens, elastin, fibronectins, matrix metalloproteinases (MMPs)[68] (Figure 8) and of particular interest to this study, the laminins.

![Figure 8: The extracellular matrix.](http://wiki.pingry.org/u/ap-biology/images/5/52/Image122.gif)

1.8.1. Laminins

Laminins are major ECM glycoproteins that are made up of three α, β and γ chains that are stabilised by disulphide bonds to form a cross shape[69] (Figure 11). These 5α, 3β and 3γ are able to assemble into numerous different heterotrimerers and the resulting trimers are named according to their chain composition such as α1β1γ1 is referred to as laminin-111 and α2β1γ2 known as laminin-212. To date, there are 17 laminin isoforms that are known[69]. These heterotrimeric proteins have various binding sites for components of the ECM such as collagen, proteoglycans, integrins and with other laminin molecules thus...
forming sheets in the basal lamina that are crucial for cell differentiation, migration, adhesion and survival[69].

One prominent binding site is the cell-surface receptor binding domain that allows laminins to bind to the 37-kDa/67-kDa laminin receptor precursor/high affinity laminin receptor (LRP/LR), thus regulating crucial cellular events, such as adhesion and signal transduction.

Figure 9: Structure of laminin-1. The cross of laminin is made up of three α, β and γ chains stabilised by disulphide bonds. It has binding sites for numerous ECM molecules including collagen IV, entactin, heparin and cell surface receptors. (Image adopted from http://themeekgroup.com/Christian/laminin.htm)
1.9. The 37-kDa/67-kDa laminin receptor precursor/laminin receptor (LRP/LR)

This laminin receptor was first isolated in 1983 from murine melanoma, human breast carcinoma and normal muscle cells by purification from the cell membrane using laminin-sepharose affinity chromatography [70-72]. This receptor was found to have a molecular mass of 67-kDa encoding for a 295 amino acid long protein with a 37-kDa molecular weight thus leading to the conclusion that the 37-kDa gene product is the precursor of the 67-kDa high affinity laminin receptor (LR), hence referred to as the laminin receptor precursor (LRP) [73]. Since the discovery of this relationship there has been numerous studies aimed at understanding how this occurs, however, the exact mechanism has not been elucidated except for proposals that this may be through homodimerization that requires post-translational modifications [74, 75]. However, a study by Landwoski illustrated that inhibition of fatty acid synthesis prevents formation of the 67-kDa LR thus implying that acylation of LR by fatty acids (palmitate, stearate and oleate) is perhaps crucial for maturation from the 37-kDa LRP[74], therefore the term LRP/LR will be used to refer to the 37kDa/67kDa laminin receptor through this manuscript.

This type II transmembrane protein (Figure 10) localises on the cell surface, nucleus[76-78] and cytosol[79, 80] acting as a receptor for numerous molecules, assisting in the maintenance of nucleic structures by binding to histones and regulation of translational processes[81], respectively. As the name suggests, its’ primary binding ligand is laminin-1 which it binds with high affinity and specificity via three regions in the C-terminal domain, namely residues 161-180[82], most C-terminal 53 residues[83] and residues 205-229[74] (Figure 12).

This LRP/LR-laminin-1 interaction is implicated in the regulation of crucial cellular processes such as cell adhesion, migration, proliferation and protease activity[84]. In addition to regulating these physiological processes by binding laminin-1, LRP/LR also acts as a receptor for extracellular matrix components i.e elastin, carbohydrates and cellular prion proteins[85]. Moreover, the LRP/LR has been implicated in numerous pathological processes by facilitating the internalization of bacteria[85], viruses[86-88] and infectious prion proteins[85]. Of particular importance to this study, is the overexpression of this receptor on a number of cancer types, which is correlated to cancer progression.
Figure 10: Structure of the 37-kDa/67-kDa laminin receptor precursor/ laminin receptor (LRP/LR). LRP/LR is a 295 aa long transmembrane protein. It is a type II membrane protein with the N-terminus and C-terminus exposed to the cytoplasmic side and extracellular space, respectively. It binds PrP, heparin, IgG antibodies and laminin. (Image adopted from[89])
1.10. Relation of the laminin receptor and cancer

High levels of LRP/LR expression were first noted in numerous cancer types, such as breast[90], lung[91], cervical[92], ovarian[93], colon[94], gastric[95], heptocellular[96] and prostate[97] cancer cells, when compared to corresponding normal cells. To date there is no data implicating particular stimuli responsible for this differential expression, however, overexpression of this receptor has been observed to augment tumor aggressiveness by affording cancer cells the enhanced ability to metastasize[98], induce angiogenesis[99] and evade cell death[99].

1.10.1. LRP/LR-laminin-1 interaction and metastasis

The first possible role of LRP/LR in cancer was noted by Rao in 1983 suggesting that the 67kDa laminin receptor plays a role in the interaction between the metastasizing cancer cell and the basement membrane[72]. During metastasis, adherence of the invasive cancer cells triggers degradation of the basement membrane, allowing the cancerous cell to invade the surrounding stroma and migrate through the bloodstream to a distant organ to form a secondary tumor[1, 100]. The two key steps of this process is the adhesion and invasion of the basement membrane, which are events mediated by the LRP/LR-laminin-1 interaction(Figure 11)[100].

Moreover, the LRP/LR-laminin-1 interaction is significantly enhanced in tumors that express the 67kDa laminin receptor at high levels thus promoting the malignancy of that tumor[101, 102]. Although the mechanism has not been clearly elucidated, it is suggested that upon adherence to the basement membrane via the LRP/LR-laminin-1 interaction, LRP/LR triggers the secretion of Type IV collagenase, a protease that mediates degradation of the main component of the basement membrane, Type IV collagen thus facilitating invasion[89]. In vitro metastatic studies have illustrated that an anti-LRP/LR specific antibody IgG1-iS18 significantly impedes adhesion and invasion of important cancer types worldwide, i.e prostate, colon, cervical, lung, breast, oesophageal and liver cancer, by interfering with the LRP/LR-laminin-1 interaction [101-103].
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**Figure 11: LRP/LR-laminin-1 interaction and metastatic cancer.** The enhanced LRP/LR-laminin-1 interaction in metastatic cells allows cells to adhere to the basement membrane, leading to the hydrolysis of type IV collagen by type IV collagenase. This allows cells to invade surrounding tissue and migrate to a secondary site to form a tumor. However, incubation of colon SW480 cells with IgG1-iS18 anti-LRP/LR specific antibody significantly impeded invasion of these cancer cells. (Image adopted from [102])

1.10.2. *The role of LRP/LR in tumor angiogenesis*

Angiogenesis known as the formation of blood vessels is imperative for wound healing, embryonic development, vascular remodelling and tissue repair by supplying cells around the body with sufficient oxygen and nutrients[104]. This process is controlled precisely in healthy individuals, however, in individuals with cancer it is deregulated in order to form additional tubular structures that will assist in facilitating metastasis by providing the tumors with routes to migrate through and initiate secondary metastasis in a distant organ[105]. Furthermore, angiogenesis also supports the viability of cancer cells by providing them with oxygen and nutrients which are crucial for cellular growth[105]. The formation of these tubular structures requires cell-ECM interactions and since LRP/LR binds the ECM protein laminin-1, resulting in proteolytic activation, a fundamental process in angiogenesis, this gave rise to speculations that LRP/LR may be involved in tumor angiogenesis[105]. Indeed, a study conducted in our laboratory confirmed that LRP/LR is involved in the angiogenic process following the observation of a significant decrease of tube formation in HUVECs (human umbilical vein endothelial cells) post treatment with anti-LRP/LR specific antibody W3(Figure 12)[106].
Figure 12: Illustration of the effect of anti-LRP/LR specific antibody W3 on angiogenic tube formation. (A) Administration of anti-LRP/LR antibody W3 to HUVE cells that had established tubular structures on Matrigel™, inhibited further degradation of the basement membrane, a prerequisite for tube formation. This halted the development of additional tubular structures, moreover, W3 antibody bound to existing tubes and blocked the LRP/LR-laminin-1 interaction, hence resulting in (B) the reversal of tube formation and cells were consequently observed as single cells on the Matrigel™ (Image adopted from [106])

1.10.3. LRP/LR and cellular viability

LRP/LR is conserved across all species and ribosomal studies in yeast have illustrated that the this receptor plays a crucial role in cellular viability by processing the 20S ribosomal RNA to 18S and in turn leading to the maturation of 80S monosome and 40S ribosomal unit that regulate cell survival[107]. The ribosomal functions of LRP/LR in mammalians have not been clearly elucidated; however, it has been observed that LRP/LR upregulates signalling pathways that are crucial for cell survival[76]. Moreover, it is hypothesized that LRP/LR associates with chromatin and the nuclear envelope during interphase, facilitating chromosomal stability that sequentially maintains cell viability[76]. It is believed that the LRP/LR attains this by binding chromatin and the nuclear envelope with the cell migration, proliferation and cell survival promoting growth factor Medkine[108]. Furthermore, Scheiman et al conducted a cell viability study in human HT1080 fibrosarcoma cells demonstrating that the LR requires the C-terminus for effective cellular viability maintainance[80].
1.10.4. *LRP/LR and the evasion of apoptosis*

LRP/LR has been implicated in cancer progression by inhibiting apoptosis. It is expected though because as stated above that LRP/LR plays a role in maintainance of cellular viability and since this receptor is expressed in high levels in several cancerous cells, its pro-cell survival role is augmented in these cancer types. siRNA constructs targeting the expression of 37kDa LRP mRNA in Hepb3 liver and HeLa cervical cancer cells stimulated the induction of apoptosis, clearly indicating the role of LRP/LR in blocking apoptosis[109]. Furthermore, a study conducted in our laboratory using siRNA LAMR1 in HeLa cervical and A549 lung cancer cells resulted in the reduction of LRP/LR expression that sequentially stimulated the induction of apoptosis by activating caspase-3, the effector caspase of apoptosis[110]. These results evidently indicate that LRP/LR is a crucial potential target for the treatment of cancers that reveal high expression levels of this receptor.
1.11. Applications of small interfering ribonucleic acid (siRNA) technology

siRNAs are 9 – 21 nucleotide long dsRNA sequences that are produced to stimulate degradation of target complementary sequences present in cellular mRNA[111]. siRNAs achieve this by either suppressing transcription (known as transcriptional gene silencing) or activating sequence specific RNA degradation (referred to as posttranscriptional gene silencing (PTGS)) or the RNAi pathway[111]. The RNAi pathway has been observed in most eukaryotic organisms such as parasites[112], flies[113], protozoa[114], humans[115] as well as plants. This mechanism utilizes the dsRNA molecules as the inducers that cleave inducer molecules thus resulting in the degradation of the target mRNA molecules[111].

The main components of the PTGS/RNAi pathway are the Dicer and RNA-Induced Signalling Complex (RISC) that are involved in the initiation of the PTGS/RNAi pathway and degradation of the target mRNA[116], respectively (Figure 13).

1.11.1. Dicer

The Dicer is a nuclease with four main domains: a dsRNA binding domain, PAZ domain, an amino terminal helicase domain and dual RNase III motifs [117-119]. Subsequent to binding of the dsRNA molecules to Dicer, the Dicer digests dsRNA forming small 22 bp sized siRNA molecules which are further digested by the RNase III nucleases into 12 – 15 bp[111, 120]. The digestion of dsRNA to siRNA occurs at two catalytic centres of the dimeric RNase III enzyme [120]. The RNase III enzyme folds on either sides of the dsRNA substrate, producing four catalytic sites with the two terminal sites containing maximum homology with the RNAse III catalytic sequences, whilst the other sites lose activity due to partial homology[121]. This enables the Dicer to process double the size of dsRNA molecules when compared to other RNase III enzymes[121].

1.11.2. RNA-Induced Signalling Complex

RISC is the nuclease activity accountable for the degradation of target mRNA in a sequence specific manner. This complex is made up of the Argonaute (Ago), Tudor-Staphylococcus nuclease (Tudor-SN) and fragile X-related (FXR) domain proteins[116].

This mechanism of RNA interference using the PTGS/RNAi pathway has not been clearly elucidated but has been summarised into three fundamental steps:

This pathway is initiated by the binding of the RNA III nuclease Dicer to dsRNA, facilitating cleavage of the dsRNA into 21 – 25 bp siRNA fragments[111]. The second step entails the incorporation of siRNAs into the complex, RISC by the dsRNA-binding protein R2D2[111], which stimulates enzymatic activation of the RISC zymogens in the presence of ATP to mediate the unwinding of the double stranded siRNA (Figure 13)[111]. Subsequently, the least stable siRNA strand of the two is discarded and only the most stable siRNA strand is left bound to the complex, hence this is the effector step[121]. Step 3 facilitates gene silencing. Whilst the single stranded siRNA is bound to the complex, the activated RISC cleaves the target mRNA endonucleolytically at the region complimentary to the siRNA[122], which is usually ten nucleotides upstream of the nucleotide paired with the 5’
end of the siRNA[123] (Figure 13). This cleavage step has been observed to operate independently of ATP[124]; however, the release of the cleaved products occurs more efficiently in the presence of ATP[113].

**Figure 13: RNA interference mechanism.** Dicer cleaves the dsRNAs into siRNAs. The siRNAs are incorporated into the RISC complex and bind to their complimentary mRNA sequences in the cells. Once bound, the target mRNA is degraded and remains non-functional. (Image adopted from Society of Toxicologic Pathology, 35 (3) 2007)
Numerous studies have focused on the application of RNA interference as a therapeutic tool for treatment of diseases that are caused by the altered expression of certain genes such as viral infections\[125\], genetic disorders\[126\], autoimmune diseases as well as cancer\[127\].

It is proposed that these diseases may be treated by modifying the activity of these genes by RNAi based therapeutics. Furthermore, it is relatively effortless introducing synthetic siRNAs \textit{in vitro} and effective gene silencing has been observed, however, delivery of these molecules \textit{in vivo} still remains challenging. This is still the case because non-specific targeting of normal cells occurs thus compromising the health of the individual. Nonetheless, RNAi based therapeutics are currently being developed for numerous viruses including, respiratory synytial viruses (RSV), hepatitis B virus (HBV) and the human immunodeficiency virus (HIV)\[125\].
1.12. Rationale, research question, aim, objectives

1.12.1. Rationale

LRP/LR has been found to be expressed in high levels in numerous cancer cells when compared to their corresponding normal cells. The high expression of this receptor affords cancer cells with the ability to enhance metastasis, angiogenesis and cellular proliferation, which are all prominent hallmarks of cancer. Moreover, the knockdown of LRP/LR has been observed to reduce viability of certain cancer cells thus indicating the fundamental role of LRP/LR in cellular viability. The high incidence and mortality rates observed worldwide with respect to cancer indicate the urgency of alternative therapeutic options and the role of LRP/LR in cancer progression is undeniable. Therefore targeting of this receptor may possibly pave a way for the elucidation of promising therapeutic tools that can be utilised for the treatment of cancer.

1.12.2. Research question

Will downregulation of LRP/LR expression using specific siRNAs or the interference of the LRP/LR-laminin-1 interaction using anti-LRP/LR antibody IgG1-iS18 reduce the viability of breast (MCF-7 & MDA-MB 231) and oesophageal (WHCO1) cancer cells, and if so, which mechanism of cell death is responsible for the observed effects?

1.12.3. Aim

To investigate whether downregulation of the LRP/LR using siRNA technology affects the cellular viability of breast (MCF-7 & MDA-MB 231) and oesophageal (WHCO1) cancer cells.

1.12.4. Objectives

- Determination of the effect of anti-LRP/LR specific antibody IgG1-iS18 on the cellular viability of MCF-7, MDA-MB 231 and WHCO1 cells since this antibody significantly impeded the adhesion and invasion, key steps of metastasis of these cancer cell lines by interfering with the LRP/LR-laminin-1 interaction
- Downregulation of LRP expression in MCF-7, MDA-MB 231 and WHCO1 cells by transfection of siRNA-LAMR1
- Analysis of the effect of siRNA-mediated downregulation of LRP/LR on the cellular viability of MCF-7, MDA-MB 231 and WHCO1 cells using an MTT assay
- Determination of the effect of siRNA-mediated downregulation of LRP/LR on the proliferative state of MCF-7, MDA-MB 231 and WHCO1 cells using a BrdU assay
- Identification of the type of cell death activated following siRNA-mediated downregulation of LRP/LR expression if an effect on cell viability is observed using
- Annexin-FITC and 7-AAD assay – to observe the exposure of phosphatidylserine (PS) during membrane blebbing, a hallmark of programmed cell death apoptosis
- Immunofluorescence microscopy – visualisation of nuclear morphological changes associated with activation of apoptosis
2. CHAPTER 2 – MATERIALS AND METHODS

2.1. Cell lines and cell culture conditions

2.1.1. Cell lines

The following cell lines were used in this study:


- Human breast adenocarcinoma cell line (MDA-MB 231 ATCC®HTB-26) – An adherent metastatic epithelial breast cancer cell line derived from a 51-year-old Caucasian female.

- Human oesophageal carcinoma cells (WHCO1) – this metastatic cell line is not commercially available; it was propagated in vitro from tumor biopsy of a patient with this type of oesophageal cancer[128].

2.1.2. Cell culture conditions

Human breast adenocarcinoma MCF-7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) high glucose (4.5 g/l) (Invitrogen Gibco®). MDA-MB 231 breast cancer cells were cultured in DMEM/Hams F12 (1:1) and WHCO1 oesophageal cancer cells were cultured in DMEM/Hams F12 (3:1), respectively. All media were supplemented with heat inactivated 10% (v/v) fetal calf serum (Sigma) and 1% (10 000 U/ml) penicillin/streptomycin (Separations) at 37°C and 5% CO₂.

2.2. Analysis of the interference of the LRP/LR-laminin-1 interaction on the cellular viability of cancer cells using the anti-LRP/LR specific antibody IgG1-iS18

4 x 10⁵ MCF-7, 3.5 x 10⁵ MDA-MB 231 and 3 x 10⁴ WHCO1 cells were seeded in the wells of a 24-well plate and incubated with 50 mg/ml of IgG1-iS18 after 24 h. Post 17 h incubation with the anti-LRP/LR specific antibody, anti-CAT antibody (negative control), 8 mM PCA (positive control – known to decrease cell viability by inducing apoptosis); cells were stained with 0.1% (v/v) crystal violet and the absorbance of the colorimetric solution was measured at 570 nm using an ELISA reader.
2.3. Downregulation of LRP expression

2.3.1. Transfection of MCF-7, MDA-MB 231 and WHCO1 cells with siRNA-LAMR1 and siRNA-scr

24 h before transfection, MCF-7, MDA-MB 231 and WHCO1 cells were seeded in the wells of a 6-well plate. 5 µl of 20 μM siRNA-LAMR1 (5’-TTGGTTCCCCATCGTAACTAA-3’) and 5 µl of 20 μM siRNA-scr (negative control) (Inqaba Biotec) were each resuspended in 195 µl of serum-free media (appendix). Concurrently, 10 µl of the DharmaFECT® transfection reagent 1 was resuspended in 190 µl of serum-free media; both solutions were incubated at room temperature (RT) for 5 min. The siRNA solutions and transfection reagent solutions were mixed gently and incubated for 20 min at RT. Media from the plated wells was removed and 400 µl of the siRNA-reagent solution was added to each well and media was topped up to 2 000 µl with antibiotic-free media. Cells were allowed to grow for 72 h in a humidified incubator at 37°C with 5% CO₂ and 95% air.

2.3.2. Whole cell lystate preparation

72 h post transfection media was aspirated out; cells were rinsed with PBS (appendix) and incubated in 250 µL of lysis buffer (appendix) for 2 min. Cells were detached using a sterile cell scraper and the resulting solution was incubated at 4°C for 15 min prior to centrifugation at 14 000 rpm for 2 min to mediate protein extraction. Resulting lysates were stored at 4°C until required.

2.3.3. Protein quantification

The amount of protein present in the whole cell lysate was quantified using the Biocichoninic acid (BCA) assay (Sigma-Aldrich). This colorimetric assay is based on the reduction of copper ions (Cu²⁺) to Cu⁺ by peptide bonds of proteins. The reduction results in a violet complex of Cu⁺ and biocinchoninic acid, thus indicating the amount of protein present relative to the intensity of the resulting solution that can be measured spectrophotometrically at 562 nm.

Bovine serum albumin (BSA) standards (Separations) (0, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL) were prepared and 25 µL of each standard was aliquoted to a 96-well plate in triplicate. Whole cell lysates obtained from the transfected cells (Section 2.3.2) were diluted (1:5) and 25 µL each lysate was pipetted into the wells of the 96-well plate in triplicate. Subsequently, BCA and copper (II) sulfate were mixed in a 1:50 ratio and 200 µL of this solution was added to each well prior to a 30 min incubation at 37°C. Absorbance of the resulting solutions was measured using an ELISA reader at 562 nm and the concentration of each protein lysate sample was extrapolated from the standard curve obtained.

Protein lysates were diluted with dH₂O to a 1 mg/ml solution in a 1:2 ratio with 2X sample loading dye (appendix) and stored at 4°C until required.
2.3.4. Sodium dodecyl sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was employed in order to separate the proteins in the cell lysate prior to detection of total LRP levels by Western blotting. Proteins were separated according to their molecular weight based on the observation that size is inversely proportional to mobility. However, proteins have different overall charges thus the protein lysates were denatured by heat in the presence of the anionic detergent SDS that confers an overall negative charge on all proteins thus allowing separation based on size only. This permits the proteins to migrate through the polyacrylamide gel that sieves the proteins by impeding the migration of larger proteins relative to smaller ones based on the amount of acrylamide used, down to the anode. In this study a 12% (v/v) polyacrylamide gel was used to in order to detect the protein of interest LRP/LR.

Following heat denaturation of the protein lysates at 95°C for 5 min in loading buffer (appendix), 10 µl of the protein samples were loaded into gel slots along the pre-stained molecular weight marker (Thermoscientific) and ran using 1X SDS running buffer (appendix) at 200V for approximately 30 min.

2.3.5. Western blotting and immunodetection

The resulting gel was electro-blotted onto a 0.45µm polyvinylidene fluoride (PVDF) membrane at 450 mV for 45 min in order to transfer the protein pattern obtained using transfer buffer (appendix) and filter paper soaked in transfer buffer. Subsequent to transfer, the blotted PVDF membrane was incubated in blocking buffer (appendix) for 1 hour at RT. The membrane was then incubated with primary antibody (anti-LRP/LR specific antibody IgG1-iS18) (Affirmed Therapeutics) and for β-actin detection the Anti-β-Actin-Peroxidase antibody (Sigma-Aldrich) was used; with both antibodies resuspended in PBS. The membranes were then incubated for an hour at RT followed by three 10 min PBS-Tween washes prior to detection of the primary LRP antibody by incubation of the membrane with the secondary antibody (anti-human IgG-HRP (horse-radish peroxidase) conjugated) (Sigma-Aldrich) resuspended in blocking buffer (appendix) for 1 h.

Membranes for both LRP/LR and β-actin detection were washed with PBS-Tween for 10 min three times and these proteins were detected using the Super WestPico Chemiluminescent substrates in a 1:1 ratio. The resulting protein bands were exposed to an X-ray film then developed and fixed onto the film using developer and fixer (both from Sigma-Aldrich), respectively.

2.3.6. Densitometry analysis

Quantification of LRP/LR expression levels was completed by densitometry analysis. An image of the X-ray film with the proteins of interest was captured using the GS-800 Calibrated Densitometer and densitometric analysis done using the Quantity One – 4.5.2 software. Intensities of the bands were calculated and the calibration curve was obtained from which the area known as Integrated Optical Density (IOD) was calculated and used to compare the LRP/LR expression levels in MCF-7, MDA-MB 231 and WHCO1 cells post
transfection with siRNA-LAMR1 relative to the non-transfected protein lysates of these cell lines. The loading control (β-actin) was not included in densitometry analysis.

2.4. Examination of the downregulation of LRP/LR expression on the viability of cancerous cells

The following assays were employed on the various tumorigenic cell lines in order to investigate the effect of LRP/LR siRNA-mediated downregulation on cellular viability.

2.4.1. Mitochondrial tetrazolium salt (MTT) assay

This assay is based on the cleavage of the yellow MTT salts by the mitochondrial succinate dehydrogenase enzyme of viable cells into water insoluble purple formazan crystals. The amount of crystals produced provided an estimate of viable cells that are able to produce the succinate dehydrogenase enzyme to carry out this cleavage.

Cells seeded in a 24-well plate were transfected and incubated in a humidified incubator at 37°C and 5% CO₂ for 72 h. Cells treated with 8 mM PCA and siRNA-scr were used as a positive and negative, respectively. 500 µl of a 1 mg/ml solution of MTT dissolved in PBS was added to each well prior to a 2 h incubation in the humidified incubator. Media was aspirated, formazan crystals were dissolved in 200 µL of DMSO and absorbance was measured at 570 nm using an ELISA plate reader.

2.4.2. Bromodeoxyuridine (BrdU) proliferation assay

This immunoassay utilizes the incorporation of bromodeoxyuridine (BrdU) incorporation into new DNA molecules of actively dividing cells as an evaluation of proliferating cells. BrdU is a thymidine analogue as a result during DNA synthesis BrdU will be incorporated into the new DNA molecules as a substitute for thymidine. Upon labeling non-transfected, siRNA-LAMR1-transfected, siRNA-scr-transfected (negative control) and 8 mM PCA-treated (positive control) cells with BrdU, an anti-BrdU antibody was used to detect incorporated BrdU and a horseradish peroxidase-conjugated secondary antibody was used in order to bind to the detector antibody, resulting in catalysis of the conversion of the fluorogenic substrate to a blue product that can be measured spectrophotometrically with an ELISA reader at 540 nm.

MCF-7, MDA-MB 231 and WHCO1 cells were seeded in a 96-well plate and transfected as previously described; with cells treated with 8 mM PCA and siRNA-scr used as a positive and negative control, respectively. Post 72 h incubation in a humidified incubator set at 37°C and 5% CO₂, 20 µL BrdU (Merck) working stock solution diluted in fresh culture media (1:2000) was pipetted to each well and incubated for 2 h in the tissue culture incubator. Contents were removed; cells were fixed and denatured with 200 µL of Fixative/Denaturing solution for 30 min at room temperature. This was to enable antibody binding to the BrdU label, thus cells were incubated with the detector anti-BrdU antibody for 1 h at room temperature, followed by a 30 min incubation with the secondary peroxidase anti-mouse IgG-
HRP conjugate antibody. 100 µL of Substrate solution was added to each well and the horseradish peroxidase catalysed the conversion of this fluorogenic substrate to a blue product and the reaction was halted with 100 µL of Stop solution after 15 min and absorbance was measured.

2.4.3. Annexin V-FITC/7AAD assay

This assay analyzes the loss of asymmetry in cell membrane phospholipids due to altered hydrophobicity and charge on the membrane surface. In healthy cells, phosphatidylserine (PS) is localized on the inner membrane, however, in apoptotic cells, PS translocates to the outer membrane thus indicating membrane alteration upon induction of apoptosis. In this assay, Annexin-V, a high affinity phospholipid-binding protein binds to PS exposed on the cell surface of apoptotic cells thus indicating cells that are undergoing apoptosis in the population. Moreover, the DNA-binding 7-AAD dye stains late apoptotic cells positively but is excluded from early apoptotic cells thus allowing for distinction between early and late apoptotic cells.

MCF-7, MDA-MB 231 and WHCO1 cells were seeded and transfected in a 24-well plate as done previously. Post 72 h incubation, cells were detached with trypsin/EDTA (Thermoscientific) and cell suspensions were rinsed with ice-cold PBS prior to centrifugation at 500 x g for 5 min. The resulting pellets were resuspended in ice-cold 1X Binding Buffer at a density of 7 x 10^6 cells/mL and all subsequent steps were performed at 4°C. 100 µL of the four cell suspensions (untreated, PCA-treated (positive control), siRNA-scr-treated (negative control) and siRNA-LAMR1-treated) were stained with 10 µL of 2.5 µg/mL concentrated Annexin V-FITC and 20µl the 0.005% 7-AAD viability dye (Beckman Coulter) in a total volume of 100 µL for 15 min in the dark. Furthermore, 100 µL of ice-cold 1X Binding buffer was added and samples were analyzed immediately using the BD Accuri flow cytometer software. Annexin V-FITC staining was analysed using the FL1 laser whilst 7-AAD staining was analysed using the FL3 laser and the following controls were used to set compensation and quadrants; unstained cells, Annexin-V-FITC stained cells, 7-AAD stained cells, Annexin V-FITC and 7-AAD stained cells as well as 8 mM PCA treated cells stained with both Annexin V and 7-AAD.

2.4.4. Immunofluorescence microscopy

MCF-7, MDA-MB 231 and WHCO1 cells were seeded onto coverslips and after 24 h were transfected with siRNA-LAMR1, siRNA-scr (negative control) and 8mM PCA (positive control). 72 h post transfection, media was aspirated and cells were fixed with 4% PFA for 15 min and washed with cold PBS. Nuclei was stained with 0.5 µL of 10 mg/ml Hoescht 33342 stain resuspended in 95 µL PBS for 5 to 10 min at RT, subsequent to mounting onto a slide using Gelmount (Sigma Aldrich) for 1 h at RT in the dark. Slides were stored at 4°C until visualization using the blue laser, under the 63X objective and the Zen 2011 software of the Zeiss LSM 710 3-Channel Confocal microscope.
3. CHAPTER 3 – RESULTS

LRP/LR has numerous cellular localisations (the cytosol, cell surface, perinuclear compartment and the nucleus) and the cell surface and total LRP/LR levels were determined in MCF-7, MDA-MB 231 and WHCO1 cells in a previous study in our laboratory. Indeed, the aforementioned cell lines expressed high total and cell surface LRP/LR levels as determined by western blotting and flow cytometry[101], respectively. Additionally, Khumalo et al illustrated that high expression of this receptor aggravated the metastatic potential of these cells lines due to the enhancement of the LRP/LR-laminin-1 interaction[101]. Furthermore, incubation of these cell lines with the anti-LRP/LR specific antibody IgG1-iS18 resulted in the inhibition of adhesion and invasion, the key steps of metastasis in the invasive MDA-MB 231 breast and WHCO1 oesophageal cancer cell lines[101].

This observation instigated the question whether IgG1-iS18 might also affect the viability of these cancer cell lines by interfering with the LRP/LR-laminin-1 interaction.

3.1. Examination of the interference of the LRP/LR-laminin-1 interaction on the cellular viability of breast and oesophageal cancer cells

Incubation of MCF-7, MDA-MB 231 and WHCO1 cells with IgG1-iS18 for 17 h had no effect on the viability when compared to both untreated (set to 100%) and anti-CAT-treated cells (Figure 14). However, incubation of these cells with 8 mM PCA) resulted in significant reduction of viability across all cell lines.

![Figure 14](image-url): The effect of the LRP/LR-laminin-1 interaction on the cellular viability of WHCO1, MCF-7 and MDA-MB 231 cells post incubation with anti-LRP/LR specific antibody IgG1-iS18. The viability of WHCO1, MCF-7 and MDA-MB 231 cells was analysed 17 h post incubation with 50 μg/μL of IgG1-iS18, anti-CAT and 8 mM PCA. IgG1-iS18 had no effect on the cellular viability of all cell lines when compared to the untreated cells set to 100%. 8 mM PCA and anti-CAT antibody were used as positive and negative controls, respectively.
Having observed no effect of the anti-LRP/LR antibody on the cellular viability of these cell lines, introduction of siRNA into MCF-7, MDA-MB 231 and WHCO1 cells was performed in order to potentially reduce the expression of LRP and determine whether reduction of LRP expression significantly influences cell viability and cell proliferation.

### 3.2. Downregulation of LRP expression

The level of LRP expression in MCF-7, MDA-MB 231 and WHCO1 cells post transfection with siRNA-LAMR1 (siRNA targeting mRNA of the 37kDa LRP) was determined by western blotting and densitometry analysis (Section 2.3).

Densitometric analysis of the western blots signals revealed that siRNA-LAMR1 transfected MCF-7, MDA-MB 231 and WHCO1 cells displayed a significant 100% (p < 0.01), 44% (p < 0.001) and 73% (p < 0.05) reduction in LRP expression, respectively, when compared to the non-transfected cells which have been set to 100% and using β-actin as a loading control (Figure 15). Transfection of all cell lines with siRNA-scr (negative control) displayed non-significant reduction in LRP expression, compared to the non-transfected cell lysates.

![Western Blot Images]

**Figure 15: LRP expression in WHCO1, MDA-MB 231 and MCF-7 cells post-transfection with siRNA-LAMR1.** The expression levels of the 37kDa LRP in WHCO1, MDA-MB 231 and MCF-7 cells was investigated post-transfection with siRNA-LAMR1 by western blotting. Densitometric analysis of the western blot signals revealed significant (p<0.05 differences in LRP expression in a) WHCO1, b) MDA-MB 231 and c) MCF-7 cells, respectively (compared to non-transfected cells).
3.3. Determination of the effect of downregulation of LRP expression on cellular viability

Significant downregulation of LRP expression resulted in significant reduction of the cellular viability of MCF-7, MDA-MB 231 and WHCO1 cells (Figure 16). The cellular viability was analysed using an MTT assay which was performed on cells incubated with 8mM PCA and transfected with siRNA-scr and siRNA-LAMR1 (Section 2.4.1).

MCF-7, MDA-MB 231 and WHCO1 cells treated with siRNA-LAMR1 displayed significant 52% (p < 0.001), 45% (p < 0.05) and 72% (p < 0.001) reduction in cellular viability, respectively (Figure 16). siRNA-scr treated MCF-7, MDA-MB 231 and WHCO1 cells exhibited no decrease in cell viability whilst 8mM PCA treated cells of these corresponding cell lines displayed significant reduction in cellular viability as expected (Figure 16).

![Figure 16](image-url)

**Figure 16**: The effect of siRNA-mediated downregulation of LRP expression on the cellular viability of WHCO1, MCF-7 and MDA-MB 231 cells. The viability of WHCO1, MCF-7 and MDA-MB 231 cells was analysed 72 h post-transfection using an MTT assay. siRNA-LAMR1 treated WHCO1, MCF-7 and MDA-MB 231 cells revealed significant 72%, 52% and 45% reduction in cellular viability, respectively, compared to untreated cells set to 100%. 8mM PCA and siRNA-scr were used as positive and negative controls, respectively.
3.4. Determination of the effect of LRP downregulation on proliferation of breast and oesophageal cancer cells

Having observed a significant decrease in the viability of these breast and oesophageal cancer cell lines post downregulation of LRP, a BrdU assay was employed in order to analyse the effects of downregulation of this receptor on cellular proliferation as the two processes are intimately linked (Section 2.4.2).

The BrdU assay revealed that the cellular proliferation of MCF-7, MDA-MB 231 and WHCO1 cells treated with siRNA-LAMR1 was significantly reduced by approximately 26% (p < 0.05), 59% (p < 0.01) and 45% (p < 0.01), respectively (compared to non-transfected cells) (Figure 17).

![Figure 17: The effect of siRNA-mediated downregulation of LRP expression on cellular proliferation.](image)

3.5. Assessment of siRNA-mediated downregulation of LRP expression on cell membrane integrity

The cell membrane integrity of MCF-7, MDA-MB 231 and WHCO1 cells was assessed 72 h post transfection with siRNA-LAMR1, siRNA-scr and treatment with 8mM PCA. The aforementioned cell lines were stained with Annexin V-FITC and the 7-AAD viability dye (Section 2.4.3).

A majority of siRNA-LAMR1 treated MCF-7, MDA-MB 231 and WHCO1 cells were located on the lower right and upper right quadrants when analysed using the flow cytometer.
(Figure 18). The lower right quadrant is indicative of early apoptotic cells that are positive for Annexin-V binding and negative for the 7-AAD dye, whereas the upper right quadrant shows late apoptotic cells that are positive for both Annexin-V and 7-AAD binding. Furthermore, the siRNA-scr treated and untreated MCF-7, MDA-MB 231 and WHCO1 cells were located on the lower left quadrant that illustrates viable cells that exclude the 7-AAD dye and are negative for Annexin-V binding (Figure 18). The shift of the siRNA-LAMR1 treated cells to the lower left and upper left quadrants indicates that these cells are undergoing early, late apoptosis and necrosis as indicated by the varying FITC and 7-AAD signals (Figure 18). Moreover, the 8mM PCA treated cells exhibited similar results across all cell lines which is anticipated because PCA is a known apoptosis inducer.

![Figure 18](image)

**Figure 18**: The effect of siRNA-mediated downregulation of LRP expression on cell membrane integrity. 72 h post-transfection, MCF-7, MDA-MB 231 and WHCO1 cells were stained with Annexin-V FITC and 7-AAD then analysed by flow cytometry. siRNA-LAMR1 treated MCF-7, MDA-MB 231 and WHCO1 cells revealed high FITC and 7-AAD signals indicative of cells undergoing early and late apoptosis, compared to untreated and siRNA-scr treated cells (8mM PCA was used as a positive control).

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

Nuclear morphology of MCF-7, MDA-MB 231 and WHCO1 cells was assessed 72 h post transfection with siRNA-LAMR1, siRNA-scr and incubation with 8mM PCA by staining with the fluorescent nuclear dye Hoescht 33342 (Section 2.4.4).

These cells were viewed using an immunofluorescence microscope, siRNA-LAMR1 treated MCF-7, MDA-MB 231 and WHCO1 cells as well as the 8mM PCA-treated corresponding
cells exhibited constricted nuclei that have lost their original nuclear morphology when compared to the untreated and siRNA-scr treated cells that exhibit normal nuclei (Figure 19). This is shown by the crescent shaped nuclei (white arrows pointing siRNA-LAMR1 treated MDA-MB 231 cells) due to the collapse of chromatin[129] (Figure 19). Furthermore, progressive condensation of the chromatin (siRNA-LAMR1 treated WHCO1 micrograph) resulted in the shrinkage of the entire nucleus as indicated by the white arrows pointing to the siRNA-LAMR1 treated MCF-7 cells (Figure 19). Additionally, post 72 h incubation with the transfections and 8mM PCA, the cell number was inconsistent between the untreated and PCA treated cells suggesting that the toxicity of PCA does indeed impact on cell viability hence there was a reduction in cell numbers observed between the micrographs.

Figure 19: The effect of siRNA-mediated downregulation of LRP expression on nuclear morphology. 72 h post-transfection, MCF-7, MDA-MB 231 and WHCO1 cells were stained with Hoescht 33324 and viewed by immunofluorescence microscopy. siRNA-LAMR1 treated MCF-7, MDA-MB 231 and WHCO1 cells displayed condensed nuclei and decreased nuclear integrity (indicated by white arrows), compared to untreated and siRNA-scr treated cells (8mM PCA used as a positive control)
4. CHAPTER 4 - DISCUSSION

The affiliation between the LRP/LR and cancer aggressiveness has been extensively investigated over the years. This huge interest was instigated by the observation of overexpression of this receptor on numerous cancer cells when compared to their normal counterparts [90, 91, 94, 130-132]. The overexpression of LRP/LR has been shown to afford cancer cells with the ability to metastasise to distant organs[98, 99], enhance angiogenesis[132] and evade apoptosis[99], which are cancer-supporting processes. LRP/LR’s implication in these hallmarks of cancer is anticipated because the LRP/LR has numerous cellular localisations (perinuclear compartment [77, 80, 133], cell surface [79], nucleus [77, 133] and the cytosol[80, 134]) interacting with various proteins, including ones that are involved in the above mentioned processes. LRP/LR is therefore considered a valuable prognostic marker for analyses of the severity of tumors[135] and additional roles of this receptor in cancer may be suggested as more investigations are carried out.

4.1. Examination of the interference of the LRP/LR-laminin-1 interaction on the cellular viability of breast and oesophageal cancer cells

A previous study in our laboratory by Khumalo et al revealed that breast MCF-7, MDA-MB 231 and oesophageal WHCO1 cancer cells express high cell surface levels as well as significantly high total LRP levels[101]. High expression of this receptor was shown to enhance adhesion and invasion of the metastatic MDA-MB 231 and WHCO1 cancer cell lines[101]. However, incubation of these cell lines with anti-LRP/LR specific antibody IgG1-iS18 significantly impeded adhesion and invasion in both cell lines, thus indicating that the LRP/LR-laminin-1 interaction is pivotal for the process of metastasis[101]. IgG antibodies are known to bind to LRP/LR via the antibody binding domain from amino acid 272 to 280 and it is hypothesized that the antibody alters the binding affinity of laminin-1 by steric hinderance, by occupying the laminin binding site on LRP/LR directly or influencing the MAPK signalling pathway through laminin and these still need to be examined[136].

The high expression of LRP/LR observed in these cells lines as well as successful inhibition of metastasis using IgG1-iS18 led to the investigation of whether this antibody will impact on the viability of these cancer cells. In this study, incubation of the cancer cell lines MCF-7, MDA-MB 231 and WHCO1 with the antibody IgG1-iS18 had no effect on the viability of these cancer cells. This result suggests that the LRP/LR-laminin-1 interaction targeted by IgG1-iS18 is not responsible or involved in the maintenance of cellular viability but mediates adhesion and invasion of cancer cells thus enhancing metastasis. It is crucial to note that the cells were not seeded onto laminin-1 coated plates, however, these cells do express laminin-1[137-139], and hence it is presumed that the LRP/LR-laminin-1 interaction occurs in this system. The secretion of this ECM component by these cells as well as other tumor and stromal cells has been shown to be enhanced by paracrine and autocrine mechanisms by radiolabelling and immunoprecipitation assays [140].
4.2. LRP silencing

Since the incubation of breast MCF-7, MDA-MB 231 and oesophageal WHCO1 cancer cells with the anti-LRP/LR specific IgG1-iS18 antibody had no effect on the viability of these cell lines, the role of LRP/LR in cellular viability was analysed post-downregulation of this receptor. Knockdown of LRP was conducted by RNA interference technology.

siRNA-LAMR1, a siRNA directed against the mRNA of LRP was utilised (purchased from Thermoscientific). This siRNA targets the open reading frame of the human LRP gene and was introduced to MCF-7, MDA-MB 231 and WHCO1 cancer cells using Transfection Reagent 1. LRP expression was significantly reduced by 43% in MDA-MB 231, 73% in WHCO1 and 100% in MCF-7 cells, as detected by western blot signals and densitometric analysis. These results indicate the high efficacy of siRNA-LAMR1 as well as the transfection method employed.

It is crucial to note that siRNA-LAMR1 targets the mRNA of the 37-kDa LRP and not that of the 67-kDa LR. However, studies have shown that the 37-kDa form is the precursor of the 67-kDa form [73]; therefore, a reduction in LRP expression correlates to a reduction in LR expression. Furthermore, the 67-kDa form cannot be targeted directly because it is not a direct product of translation or transcription but produced as result of post-translational modification of the 37-kDa LRP form that is still elusive[75, 141]. It is suggested that conversion of the 37-kDa LRP form to the 67-kDa LR form requires acylation at Ser 2 by fatty acids (palmitate, stearate and oleate)[141].

Moreover, only the 37-kDa form is detected by western blotting and this might be due to the low concentrations of the 67-kDa form in cytoplasmic extractions [79]. Although it is clear that the control siRNA-scr had no effect on the expression of LRP/LR and that siRNA-LAMR1 is specific relative to the control it is crucial to note that siRNA-LAMR1 may have had off-target effects, however it is certain that this siRNA targeted the C-terminus of LRP/LR as this is the region responsible for the maintenance of cellular viability as observed below. Furthermore, the effects of another LRP/LR-specific siRNA targeting a different region on the receptor in order to observe whether similar effects will occur thus controlling for and assessing the specificity of siRNA-LAMR1.

4.3. Cellular viability

The effect of downregulation of LRP expression on the viability of breast MCF-7, MDA-MB 231 and oesophageal WHCO1 cancer cells was examined. Downregulation of LRP expression was accompanied by a significant reduction in the viability of MCF-7, MDA-MB 231 and WHCO1 cancer cells by 43%, 46% and 72%, respectively, thus indicating the crucial role of LRP/LR on cellular viability. MCF-7, MDA-MB 231 and WHCO1 cells transfected with siRNA-scr exhibited no differences on cellular viability (compared to non-transfected cells), further indicating the prominent effect of siRNA-LAMR1.

Evasion of apoptosis is one prominent characteristic of cancer cells and it is evident that high levels of LRP expression afford cancer cells the ability to maintain cellular viability and in
turn enhance propagation of the tumour. LRP/LR is indeed a pathologically significant protein in this disease and it has been hypothesized that LRP/LR maintains cellular viability by retaining chromosomal stability in cells by binding to the growth factor Midkine (MK)\cite{108}. MK with a molecular weight of 13 000 is a heparin-binding growth factor that is involved in modulating numerous functions such as cell migration, proliferation, fibrinolysis and angiogenesis\cite{142}. In normal individuals the serum level of MK is less than 0.6 nm/ml, however, in cancer patients with breast, bladder, lung, stomach, prostate and oesophageal cancer the levels of this growth factor are elevated\cite{142}. By binding to Medkine, LRP/LR is thought to associate with the nuclear envelope and chromatin material, connecting the latter to the former thus retaining chromosomal stability\cite{108}. Furthermore, studies have illustrated that MK cause cells to upregulate the expression levels of PKB, mTor and Bad which are components crucial for maintenance of cell survival and inhibits apoptosis by inhibiting the expression of caspase-3, the effector caspase\cite{142}. Elevated blood levels of MK have been observed in the cell lines of this study as well, breast MCF-7, MDA-MB 231 and oesophageal WHCO1 cancer cells, and also regarded as a diagnostically useful marker as MK levels correlate with disease stage and prognosis in these cancers\cite{142}. This proposed mechanism partly explains the results observed when the various cancer cell lines were incubated with IgG1-iS18 which targets the LRP/LR-laminin-1 interaction. This mechanism is dependent on the expression of LRP/LR and not the LRP/LR-laminin-1 interaction, hence, IgG1-iS18 had no effect on the cellular viability of the above mentioned cancer cell lines.

4.4. Cellular proliferation

Cellular proliferation was assessed in order to elucidate whether the observed reduced cell viability of MCF-7, MDA-MB 231 and WHCO1 cells could be due to the inhibition of cellular division. Proliferation was similarly reduced following downregulation of LRP expression by 26%, 45% and 59% in MCF-7, WHCO1 and MDA-MB 231 cells, respectively. This is comprehensible because LRP is physiologically involved in cellular proliferation and also binds to MK, which is a growth factor known to mediate cell proliferation as well as migration and survival \cite{108}. Therefore, reduced expression of LRP/LR may possibly result in a lowered LRP/LR-MK interaction thus resulting in hampered cell proliferation and survival.

Furthermore, as a ribosomal protein LRP/LR has been shown to be crucial for protein translation by associating with the 40S ribosomal subunit, which is a process required for the progression of the cell through the G1 to S phase hence a reduction in cell proliferation is observed upon downregulation of this receptor \cite{143}. Additionally, LRP/LR expression may promote the translation of proteins required for cell-cycle progression, whilst reduced expression of this receptor may induce translation of cell cycle arrest proteins. This may indicate that LRP/LR’s ribosomal functions are crucial for the regulation of cell proliferation, however, to ascertain this a cell cycle study may be conducted. The cell cycle RT\textsuperscript{2} Profiler PCR Array in order to investigate whether cell cell cycle-related gene expression is altered between siRNA- LAMR1-transfected and non-transfected cells focusing on cyclins, p21 and survivin expression levels.
4.5. Apoptosis

Apoptotic assays were conducted in order to elucidate whether the reduction in cellular viability of MCF-7, MDA-MB 231 and WHCO1 cells is due to this form of programmed cell death. Indeed apoptosis was responsible for the observed decrease in cellular viability as illustrated by the nuclear morphological changes following downregulation of LRP/LR using siRNA-LAMR1.

The loss of nuclear integrity is no surprise because LRP/LR localises in the perinuclear compartment [77, 133, 143] and the nucleus [77, 133]. It is believed that LRP/LR binds to histones thus assisting in the maintenance of nuclear structures[81], therefore, loss or reduced expression of this receptor leads to instability of nuclear structures which triggers induction of apoptosis which is illustrated by Hoescht which binds to dsDNA thus allowing for visualisation of DNA that may be compromised which is indicative of apoptosis induction.

Apoptosis occurrence was further exhibited by the Annexin-V FITC/7-AAD assay carried on the above mentioned cell lines (Section 2.4.3). Annexin-V is an intracellular protein that binds to PS, which is located in the intracellular leaflet of the plasma membrane of viable cells[144]. In viable, intact cells the Annexin-V cannot bind to PS as it is excluded, however, during apoptosis the cell membrane integrity is compromised and the phospholipids lose their asymmetry and translocate to the outer leaflet of the membrane[145]. Exposure allows the Annexin-V to bind in the presence of calcium thus stain cells undergoing apoptosis[145]. This is observed in Figure 18 where the untreated and siRNA-scr treated cells are stained negative for Annexin-V binding (lower right quadrant) whereas the 8mM PCA and siRNA-LAMR1 treated cells are stained positive for Annexin-V binding (lower and upper right quadrants). This shift illustrates that as the expression of the LR is downregulated in MCF-7, MDA-MB 231 and WHCO1 cells, cell membrane alterations (membrane blebbing) occur as the cells experience apoptosis. It is crucial to note that the Annexin-V is conjugated to FITC for detection purposes and studies have shown that addition of this fluorochrome does not impact on the phospholipid binding properties of Annexin hence it is appropriate for analysis of cell membrane asymmetry in cells[145].

Additionally, Annexin-V binding cannot distinguish between early and late apoptotic cells[145], hence the 7-AAD viability dye was utilised as well. Similarly to Annexin-V, 7-AAD does not stain viable cells as it cannot be incorporated to into intact cells. This dye undergoes a spectral shift upon interaction with DNA emitting at 647 nm which is appropriate for flow cytometric analysis[146]. Following membrane alterations, 7-AAD enters the cell and passes through to the nucleus to stain DNA [147, 148]. Early apoptotic cells that are only experiencing proteolytic digestion exclude 7-AAD dye thus this dye only stains cells that have progressed through the apoptotic pathway and have experienced membrane alterations. The early and late apoptotic cell populations are observed in the siRNA-LAMR1 treated MCF-7, MDA-MB 231 and WHCO1 cells (lower and upper right quadrants, respectively) as displayed in the micrographs.
Furthermore, it was observed that a majority of siRNA-LAMR1 treated MCF-7 and WHCO1 cells experience late apoptosis with 43% and 57%, respectively (Figure 18). However, only 26% of siRNA-LAMR1 treated cells were undergoing late apoptosis with a majority (45%) experiencing necrosis (upper left quadrant). There is no particular reason for this observation; however, one can speculate that the MDA-MB 231 breast cancer cell line may be more susceptible to LRP/LR downregulation as this receptor plays a more prominent role in this cell line; which may be attributed to the fact that MDA-MB 231 is a triple negative breast cancer (TNBC) cell line, not expressing progesterone (PR), estrogen (ER) and the Human Epidermal Growth Factor Receptor 2 (HER 2) [149, 150]. Thus propagation of this cancer is independent of the presence of these receptors, therefore the high expression of LRP/LR may be crucial for survival, growth and progression when compared to other cell lines, and hence this cell line is more susceptible to LRP knockdown and experienced necrotic cell death. Similarly, this data may suggest that LRP/LR predominantly induces necrosis rather than late apoptosis in this cell line. Furthermore, the proliferation and cell viability studies, BrdU and MTT assays indicate significant hampering of these two crucial processes following knockdown of LRP/LR expression by about 59% and 45%, respectively, which further supports the observation of a majority of the MDA-MB 231 breast cancer cell line experiencing necrosis. However, it is also important to note that all experiments were carried out 72 h post transfection with siRNA-LAMR1 and analysis after shorter incubation times could yield different results. Moreover, further experiments may be conducted in order to elucidate whether the observed effects are specific or more pronounced in TNBC ER+ or TNBC PR+ cell lines. A breast cancer-focused investigation starting with determination of expression profiles of genes in a collection of breast cancer cells by RNA isolation and quantitative real-time PCR which will allow for characterisation of the breast cancer cell lines into either TNBC ER+ or TNBC PR+. Known TNBC PR+ breast cancer cell lines (i.e T47D) and TNBC ER+ (MDA-MB-361) [151]will then be transfected with siRNA-LAMR1 for knockdown of LRP/LR expression and similar assays (i.e viability MTT, proliferation BrdU, apoptosis assays) will be conducted with these cells and appropriate conclusions will be made from the observations.

To further demonstrate apoptosis in the above cell lines, the nuclear morphology was investigated by Hoescht staining. Disruptions in the nuclear morphology of siRNA-LAMR1 treated breast MCF-7, MDA-MB 231 and oesophageal WHCO1 cancer cells were clearly evident. Nuclear morphological changes and membrane alterations are representative of apoptosis thus indicating the crucial role of the laminin receptor in cancer progression by inhibiting the induction of apoptosis. Moreover, LRP/LR may be supporting cancer in numerous functions because by assisting in the maintenance of nuclear structures it plays a role in regulating gene expression and since cancer is a gene-regulated disease the possibilities are endless. Thus further studies may be carried out to discover these functions.

It is clear that apoptosis is the mode of cell death in all three cell lines; however, the specific apoptotic pathway that is responsible for the reduction in cellular viability cannot be elucidated from these results hence further investigations can be carried. However, from literature the most common pathway observed in the caspase-3 deficient MCF-7 cells is the
death receptor pathway whereas for the MDA-MB 231 breast cancer cells it is the mitochondrial pathway and with WHCO1 oesophageal cancer cells both pathways are similarly favoured[152, 153]. This does not mean the same will be observed upon induction of cell death by downregulation of LRP/LR expression different apoptotic stimuli may activate either pathway, however this information and experimental investigation such as carrying out a caspase-8 and -9 assay can provide insight on the apoptotic pathway responsible in each cell line following knockdown of LRP/LR.

4.6. LRP/LR as an alternative therapeutic tool for cancer treatment

LRP/LR is a protein with multiple cellular localisations and functions, both physiological and pathological. Its implications in cancer seem to be increasing, ranging from enhanced metastasis, invasion, cellular proliferation and cellular viability due to high expression in numerous cancers. The above mentioned processes are prominent hallmarks of cancer thus targeting of LRP/LR can be deemed a possible therapeutic tool for the treatment of cancers.

Numerous in vitro studies targeting LRP/LR for the inhibition of these processes have been successful in various cancer cells using anti-LRP/LR specific antibodies, pentosan polysulfates, heparin mimetics to name a few. Moreover, a similar study investigating the effect of siRNA-mediated knockdown of LRP/LR on cellular viability of lung and cervical cancer cells has been carried out in our laboratory[110]. The expression levels of LRP/LR were significantly downregulated in both A549 lung and HeLa cervical cancer cells approximately 80% and 60%, respectively, when compared to non-transfected controls of these cell lines[110]. Furthermore, reduction in cellular viability of these cell lines was observed, however, the extent of LRP downregulation does not correlate to the extent of reduced cellular viability which is observed as well in the current study[110]. With regards to these two studies, the most significant reduction in LRP/LR expression was observed in MCF-7 breast and HeLa cervical cancer cells, however, this may vary amongst different cell lines thus one cannot speculate that similar results will be observed in other breast and cervical cancer cell lines[110]. Therefore, it is noteworthy to comprehend that not all cancers will be responsive to siRNA treatment and that the efficacy of this method will vary amongst different cancer types. Hence it is crucial to investigate how other cancers may respond to this method of treatment, thus provide insight on whether targeting of this receptor may be regarded an alternative broad spectrum therapeutic tool for the treatment of cancer.

Therefore, LRP/LR directed tools can be utilised for the treatment of cancers that have been proven to be responsive to this mode of treatment, however, administration in the human body may pose difficult as this receptor is imperative for numerous physiological processes (cell differentiation, migration, adhesion and growth). Thus knockdown of LRP/LR may lead to inhibition of these crucial cellular processes causing deleterious homeostatic disruptions that could propagate the tumor further. Therefore target of solely cancer cells is crucial for use of anti-LRP/LR tools.
CHAPTER 5 – CONCLUSION AND FUTURE WORK

5.1. Conclusion

This study has illustrated that knockdown of LRP expression in breast MCF-7, MDA-MB 231 and oesophageal WHCO1 cancer cells results in decreased cellular viability through the inhibition of cellular proliferation. The decrease in cell viability was observed to be due to the induction of apoptosis as indicated by the loss of nuclear morphology and exposure of the phosphatidylinerine following membrane blebbing of MCF-7, MDA-MB 231 and WHCO1 cells as shown by immunofluorescence microscopy and the Annexin-V FITC assay, respectively.

Therefore these results strongly suggest the role of LRP/LR on the maintenance of cell viability of these cancers and targeting the mRNA of LRP with specific siRNA may potentially be used as an alternative therapeutic tool for the treatment of breast and oesophageal cancer cells.

5.2. Future work

The data shown addresses only two cancer types, namely breast and oesophageal cancer, therefore investigation of the effects of LRP downregulation on other prominent cancer types should be considered because various cancers behave differently, hence it cannot be assumed that this will hold true for all cancers.

Additionally, research focusing on the effect of LRP downregulation on normal tissues could be carried out and if minimal effects are observed, investigations in animal models could be considered (i.e. nude mice system). siRNAs could be administered via the tail vein of the mice following injection of cancerous cells into the mice. The progression of the cancer will be monitored for a certain period with specific analysis to the number as well as size of colonies as an indication of tumor progression.

Furthermore, successful animal trials could pave way for clinical trials once appropriate delivery systems into the human body have been elucidated. A lentiviral or AAV delivery system may be utilised considering that LRP/LR binds numerous viruses i.e Sindbis, Dengue and AAV. Moreover, combination therapy of anti-LRP/LR specific antibodies and siRNAs could be appropriate for the targeting of metastasis, tumor angiogenesis and induction of apoptosis, respectively in these cancer cells.
6. CHAPTER 6 – REFERENCES

89. Mbazima, V., et al., Interactions between PrP(c) and other ligands with the 37-kDa/67-kDa laminin receptor. Front Biosci, 2010. 15: p. 1150-63.


7. CHAPTER 7 - APPENDIX

Determination of total LRP levels by Western blotting

Lysis buffer

Mix the following in dH₂O:

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

Store solution at 4 °C
2X sample loading dye
Mix the following in dH₂O
Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS)

1X SDS running buffer
Mix the following in dH₂O
0.2 M Tris
0.19 M Glycine
3 mM SDS
Store at room temperature (RT)

40% acrylamide: Bis-Acrylamide
Mix Acrylamide and Bis-Acrylamide (19:1) in dH₂O
Dissolve by heating, filter and store in dark bottle at 4 °C

Stacking buffer
Mix the following in dH₂O
500 mM Tris
pH to 6.8 with hydrochloric acid (HCl)
Store at 4 °C

Separation buffer
Mix the following in dH₂O
1.5 M Tris
pH to 8.8 with HCl
Store at 4 °C

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

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

12% Separation and 5% Stacking gel were employed for the separation of the protein of interest, the 37kDa LRP

12% Separation gel
Mix the following in dH₂O
9.9% (v/v) 40% Acrylamide:Bis-Acrylamide
25% (v/v) Separation buffer
1% (v/v) 10% SDS
1% (v/v) 1% APS
0.4% (v/v) TEMED

5% Stacking gel
Mix the following in dH₂O
12.5% (v/v) 40% Acrylamide:Bis-Acrylamide
12.5% (v/v) Stacking buffer
1% (v/v) 10% SDS
1% (v/v) 1% APS
0.1% (v/v) TEMED

Pouring gels
Pour the 12% separation gel and overlay with ethanol until polymerised. Discard the ethanol, pour the 5% stacking gel and insert the comb immediately.

Transfer buffer
Mix the following in dH₂O
20 mM Tris
150 mM Glycine
20% Methanol
Store at 4 °C

Blocking buffer
3% BSA-PBS-Tween
Dissolve 3% BSA in PBS Tween
Store at 4°C

1X PBS
Mix the following in dH20:
137 mM NaCl
12 mM Phosphate
2.7 mM KCl
pH 7.4
Store at RT

PBS-Tween
Add 0.001% Tween to 1 X PBS
Store at 4 °C

Transfection per well (24 well plate)
MCF-7, MDA-Mb 231 and WHCO1 cells were seeded in the wells of a 24 well plate. 24 h later, 1.25 μl of 10 μM siRNA-LAMR1/ siRNA-scr and 2.5 μL of DharmaFECT® 1 transfection reagent were each concurrently diluted in serum-free DMEM to a final volume of 50 μL each, the contents of each tube was gently mixed and allowed to incubate for 5 min at RT. The diluted siRNA-LAMR1 solution was then added to the diluted DharmaFECT® 1 transfection reagent, mixed gently and incubated for 20 min at RT. The culture medium was removed from the plated cells and 400 μL DMEM containing 10% (v/v) FCS added to the siRNA-reagent solution which the cells were then grown in for 72 h in a humidified incubator at 37 °C with 95% air and 5% CO₂.
8mM PCA

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