Knockdown of LRP/LR induces apoptosis in pancreatic cancer and neuroblastoma cells through activation of caspases

Carryn J. Chetty[#], Eloise Ferreira[#], Katarina Jovanovic[‡], Stefan F. T. Weiss*

School of Molecular and Cell Biology, University of the Witwatersrand, Private Bag 3, Wits 2050, Johannesburg, Republic of South Africa

*Correspondence and requests for materials should be addressed to S.F.T.W. (Stefan.weiss@wits.ac.za)

[‡]present address: Ocular Biology and Therapeutics, UCL Institute of Ophthalmology, 11-43 Bath Street, London EC1V 9EL, UK

[#] These authors contributed equally to this work.

Abstract

The 37kDa/67kDa laminin receptor (LRP/LR) serves various physiological and pathological roles such as enhancing tumour-related processes including metastasis, angiogenesis, cellular viability and telomerase activation in cancerous cell lines. The present study investigates the effect of siRNA mediated downregulation of LRP/LR on pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells. MTT and BrdU assays revealed that siRNA mediated downregulation of LRP resulted in a significant reduction in cell viability and cell proliferation. In addition, knock-down of LRP resulted in phosphatidylserine externalisation, diminished nuclear integrity and significantly enhanced caspase-3 activity, which is indicative of apoptosis. .LRP downregulation resulted in a significant increase in caspase-8 activity in IMR-32 cells and enhanced caspase-8 and 9 activity in AsPC-1 cells. These data recommend siRNA mediated

Abbreviations: AsPC-1, Pancreatic cancer cells; BCA, bicinchoninic acid; BrdU, Bromodeoxyuridine; EDTA, Ethylenediaminetetraacetic acid; ELISA, Enzyme-linked Immunosorbent Assay; EMEM, Eagle Minimum Essential Medium; FBS, Fetal bovine serum; FITC, Fluorescein isothiocyanate; HRP, Horse radish peroxidase; hTERT, Human telomerase reverse transcriptase; IgG, Immunoglobulin class G; IMR-32, neuroblastoma cells; IgG1-iS18, Full Length Immunoglobulin Antibody; LR, Laminin Receptor; LRP, Laminin Receptor Precursor; PAGE, Polyacrylamide gel electrophoresis; PCA, Protocatechuic acid; siRNA, small interfering RNA; SDS, Sodium Dodecyl Sulphate

knock-down of LRP as a potential therapeutic avenue for the treatment of pancreatic cancer and neuroblastoma.

Keywords

37kDa/67 kDa laminin Receptor; apoptosis; small interfering RNAs; pancreatic cancer; neuroblastoma

Introduction

Laminins are glycoproteins that play a major role in the formation of the extracellular matrix[1]. Both integrin and non-integrin laminin receptors have been identified as mediators used by laminins to carry out their functions, which include cell migration[2], adhesion[3], growth[4], cell differentiation, proliferation[5] and cell signaling[6]. One major receptor and binding partner for laminin is the 37kDa/67kDa laminin receptor precursor/high-affinity laminin receptor (LRP/LR)[7].

LRP/LR is a major extracellular matrix/non-integrin laminin receptor with high specificity and affinity for laminin-1 [8]. LRP/LR is predominantly found as a transmembrane receptor but also localizes in the cytosol[9] and the nucleus[10] – facilitating translational processes and the maintenance of nuclear structures, respectively. It plays a role in physiological processes such as cellular adhesion, migration, proliferation, maintenance of cellular viability[11], cell cycle[12], protein synthesis[12] and processing of ribosomal RNA[13].

Numerous studies have implicated LRP/LR as a key contributor to the pathogenesis of certain viral and bacterial infections [14, 15], prion-protein related diseases such as Transmissible

Spongiform Encephalopathies [16], neurodegenerative diseases such as Alzheimer's disease [17, 18], and several cancer types [19-22]. Blockage of the interaction between Laminin-1 and LRP/LR by use of the anti-LRP/LR specific antibody IgG1-iS18 has been shown to diminish levels of adhesion and invasion of various cell lines *in vitro* [19-25]. Therefore, it is evident that LRP/LR is crucial for metastatic processes and is a promising target for metastatic cancer therapeutics. In addition to metastasis, LRP/LR is also suggested to enhance tumour angiogenesis [26] and recently Naidoo *et al.* [27] and Otgaar et al. [28]elucidated a novel role for LRP/LR in mediating telomerase activity through the enhancement of hTERT activity. In particular, knock-down of LRP/LR resulted in impediment of telomerase activity in metastatic breast cancer cells, combating the pro-tumourigenic activities of LRP/LR[27]. Very recently, we showed that LRP::FLAG enhances telomerase activity concomitant with reduced senescence markers, indicating that LRP plays a profound role in the ageing process[28]. Due to the involvement of LRP/LR in these aforementioned numerous tumourigenic processes, the role of this receptor in cell survival and viability has become an attractive topic of research[29]. Recent studies showed reductions in the viability of liver (Hep3b)[30], lung (A549)[31], cervical (HeLa)[31] , breast (MCF-7 and MDA-MB231)[32] and oesophageal cancer (WHCO1)[32] cells after siRNA-mediated downregulation of LRP/LR – thus suggesting that LRP/LR plays a pivotal role in the maintenance of tumour cell viability. Furthermore it has been shown that LRP/LR plays an important role in apoptosis [30-32].

Apoptosis is a process of programmed cell death necessary for the maintenance of tissue homeostasis and embryonic development [33]. Three main types of biochemical changes have been observed in apoptosis, namely caspase activation, DNA/protein breakdown, and membrane changes that result in phagocytosis [34]. There are two main pathways by which caspases can be

activated and thereby induce apoptosis, namely the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway [34]. Recent studies made use of siRNA technology to downregulate LRP/LR and assess the effects of this down-regulation on apoptosis [31, 32]. siRNA-mediated LRP/LR down-regulation was shown to significantly reduce cellular viability of lung (A549) and cervical cancer (HeLa) cells and increase the levels of caspase-3 activity in both cell lines, thus suggesting that LRP/LR is pivotal in the maintenance of cellular viability as well as the evasion of apoptosis in tumour cells.

Therefore, the present study aimed to assess the role of LRP/LR on the viability and survival of highly aggressive pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells by using an siRNA approach. Additionally, both the intrinsic and extrinsic apoptotic pathways were evaluated following siRNA mediated downregulation of LRP/LR. This study showed that LRP/LR knockdown significantly reduced the viability of pancreatic cancer and neuroblastoma cells, and suggests that the observed reduction in cellular viability is due to apoptosis.

Materials and Methods

Cell culture. AsPC-1 and IMR-32 cells were obtained from the Fox Chase Cancer Center (USA). AsPC-1 cells were cultured in RPMI 1640 supplemented with 10% Fetal Calf Serum (FCS), 1% penicillin/streptomycin, 2mM L-glutamine, 10mM HEPES and sodium pyruvate. IMR-32 cells were cultured in Eagle Minimum Essential Medium (EMEM) supplemented with 10% FCS, 1% penicillin/streptomycin, 2mM L-glutamine and 1% non-essential amino acids. Cell lines were maintained in a humidified incubator at 37ºC with 5% CO_2 and were sub-cultured when 80% confluency was reached.

siRNA-mediated down-regulation of LRP/LR. Cells were seeded into 6 well plates and were transfected at approximately 50-70% confluency. Transfections were performed using siRNA-LAMR1 to downregulate LRP/LR and siRNA-scrambled was employed as a negative control (Dharmacon). The Dharmafect1 Transfection reagent was used according to the manufacturer's instructions. Additionally, AsPC-1 and IMR-32 cells were transfected with Lipofectamine 3000 reagent (Thermo Scientific) and either esiRNA-RPSA (targeted to LRP/LR) or esiRNA-RLUC (negative control) (Sigma-Aldrich), in order to confirm that downregulation was not due to off-target effects. Thereafter, the plates were incubated at 37°C for a period of 72 hours prior to downstream experiments being performed.

Western blotting. Endogenous LRP/LR levels as well as siRNA-treated LRP/LR levels were determined by employing Western blotting. In order to prepare cell lysates, cells were incubated in 500µl of lysis buffer (10mM Tris/HCl pH 7.5, 10mM EDTA, 100mM NaCl, 0.05% (w/v) deoxycholic acid (DOC), 0.5% (v/v) Nonidet-P40) for 15 minutes at 4°C. This was followed by centrifugation at 14000 rpm for 2 minutes, after which the supernatant containing the extracted proteins was retained. Cell lysates were separated on 12% SDS-PAGE gels (Bio-Rad). Proteins were transferred to PVDF membranes for 45 min at 300V and 350A. Thereafter, the membranes were blocked for 1 hour using blocking buffer (3% BSA in PBS-Tween) followed by an 1 hour incubation of the membranes in the primary antibody (anti-LRP/LR specific antibody IgG1-iS18) diluted 1:5000 in blocking buffer. Three washes in washing buffer (10 min each) were performed before incubation of the membranes in secondary antibody (anti-human IgG-HRP conjugated antibody) diluted 1:10000 in blocking buffer for 1 hour. Three washes were again performed as previously mentioned before chemiluminescent substrate was added for detection of proteins. X-ray films were used to capture the chemiluminescent reaction. β-actin (mouse

monoclonal anti-β-actin peroxidase; Sigma) served as a loading control. Densitometry was performed to quantify protein levels with ImageJ™ software.

MTT assay. Both AsPC-1 and IMR-32 cells were seeded at a density of $1x10^4$ cells per well in a 24-well plate and transfected 24 hours later. Seventy two hours post transfection, 100µg of MTT was added to each well, and incubated for 2 hours at 37°C. Post incubation, the MTT-containing medium was discarded from each well and the remaining formazan crystals were dissolved in 500µl of DMSO. The absorbance was measured at 570nm using an ELISA plate reader. Controls included untreated cells, negative control siRNA treated cells, and positive control protocatechuic acid (PCA) treated cells.

BrdU assay. BrdU assays were performed using a BrdU Cell Proliferation Kit (Calbiochem) according to the manufacturer's instructions. In brief, cells were seeded at a density of 1×10^4 cells in a 24-well plate and transfected. Post transfection (72 hours), 80µl of a working stock of BrdU was added to each well followed by a 24 hour incubation period at 37°C. Following aspiration of contents, 800µl of fixative solution was added to each well and incubated for 30min at room temperature. After removal of the fixative, cells were subjected to an hour incubation in 100µl of anti-BrdU antibody. Cells were washed thrice with wash buffer and incubated for 30min, at room temperature, with 400µl of the peroxidase anti-mouse IgG HRP-conjugated antibody. After cells were washed three times, 400µl of substrate solution was added followed by a subsequent incubation period of 15min. Absorbance was measured within 30min of addition of stop solution (400µl) at dual wavelengths of 450 and540 nm. Untreated cells were used as a control and siRNA-scr and PCA were used as negative and positive controls, respectively.

Confocal microscopy. AsPC-1 and IMR-32 cells were seeded onto coverslips at a density of $1x10^4$ cells prior to siRNA transfection. Thereafter, 72 hours post transfection, cells were fixed in

4% paraformaldehyde for 15 min followed by three washes with PBS. Post washing, cells were incubated with Hoechst 33342 nuclear stain diluted in PBS (1:100) for 5 to 10 min in the dark. The coverslips were then washed 2-3 times in PBS and thereafter, coverslips were mounted (cell side down) onto a clean microscope slide using mounting fluid. After setting for 45 min in the dark, slides were stored at 4ºC until ready to view with a confocal microscope (Zeiss LSM 780). Controls included untreated cells, cells treated with siRNA-scr (negative control) and PCA (positive control).

Annexin V-FITC/7AAD assays. The Annexin V-FITC/7-AAD assay (Beckman Coulter) was performed according to the manufacturer's instructions. Briefly, post siRNA transfection, cells were harvested with trypsin/EDTA and washed with cold PBS by centrifugation at 500 x *g* for 5 min. The cells were resuspended in 1 x Annexin-binding buffer and 10µl of Annexin V-FITC solution and 20µl of 7-AAD viability dye was added to 100µl of the cell suspensions. The cell suspensions were then incubated on ice for 15 min in the dark. Finally, samples were supplemented with 400µl of cold 1X Annexin binding buffer prior to flow cytometric analysis. Untreated cells, siRNA-scr treated cells, and PCA treated cells were used as controls. Samples were analysed using the BD Accuri C6 flow cytometer, where the FL-1 laser was used to detect Annexin V- FITC staining and FL-3 was used for detection of 7-AAD staining. Compensation and quadrants were set using the following controls: unstained cells, Annexin V-FITC stained cells, 7-AAD stained cells, cells stained with both Annexin V-FITC and 7-AAD, and PCA-treated cells stained with both Annexin V-FITC and 7-AAD.

Caspase-3, -8 and -9 activity assays. Caspase 3, Caspase 8 and Caspase 9 Activation Assays (Abcam) were performed according to manufacturer's instructions. In brief, 72 hours after siRNA transfection, $1x10^6$ cells were pelleted by centrifugation at 1200 rpm for 10 min.

Thereafter, cells were re-suspended in 50µl of cell lysis buffer and incubated on ice for 10 min. This was followed by centrifugation of cells at 10000 x *g* for 1 min. Following this, the protein concentration of the supernatant was determined by use of a BCA™ assay and subsequently, 200µg of protein was diluted to 50µl with cell lysis buffer and added to appropriate wells of a 96-well plate. The 2X reaction buffer was then supplemented with DTT (10µl DTT/1ml reaction buffer) and 50µl of this solution was added to each sample, followed by the addition of 5µl of peptide substrate. After a 2 hour incubation period at 37ºC, the absorbance was measured at 405nm using an ELISA plate reader. In addition to untreated cells, siRNA-scr treated and PCA treated cells were used as negative and positive controls, respectively.

Statistical evaluation. The two-tailed Student's *t*-test with a confidence interval of 95% was used in order to analyse the data, with p-values of less than 0.05 being considered as significant. This statistical analysis was done using Graphpad Prism version 5.03.

Results

siRNA technology leads to down-regulation of LRP expression in pancreatic cancer and neuroblastoma cells. In order to determine the effect of LRP/LR expression on cellular viability, siRNA-mediated knockdown of LRP/LR was performed. Post transfection of pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells with siRNA-LAMR1 (specifically targeting the mRNA of the 37kDa LRP), Western blot analysis and subsequent densitometry was performed. Densitometric analysis revealed that LRP was successfully down-regulated in both AsPC-1 (Fig.1a and 1b) and IMR-32 (Fig.1c and 1d) cells by transfection with the afore-mentioned siRNA. It was observed that siRNA-LAMR1 transfected AsPC-1 and IMR-32 cells exhibited a 90% and 71% reduction in LRP expression, respectively, when compared to non-transfected cells, set to 100%. Moreover, transfection of AsPC-1 cells with siRNA-scr (negative control scrambled siRNA) showed no significant LRP knockdown when compared to non-transfected cells (Fig.S1a and S1b). Likewise, the same effect was observed when IMR-32 cells were transfected with siRNA-scr (Fig.S1c and S1d).

siRNA-mediated down-regulation of LRP expression results in significantly reduced viability of pancreatic cancer and neuroblastoma cells. After siRNA-mediated knockdown of LRP, the effect thereof on cellular viability was analysed. Following treatment of AsPC-1 and IMR-32 cells with siRNA-LAMR1, MTT assays revealed that cellular viability was significantly reduced in comparison to controls. This signifies that siRNA-mediated LRP knockdown leads to the observed decreases in cellular viability in both of the tumourigenic cell lines.

It was seen that AsPC-1 and IMR-32 cells treated with siRNA-LAMR1 exhibited an 82% and 75% reduction in cellular viability, respectively, when compared to non-transfected cells (Fig.1e). Moreover, no significant reduction in cellular viability was observed for both cell lines

when treated with the negative control siRNA-scr and compared to non-transfected cells (Fig.1e). Protocatechuic acid (PCA) was used as a positive control treatment as it is a known apoptosis inducer.

Use of an alternative siRNA confirms that LRP down-regulation is responsible for reductions in cellular viability in pancreatic cancer and neuroblastoma cells. In order to ascertain whether the observed reduction in cellular viability occurred as a result of siRNA-LAMR1-mediated LRP down-regulation and not an off-target effect, an additional siRNA targeting a different region of LRP was used. After transfection of cells with the alternative siRNA (esiRNA-RPSA), Western blot analysis and densitometry was performed. By comparison to non-transfected cells, pancreatic cancer (AsPC-1) cells showed a significant knockdown of 83% in LRP expression (Fig.2a and 2b). Similarly, neuroblastoma (IMR-32) cells exhibited a significant down-regulation of 64% in LRP expression when compared to non-transfected cells (Fig.2c and 2d). Additionally, treatment of AsPC-1 cells with esiRNA-RLUC (negative control siRNA) displayed no significant difference in LRP expression when compared to non-transfected cells (Fig.S2a and S2b). Likewise, the same effect was observed for IMR-32 cells treated with esiRNA-RLUC (Fig.S2c and S2d).

To assess the effects of esiRNA-RPSA-mediated LRP knockdown on the viability of AsPC-1 and IMR-32 cells, MTT assays were performed. It was found that treatment of cells with this alternative siRNA resulted in significantly reduced cellular viability in both tumourigenic cell lines under study. Specifically, AsPC-1 cells showed an 82% reduction in cellular viability post siRNA treatment whilst IMR-32 cells exhibited a 60% reduction when compared to non-transfected cells (Fig.2e). Moreover, treatment of both cell lines with the negative control siRNA

(esiRNA-RLUC) did not result in a significant change in cellular viability when compared to non-transfected cells (Fig.2e).

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

It was observed that AsPC-1 and IMR-32 cells showed significant reductions of 76% and 44% in cellular proliferation, respectively, after being treated with siRNA-LAMR1 and compared to non-transfected cells (Fig.3). Furthermore, treatment of both cell lines with the negative control siRNA (siRNA-scr) showed no significant differences in cellular proliferation when compared to non-transfected cells (Fig.3).

siRNA-mediated knockdown of LRP expression results in nuclear morphological changes indicating apoptotic induction. To examine if apoptotic induction led to the reductions in cellular viability post siRNA-LAMR1 treatment, nuclear morphology was investigated by confocal microscopy. Compared to the nuclei of non-transfected cells (Fig.4a), siRNA-LAMR1 treated pancreatic cancer cells (AsPC-1) displayed nuclear morphology indicative of apoptosis (Fig.4d). Similarly, comparison of nuclei of siRNA-LAMR1 treated neuroblastoma cells (IMR-32) (Fig.4h) to the nuclei of non-transfected cells (Fig.4e) revealed nuclear morphological changes such as nuclear shrinkage, membrane blebbing and the formation of apoptotic bodies. Moreover, treatment of both cell lines with the negative control siRNA-scr did not result in changes in nuclear morphology when compared to non-transfected cells (Fig.4b and 4f). PCA

was used as a positive control for studying nuclear morphological changes under apoptotic conditions (Fig.4c and 4g).

siRNA-mediated down-regulation of LRP expression leads to phosphatidylserine externalization and apoptotic induction. Confocal microscopy suggested that knockdown of LRP expression in pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells causes nuclear morphological changes that are characteristic of cells undergoing apoptosis. In order to confirm that this observation of apoptotic induction holds true for both the cell lines in question, an Annexin V-FITC/ 7-AAD assay was performed.

It was observed that transfection of AsPC-1 cells with siRNA-LAMR1 resulted in 76% of cells undergoing apoptosis (Fig.5d) when compared to non-transfected cells (Fig5a). Treatment of IMR-32 cells with siRNA-LAMR1 induced apoptosis in 42.7% of cells (Fig.5h) when compared to non-transfected cells (Fig.5e). Furthermore, treatment of both cell lines with the negative control siRNA-scr did not induce apoptosis (Fig.5b and 5f). Protocatechuic acid (PCA) was used as a positive control (Fig.5c and 5g).

siRNA-mediated knockdown of LRP expression leads to significantly increased caspase-3 activity. To further confirm that down-regulation of LRP induces apoptosis in pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells, caspase-3 activity was assessed. It was found that after transfection with siRNA-LAMR1, AsPC-1 cells displayed a significant 10-fold increase in caspase-3 activity when compared to non-transfected cells (Fig.6a). IMR-32 cells showed a 6-fold increase in caspase-3 activity after treatment of cells with siRNA-LAMR1 and compared to non-transfected cells (Fig.6a). Additionally, treatment of both cell lines with the negative control siRNA-scr showed no significant differences in caspase-3 activity when compared to non-transfected cells (Fig.6a).

Pancreatic cancer and neuroblastoma cells exhibit significantly increased caspase-8 activity after siRNA-mediated down-regulation of LRP expression. Since caspase 3 is activated in both the intrinsic and extrinsic pathways of apoptosis, caspase-8 activity assays were performed to further understand the role LRP/LR plays in promoting cell survival of cancerous cells. This assay was used to gain further insight into whether transfection with siRNA-LAMR1 results in activation of the caspase-8-mediated extrinsic pathway in both cell lines. It was observed that AsPC-1 and IMR-32 cells treated with siRNA-LAMR1 experienced a significant 3-fold and 4-fold increase in caspase-8 activity, respectively, when compared to non-transfected cells (Fig.6b). Moreover, transfection of both cell lines with the negative control siRNA-scr revealed no significant differences in caspase-8 activity when compared to non-transfected cells (Fig.6b).

Pancreatic cancer cells show a significant increase in caspase-9 activity after siRNA-mediated LRP knockdown. Caspase-9 activity assays were used to establish whether pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells undergo apoptosis through the caspase-9-mediated intrinsic pathway after siRNA-mediated LRP downregulation. It was found that AsPC-1 cells exhibited a significant 3-fold increase in caspase-9 activity after transfection with siRNA-LAMR1, when compared to non-transfected cells (Fig.6c). No significant difference was however observed in caspase-9 activity in IMR-32 cells after transfection when compared to non-transfected cells (Fig.6c). Furthermore, treatment of both cell lines with the negative control siRNA-scr resulted in no significant differences in caspase-9 activity when compared to non-transfected cells, as expected (Fig.6c).

Discussion

The overexpression of LRP/LR in cancerous cell lines and its involvement in numerous tumourigenic processes has made this receptor an attractive target for study. Specifically, tumour progression is enhanced by the LRP/LR-assisted promotion of processes such as tumour angiogenesis, metastasis and apoptotic evasion – three out of the six hallmarks of cancer[35]. The widespread cellular localization of LRP/LR allows this receptor to serve several physiological functions such as cell adhesion, cell migration and proliferation, cell viability, regulation of the cell cycle, protein synthesis and ribosomal RNA processing. Due to LRP/LR over-expression, these functions may be exploited in tumour cells, leading to the occurrence of the afore-mentioned tumour-promoting processes.

In order to determine the role played by LRP/LR in the maintenance of cellular viability, siRNAs were used to downregulate LRP expression in AsPC-1 and IMR-32 cells (Fig.1 and 2) and the effect on cell viability was assessed. Treatment of the cells with siRNA-LAMR1 and esiRNA-RPSA resulted in significant reductions in viability which correlated with siRNA-mediated LRP knockdown expression levels (Fig. 1e and 2e). This observation suggests that LRP plays a crucial role in cell survival of these two cancer cell lines.

Since it is known that, in addition to viability, LRP/LR plays a role in proliferation, we wanted to establish whether downregulation of LRP/LR would further impair cellular division of AsPC-1 and IMR-32 cells. After transfection with siRNA-LAMR1, AsPC-1 and IMR-32 cells showed reductions in cellular proliferation that correlated with the LRP expression levels observed after downregulation. This suggests that LRP/LR plays a role in cellular division which might in addition facilitate cellular viability maintenance. LRP/LR has also been identified in interactions with certain proteins that govern cell cycle progression, such as cyclins A2 and B1, CDK's 1 and

2, and p2 [11], thus providing not only an explanation for the hampered cellular growth but also the decreased proliferation observed upon siRNA treatment.

Next we wanted to determine whether apoptosis was the form of cell death responsible for the observed reduction in the viability of AsPC-1 and IMR-32 cells after LRP downregulation. After treatment of these two cell lines with siRNA-LAMR1, confocal microscopy revealed evident alterations in nuclear morphology – such as nuclear shrinkage, nuclear membrane blebbing and the formation of apoptotic bodies (Fig.4). Due to the maintenance of nuclear structures by the binding of nuclear and perinuclear LRP/LR to histones[36], it is clear that knockdown of this receptor leads to diminished nuclear integrity and altered nuclear morphology. These siRNA-LAMR1-induced changes to the nuclear morphology of AsPC-1 and IMR-32 are indicative of apoptotic induction in these cell lines.

Further confirmation and quantification of apoptotic induction was achieved by use of an Annexin-V-FITC/ 7-AAD assay. It was observed that non-transfected and negative control siRNA-scr transfected AsPC-1 and IMR-32 cells did not display Annexin-V and/or PI, which is representative of live cells. However, 8mM PCA-treated and siRNA-LAMR1-treated AsPC-1 and IMR-32 cells exhibited an increase in Annexin-V or Annexin-V and PI which is indicative of early and late stage apoptosis respectively. The shift from negative to positive Annexin-V staining signifies that siRNA-LAMR1-mediated LRP down-regulation in AsPC-1 and IMR-32 cells triggers loss of membrane asymmetry and PS externalization on the outer leaflet of the plasma membrane which are common features of apoptotic cells [37].

Nuclear morphological studies as well as Annexin V assays proved that siRNA-mediated knockdown of LRP expression leads to apoptotic induction in AsPC- 1 and IMR-32 cells. Additionally, caspase-3 assays were performed in order to further ascertain the occurrence of

apoptotic induction as well as the involvement of caspases after treatment of these cell lines with siRNA-LAMR1. Caspase-3 is an effector caspase that exhibits increased activity in cells that are actively undergoing apoptosis. After treatment of AsPC-1 and IMR-32 cells with siRNA-LAMR1, there was a marked increase in caspase-3 activity in both cell lines when compared to non-transfected cells. These findings clearly indicate that siRNA-mediated LRP down-regulation induces apoptosis in these two tumourigenic cell lines. This observation of apoptotic induction after siRNA-mediated LRP knockdown may also be attributed to the interaction of LRP/LR with focal adhesion kinase (FAK). A recent study showed that after LRP/LR binds to laminin, interactions can occur between LRP/LR and FAK[38]. These interactions were implicated in the activation of PI3-kinase/AKT and MEK/ERK 1/2 cell survival pathways and also the up-regulation of Bcl-2 - an anti-apoptotic protein[38]. Alternatively, down-regulation of LRP by siRNAs may have led to apoptotic induction due to the ribosomal functions of LRP/LR. It is known that LRP/LR plays a role in the biogenesis of ribosomes by facilitating 21S pre-rRNA processing into mature 18S rRNA[13], and also associates with and acts as a component of the 40S ribosomal small subunit where it assists with protein translation and synthesis[39-41]. Therefore, knockdown of LRP in this study might interfere with ribosome formation and consequent translation of proteins that are critically involved in proper cell functioning and survival, thereby leading to cell death.

As mentioned before LRP/LR plays a role in the PI3-kinase/AKT and MEK/ERK 1/2 cell survival pathways. Since both these pathways are involved in inhibiting the intrinsic as well as extrinsic apoptotic pathways, caspase-8 and caspase-9 assays were performed to assess which apoptotic pathway is induced after siRNA-LAMR1-mediated knockdown of LRP/LR. It was observed that both of the tumourigenic cell lines exhibited increased caspase-8 activity after

treatment with siRNA-LAMR1, when compared to non-transfected cells indicating that the extrinsic apoptotic pathway is activated [42].

In addition, it was observed that AsPC-1 cells treated with siRNA-LAMR1 exhibited a significant increase in caspase-9 activity in comparison to non-transfected cells. Caspase-9 is intricately involved in the mitochondrial (intrinsic) pathway[42], thus suggesting that siRNA-mediated LRP knockdown also induces apoptosis in AsPC-1 cells via the intrinsic pathway.

Interestingly, it has been shown by Celay et al. [43]that activation of the apoptotic intrinsic pathway is less probable after treatment of IMR-32 cells with 9-cis Retinoic Acid (RA). Treatment of IMR-32 and SH-SY5Y cells with 9-cis RA leads to an increase of BIRC3 and BIRC8 (an inhibitor of APAF1, BAX and Caspase 9) and to a decrease of APAF1 and BCL10 (an activator of Caspase 9), respectively, suggesting a lesser formation of the apoptosome and a blockade of the intrinsic pathway. Moreover, RA causes downregulation of the 67 kDa Laminin Receptor which correlates with reduced biological aggressiveness of human neuroblastoma cells[44]. We therefore suggest that the decrease in caspase 9 activation observed after treatment of RA is possibly due to the downregulation of the 67 kDa Laminin Receptor. This might explain why siRNA mediated downregulation of LRP induces caspase 8 activation in both cell lines whilst caspase 9 activation occurs only in AsPC-1 cells.

Besides its normal roles in mediating cell viability, cellular adhesion and functioning as a member of the ribosomal translational machinery, LRP/LR has been implicated in a number of pathological processes including cancer and metastasis [45]. However, the induction of apoptosis in response to downregulation of LRP/LR, as shown here and previously for MCF-7, MDA-MB231and WHCO1 cells [32], suggests that this may not be a practical approach without specific targeting to avoid inducing apoptosis in non-tumourigenic cells. Interestingly, though, we have

previously shown that shRNA mediated downregulation of LRP/LR had no significant effect on the viability of non-cancerous HEK293 cells suggesting that this protein might be crucial for cell survival in certain cell types only[17]. As such, modifying the expression of LRP/LR is an attractive therapeutic target for the treatment of cancer.

Conclusion

The present study has demonstrated that the siRNA-mediated knockdown of LRP expression significantly reduced the viability of pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells through hampering of cell proliferation and through the induction of apoptosis. In IMR-32 cells, apoptosis was solely induced through the death receptor (extrinsic) pathway while apoptosis occurred via both the intrinsic and extrinsic pathways in AsPC-1 cells. It is likely that the PI3K/AKT and MAPK/ERK pathways are involved however the exact mechanism of action warrants further investigation. These findings exemplify the critical role played by LRP/LR in the maintenance of cellular viability in both of these tumourigenic cell lines. Furthermore, this *in vitro* study highlights the potential of using siRNAs targeting LRP therapeutic tools for the treatment of pancreatic cancer and neuroblastoma. Future research will therefore include investigation of the effect of LRP/LR knock-down strategies in in vivo cancer models.

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Conflict of interest

The authors declare that they have no conflict of interest

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Figure Legends

Figure 1: LRP expression in pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells after siRNA transfection, and the subsequent effect of siRNA-mediated LRP downregulation on cellular viability. a) and **b**) Transfection of AsPC-1 cells with siRNA-LAMR1 resulted in significant 90% knockdown of LRP when compared to non-transfected cells. The value for the non-transfected cells was set to 100%. **p=0.0086. **c)** and **d**) Densitometry revealed that there was a significant 71% reduction of LRP expression after transfection of IMR-32 cells with siRNA-LAMR1 when compared to non-transfected cells. The value for non-transfected cells was set to 100%. *p=0.0117. **e**) MTT assays were used to analyse the viability of AsPC-1 and IMR-32 cells after treatment with siRNA-LAMR1. The value for non-transfected cells was set to 100% for both cell lines and by comparison, AsPC-1 and IMR-32 cells treated with siRNA-LAMR1 displayed a significant reduction of 82% and 65% in cellular viability, respectively. Cells treated with the negative control siRNA-scr showed no significant differences in cellular viability when compared to non-transfected cells for both cell lines. Protocatechuic acid (PCA) was used as a positive control. Graph is representative (mean ± STD) of an average of experiments performed in triplicate and repeated three times. **p=0.0017, ***p=0.0008, N.S.: p>0.05, non-significant.

Figure 2: Detection of LRP expression in pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells after transfection with esiRNA-RPSA, and the subsequent effect thereof on cellular viability. a) and **b**) After transfection of AsPC-1 cells with esiRNA-RPSA, there was a significant reduction of 83% in LRP expression when compared to non-transfected cells. β-actin was used as a loading control. **p=0.0057. **c)** and **d**) Densitometry revealed that after treatment of IMR-32 cells with esiRNA-RPSA, there was a significant reduction of 64% in LRP

expression when compared to non-transfected cells. Non-transfected cells were set to 100% to allow for comparison and β-actin was used as a loading control. *p=0.025. **e**) After downregulation of LRP with esiRNA-RPSA, MTT assays were performed to assess cellular viability. Compared to non-transfected cells (set to 100%), AsPC-1 and IMR-32 cells showed a reduction of 82% and 60% in cellular viability, respectively. No significant differences were observed in the viability of cells treated with the negative control siRNA (esiRNA-RLUC) when compared to non-transfected cells for both cell lines. PCA was used as a positive control. Data shown are representative (mean ± STD) of three biological replicates, each performed in triplicate. **p=0.0014, ***p=0.0005, N.S.: p>0.05 therefore non-significant.

Figure 3: The effect of siRNA-mediated LRP knockdown on the proliferation of pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells. After downregulation of LRP, AsPC-1 cells showed a 76% reduction in cellular proliferation when compared to non-transfected cells (set to 100%). IMR-32 cells showed a decrease of 44% in proliferation after siRNA-LAMR1 treatment by comparison to non-transfected cells. Treatment of both cell lines with the negative control siRNA-scr revealed no significant changes in cellular proliferation when compared to non-transfected cells. Protocatechuic acid was used as a positive control and data shown are representative (mean ± STD) of three biological replicates, each performed in triplicate. **p=0.0053 and 0.0031 for AsPC-1 and IMR32 cells, respectively. N.S.: p>0.05, non-significant.

Figure 4: The effect of siRNA-mediated LRP knockdown on nuclear morphology of pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells. a) Non-transfected AsPC-1 cells showed normal rounded nuclei with uncompromised integrity. **b**) AsPC-1 cells treated with the negative control siRNA-scr displayed nuclei similar to that of the non-transfected cells. **c**)

PCA-treated AsPC-1 cells showed membrane blebbing and apoptotic body formation (indicated by white arrows). **d**) siRNA-LAMR1 transfected AsPC-1cells exhibited diminished nuclear integrity and the formation of apoptotic bodies (shown by white arrows). **e**) Nuclear integrity is maintained in non-transfected cells, evident by the normal rounded shape of nuclei of the IMR-32 cells. **f**) Negative control siRNA-scr transfected IMR-32 cells showed normal rounded nuclei with uncompromised integrity. **g**) PCA (positive control) treated IMR-32 cells showed nuclear shrinkage (indicated by white arrows). **h**) siRNA-LAMR1 transfected IMR-32 cells showed compromised nuclear integrity, made clear by the observation of nuclear shrinkage and apoptotic body formation (shown by white arrows). All images were obtained at a magnification of 63X. Scale bar applies to all images.

Figure 5: Apoptotic induction in pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells after siRNA transfection. a) and **e**) Non-transfected cells show that majority of cells lie in the lower left quadrant (Q1-LL) which is indicative of normal living cells. **b)** and **f**) Treatment of cells with the negative control siRNA-scr also resulted in majority of the cells appearing in the lower left quadrant. **c)** and **g**) Protocatechuic acid (PCA) was used as a positive control and resulted in most of the cells appearing in the upper right quadrant (Q1-UR), which is representative of cells undergoing late apoptosis. **d)** and **h**) Transfection of cells with siRNA-LAMR1 led to 0.8% of AsPC-1cells and 7.5% of IMR-32 cells undergoing early apoptosis, as shown in the lower right quadrant (Q1-LR), and 75.2% of AsPC-1cells and 35.2% of IMR-32 cells undergoing late apoptosis – therefore a total of 76% of AsPC-1 and 42.7% of IMR-32 cells underwent apoptosis after siRNA-LAMR1 treatment. Plots are indicative of an average of experiments performed in triplicate and repeated three times.

Figure 6: The effect of siRNA-mediated LRP down-regulation on caspase-3, -8 and -9 activity in pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells. **a**) Caspase-3 activity assays showed that after treatment of cells with siRNA-LAMR1, AsPC-1 and IMR-32 cells showed a significant 10-fold and 6-fold increase in caspase-3 activity, respectively, when compared to non-transfected cells (which were set to 100%). No significant difference was observed when both cell lines were treated with the negative control siRNA-scr and compared to non-transfected cells. Prototechuic acid (PCA) was used as a positive control. *p=0.0109 and 0.0154 for AsPC-1 and IMR-32, respectively. N.S: p>0.05, non-significant. **b**) siRNA-LAMR1 transfected AsPC-1 and IMR-32 cells showed a significant 3-fold and 4-fold increase in caspase-8 activity, respectively, when compared to non-transfected cells (set to 100%). Treatment of both cell lines with the negative control siRNA-scr resulted in no significant difference in caspase-8 activity when compared to non-transfected cells. *p=0.0357, **p=0.0053, N.S: p>0.05, non-significant. **c**) siRNA-LAMR1 transfected AsPC-1 cells showed a significant 3-fold increase in caspase-9 activity when compared to non-transfected cells (set to 100%). Treatment of IMR-32 cells with siRNA-LAMR1 showed no difference in caspase-9 activity by comparison to non-transfected cells. Both tumourigenic cell lines displayed no significant difference in caspase-9 activity after treatment with the negative control siRNA-scr when compared to non-transfected cells. **p=0.0016, N.S.: p>0.05, non-significant. Data shown are representative (mean ± STD) of three biological replicates, each performed in triplicate.