Knock-Down of LRP/LR Influences Signalling Pathways in Late Stage Colorectal Carcinoma Cells

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Research article

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Abstract

Background

The 37kDa/67kDa laminin receptor (LRP/LR) is involved in several tumourigenic-promoting processes including cellular viability maintenance and apoptotic evasion. Thus, the aim of this study was to assess the molecular mechanism of LRP/LR on apoptotic pathways in late stage (DLD-1) colorectal cancer cells upon siRNA-mediated down-regulation of LRP/LR.

Methods

siRNAs were used to down-regulate the expression of LRP/LR in DLD-1 cells which was assessed using western blotting and qPCR. To evaluate the mechanistic role of LRP/LR, proteomic analysis of pathways involved in proliferation and apoptosis were investigated. The data from the study was analysed using a one-way ANOVA, followed by a two-tailed student's t-test with a confidence interval of 95%.

Results

Here we show that knock-down of LRP/LR led to significant changes in the proteome of DLD-1 cells, exposing new roles of the protein. Moreover, analysis showed that LRP/LR may alter components of the MAPK, p53-apoptotic and autophagic signalling pathways to aid colorectal cancer cells in continuous growth and survival. Knock-down of LRP/LR also resulted in significant decreases in telomerase activity and telomerase-related proteins in the DLD-1 cells.

Conclusions

These findings show that LRP/LR is critically implicated in apoptosis and cell viability maintenance and suggest that siRNA-mediated knock-down of LRP/LR may be a possible therapeutic strategy for the treatment of colorectal cancer.

Background

Colorectal cancer has been ranked as the 3rd most common cancer type in 2018, and was found to be among the top five most frequent locations for cancer development in men and women globally [1]. Due to the increasing prevalence and mortality rates of colorectal cancer, it is crucial to develop a novel treatment strategy to combat this disease.

Recently, research into the 37kDa/67kDa laminin receptor (LRP/LR) has gained a large amount of interest as it plays several physiological roles within the cytoplasmic, perichromosomal and perinuclear areas as well as on the plasma membrane of cells [2]. In particular, LRP/LR is found to aid in ribosomal processing and protein translation in the cytoplasm [3]. While in the nucleus, the receptor is seen to interact with histones, thereby aiding in nuclear structure maintenance [4, 5]. LRP/LR also interacts with components of the cytoskeleton such as actin and tubulin, by acting as a linker for ribosomes to
microtubules, resulting in functional protein synthesis [6]. In addition to these physiological roles, LRP/LR has also been shown to have pathological roles in several diseases including micro-organism infections [7], Transmissible Spongiform Encephalopathies (TSEs) [8], prion disorders [9], Alzheimer's Disease (AD) [10-13] and age-related disorders [14]. The receptor has also been shown to play a major role in cancer since it is often overexpressed on the surface of several cancer cells [15-19]. This over-expression is said to enhance the adhesion of tumour cells to the basement membrane and disseminate these tumours – both fundamental events of metastasis [15, 16, 18-21] as well as promote tumour angiogenesis [22].

Another key role that LRP/LR has been identified to possess, is the critical maintenance of tumourigenic cell viability. This has been shown through several studies where down-regulation of LRP/LR through siRNA technology results in the reduction in cellular viability in liver (Hep3B) [23], cervical (HeLa) [24], lung (A549) [24], breast (MCF-7 and MDA-MB231) [25], oesophageal (WHCO1) [25], neuroblastoma (IMR-32) [26] and pancreatic (AsPC-1) [26] cancer cells, early (SW-480) and late (DLD-1) stage colorectal cancer [27] as well as early (A375) and late (A375SSM) stage malignant melanoma cells [28]. This reduction of cell viability was due to the induction of apoptosis, evident by increased caspase-3 activity observed in several of the mentioned cell lines [23-26, 28]. Apoptosis, also known as programmed cell death, is a vital process required for proper cell development, damaged cell elimination and homeostatic maintenance in every organism. Once a cell undergoes apoptosis, it experiences several biochemical alterations including caspase activation [29]. Caspases are activated through either the death receptor extrinsic (caspase-8) pathway or the mitochondrial intrinsic (caspase-9) pathway [29], which ultimately leads to apoptosis. Upon siRNA-mediated down-regulation, recent studies show that pancreatic (AsPC-1) [26], neuroblastoma (IMR-32) [26], early (SW-480) and late (DLD-1) stage colorectal cancer [27] cells as well as early (A375) and late (A375SSM) stage malignant melanoma [28] cells either undergo both the extrinsic and intrinsic apoptotic pathways or either one of them. Of particular interest, early and late stage colorectal cancer cells were found to undergo both caspase-8 and -9 activation [27]. This is highly likely as the extrinsic and intrinsic apoptotic pathways are able to interconnect at numerous levels and as a result, become influenced by similar factors.

In addition to LRP/LR's roles in cancer, the receptor has been found to contribute to telomerase activity in breast cancer cells [30]. Telomerase is a ribonucleoprotein with reverse transcriptase activity principally responsible for maintaining and elongating telomeres; tandem TTAGGG repeats found on the ends of chromosomes which help prevent chromosomal degradation [31]. Telomerase is also found to play an important role in immortalization and cellular senescence, thus it plays a significant role in the ageing process as well as the cancerous state. [32] In addition to telomere extension, telomerase has various extra-telomeric functions including DNA repair and mtDNA protection as well as several others which are found to favour cell viability preservation and cell proliferation. Moreover, a study showed that there is increased telomerase activity in approximately 90% of malignant human tumours [33]. In particular, a major role of hTERT, the catalytic subunit of telomerase, involves activating and regulating several cellular pathways as well as acting as a transcriptional activator for several proliferative genes [34]. It has also been shown that there is a direct association between hTERT and an increased resistance to apoptosis [35, 36] and that the knock-down of hTERT through RNA silencing has been found to activate
the pro-apoptotic protein Bax, triggering the mitochondrial cell death pathway [37]. Interestingly, it has been shown that there is a relationship between LRP/LR and telomerase. More importantly, that the two proteins have interlinked roles in various diseases such as cancer and neurodegenerative disorders [38]. Naidoo et al (2015) showed that telomerase and LRP/LR co-localize in the perinuclear compartment of breast cancer cells [30] and it has recently been found that LRP/LR enhances telomerase activity [12, 14]. Moreover, additional studies have revealed that the knock-down of LRP/LR significantly reduces telomerase activity [30, 39]. This suggests that LRP/LR may be influencing telomerase and that the knock-down of LRP/LR may be a potential therapeutic intervention for cancer through the resultant down-regulation of telomerase activity.

Telomeres together with telomere-related proteins work concurrently to protect and “cap” genomic DNA by preventing damage and degradation [40]. In particular, telomere-related proteins include: TRF-1, TFR-2, RAP-1, POT-1, TPP-1, and TIN-2, forming the complex known as “shelterin” which has a high specificity for telomeres [41]. The functions of the shelterin complex include additional telomere protection such as: the maintenance of telomere length, development of t-loop formation, recruitment of telomerase to the ends of telomeres and lastly, protection of chromosomal ends as being DNA damage [41]. Two important proteins making up the shelterin complex are the telomeric repeat-binding factor-1 (TRF-1) and -2 (TRF-2) since telomere sequences are found to be directly bound to these two proteins [42]. Once the telomere repeats bind to these proteins, they are responsible for directly protecting telomeres as well as regulating telomere length [43, 44]. Interestingly, it has been found that both TRF-1 and TRF-2 are over-expressed in several cancer types, however whether the increased expression contributes to cancer progression is still unclear [43, 44]. Thus, the expression of these proteins and their role in tumourigenesis has not been clearly understood, making them important points of interest in relation to LRP/LR, a receptor known to contribute to cancer development.

Although these findings have given some insight into LRP/LR and its mechanism in cancer and apoptosis, more information is still needed to fully understand the role that LRP/LR plays in the evasion of apoptosis in cancer. Thus, these outcomes encouraged the question of whether siRNA-mediated down-regulation of LRP/LR influences the expression of specific proteins involved in apoptosis and other cell signalling pathways in colorectal carcinoma cells.

**Methods**

**Cell cultivation**

The DLD-1 colorectal cancer cell line was obtained from Fox Chase Cancer Centre (FCCC). The cell line was cultured in Roswell Park Memorial Institute (RPMI) 1640 culture medium, supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) non-essential amino acids and 1% (v/v) Penicillin/Streptomycin/ in 75 cm$^3$ culture flasks. Cells were sub-cultured twice a week by discarding the media and washing the cells in PBS to remove any residual media. This was followed by an incubation in 1 X Trypsin/EDTA at 37
°C with 5% CO2 in a humidified atmosphere for 5 minutes. The Trypsin/EDTA was inactivated with culture medium and cells were seeded at appropriate concentrations and dilution factors when necessary.

Transfection procedure

Before transfections could take place, cells were seeded on 6-well or 24-well tissue culture plates at specific concentrations, depending on the experiment being performed. Cells were allowed to reach 50-70% confluence prior to transfection. Thereafter, serum-free Opti-MEM® media was used to dilute the experimental Dharmacon™ ON-TARGETplus SMARTpool Human-RPSA siRNA (targeted to LRP/LR) as well as the negative control MISSION® esiRNA-RLUC siRNA. In order for the transfection procedure to take place, appropriate amounts of DharmaFECT® transfection reagent were also added to the cells. This was followed by incubation at 37°C for 72 hours in order to allow the transfection to take place.

SDS-PAGE and Western blotting

In order to determine the levels of several proteins upon siRNA-mediated down-regulation of LRP/LR, western blotting was performed. The Trans-Blot® Turbo™ Transfer System was used for electroblotting with set parameters at 25V for 30 minutes. Following this, the membranes were blocked for an hour using 3% (w/v) BSA (Bovine Serum Albumin) in 1 X PBS-Tween (0.1% (v/v) Tween 20 and PBS). Thereafter, the membranes were incubated overnight in the appropriate primary antibody diluted in blocking buffer [IgG1-iS18 (1:1000), anti-β actin peroxidase, (1:1000), anti-rabbit TRF-1 (1:1000), anti-rabbit TRF-2 (1:1000), anti-rabbit pTERT (1:1000) and anti-mouse hTERT (1:1000)]. The membranes then underwent three 10-minute washes in 1X PBS-Tween. Thereafter, the membranes were incubated for 1 hour at room temperature in the appropriate anti-mouse or anti-rabbit secondary antibody with a horseradish peroxidase (HRP) conjugate which was diluted in 3% (w/v) BSA in 1X PBS-Tween. This was followed by three more 10-minute washes in 1X PBS-Tween before the chemiluminescent substrate was added and proteins were detected using the ChemiDoc™ imaging machine. In addition, the 42 kDa β-actin antibody was used for the detection of β-actin which served as a loading control. The Bio-Rad Image Lab 5.1 Image acquisition and analysis software was used to analyse the blot features and capture the image data.

Cell cycle analysis – Flow cytometry

A total of 100 000 DLD-1 colorectal cancer cells were seeded per well in 6-well tissue culture plates prior to transfection for 72 hours. The cells were then harvested and washed with 1X PBS followed by a centrifugation at 420 x g for 10 minutes. Thereafter, cells were fixed in cold 70% ethanol in a dropwise manner while vortexing, in order to ensure all cells were fixed. Fixation took place for at least 24 hours at 4°C. Following this, cells were washed with 1 X PBS after which they were centrifuged twice at 420 x g for 10 minutes in order to rid the cells of the ethanol. Following the supernatant being discarded, cells were incubated with 200 µl of Guava® cell cycle reagent containing Propidium Iodide for 30 minutes in
the dark. These resulting suspensions were evaluated using the BD Accuri C6 flow cytometer and software. Pulse processing was employed for the exclusion of cell doublets, using the pulse area vs. pulse width/height. Since the maximum emission of PI is in the orange range of the spectrum, the FL2 laser using a 562-588 nm band pass filter was utilized to detect the dye. Thereafter, the percentage of cells within each cell cycle was determined using markers set within the analysis program.

**Telomerase activity assay - Quantitative polymerase chain reaction (qPCR)**

**Sample preparation**

A total of 750 000 DLD-1 cells were seeded per well in 6-well tissue culture plates prior to transfection for 72 hours. The cells were then harvested and centrifuged at 1500 x g for 10 minutes. The resultant pellets were resuspended in 250 µl of CHAPS ((3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate)) lysis buffer) followed by 30-minute incubation on ice. Thereafter, samples were centrifuged for 20 minutes at 15000 x g at 4°C in a microcentrifuge where the resulting supernatant was snap frozen on dry ice. The protein concentration was then quantified using Nanodrop spectroscopy (Nanodrop® ND-1000) and the proteins were diluted to 500 ng/µl.

**Telomerase activity detection**

Relative telomerase activity was quantified by the TRAPEze® RT1 Telomerase detection Kit (Merck Millipore), following the manufacturers protocol. All samples were then subjected to experimental analysis by qPCR accompanied by a positive control (human embryonic kidney (HEK293) cell extracts with confirmed telomerase activity). Three negative controls were used: a minus telomerase control, consisting of only CHAPS Lysis Buffer to ensure the buffer had not been contaminated and had no telomerase activity; a no template control (water) consisting of only nuclease free/PCR Grade Water so as to normalise against primer dimer formation of the AmpliFluor primers in the absence of telomerase activity, and lastly, since telomerase is a heat-sensitive enzyme, a heat-treated telomerase negative control was also included so as to evaluate each sample for heat sensitivity. Thereafter, 10 µl of each 500 ng/µl sample was incubated at 85°C for 10 minutes prior to detection, in order to inactivate telomerase. All reactions were performed in triplicate in 96-well qPCRplates. The mastermix contained the following: OneTaq® HotStart Taq Polymerase (5 U/µl), nuclease-free water and the TRAPEze RT reaction mix. All samples were analysed via qPCR with the CFX Maestro™ thermo cycler with the following cycling parameters applied: One cycle of 37°C for 30 minutes, 95°C for 2 minutes and 45 cycles of 95°C for 15 seconds, 59°C for 60 seconds and 45°C for 10 seconds. Telomerase activity was thereafter calculated from the standard curve generated by 1:10 serial dilutions (20–0.0002 amoles) of the provided TSR8 control template as per Merck Millipore instructions. Negative controls were included. The data was then analysed with CFX Maestro™ software version 1.0. All values were normalised against the negative controls, whereby, all negative control values (including signals for CHAPS only, no template control and
heat-treated, respectively) were subtracted from the signal of each sample and thereafter, the mean value calculated for all biological repeats.

**Proteome Profiler Antibody Arrays™**

In order to determine whether proteins involved in the apoptotic and MEK/ERK signalling pathways are affected post siRNA-mediated down-regulation of LRP, Proteome profiler antibody arrays were performed as per the supplier's instructions (R&D systems). A total of 750 000 DLD-1 cells were seeded per well in 6-well tissue culture plates prior to transfection for 72 hours. The cells were harvested, and cell counts were performed. Thereafter, 300-600 µl of cold lysis buffer (the lysis buffer for the apoptotic array contained a protease inhibitor cocktail) was added to each sample, followed by a 30-minute incubation at 4 °C. Each cell suspension was then centrifuged at 10 000 x g for 5 minutes where the resultant supernatant was used as the experimental protein lysate. A BCA assay was performed, each lysate was diluted, and 200-600 µg of protein was loaded on each membrane. Following this, each membrane was placed into the 8-well tray provided with each kit, followed by incubation with 1 ml of 1X Array buffer at room temperature for 1 hour, in order to ensure no non-specific binding to the membrane surface. The 1X Array buffer was then removed, and the membranes were incubated with 1 ml of protein sample overnight at 4ºC with gentle shaking. Thereafter, each membrane was placed into separate containers where they were washed three times with 20 ml 1X Wash Buffer for 10 minutes. Arrays were incubated with the detection antibody cocktail solution for 1 hour at room temperature and then washed three times with 1X Wash buffer. This was followed by incubation with 1X Streptavidin-HRP at room temperature for 30 minutes. The arrays were washed three times with washing buffer, and protein spots were visualized using the chemiluminescence detection reagents supplied in the Array Kits. The intensity score of each duplicated array spot was measured with the ImageLab (version 5.1) software and the average intensity was calculated by subtracting the averaged background signal and PBS spots (negative control). The identity and the respective coordinates of all the antibodies on the arrays can be found in the Supplementary Data section.

**Quantification of mRNA expression levels – Quantitative reverse transcriptase polymerase chain reaction (RT-qPCR)**

**RNA Extraction**

A total of 750 000 DLD-1 cells were seeded per well in 6-well tissue culture plates prior to transfection for 72 hours. To determine mRNA expression levels, RNA was first extracted from the DLD-1 samples. The extraction procedure followed the Quick-RNA™ MiniPrep kit (manufacturer’s protocol. Briefly, cells were trypsinized and collected by centrifugation. The cell pellets were then resuspended and lysed in the provided lysis buffer, followed by centrifugation. Thereafter, the supernatant containing the RNA was further purified by incubating the samples in DNase buffer for 15 minutes in order to eliminate any trace
contaminants of DNA. The samples were then loaded onto spin columns specific for RNA extraction and washed twice using 400 µl wash buffer. Lastly, the purified RNA samples were eluted using 80 µl nuclease-free water. The resultant sample concentrations were determined using Nanodrop spectroscopy (Nanodrop® ND-1000).

Thereafter, a 1% (w/v) agarose gel containing a nucleic acid gel stain was used for gel electrophoresis (agarose, in 1 x TBE buffer consisting of Tris, boric acid and EDTA) to evaluate the RNA integrity. The samples were loaded with 6 x loading dye and Low range DNA ladder was used as a molecular weight marker. The DNA was separated at 100 V for approximately 30 min in TBE buffer. The RNA was visualized using ChemiDoc™ system in order to view total/intact RNA as well as any DNA contamination (Refer to Supplementary data section).

**cDNA synthesis**

Once the absence of DNA contamination was confirmed, the samples were converted into cDNA. The SensiFAST™ cDNA synthesis kit was used as per the manufacturers instructions whereby a mastermix was prepared containing appropriate volumes of: 5X TransAmp buffer, reverse transcriptase and nuclease-free water. Thereafter, 1 µg of mRNA was added to each PCR tube prepared. A no reverse transcriptase control was also prepared for each sample, containing each reagent except reverse transcriptase. Thereafter, the samples were analyzed via qPCR using the CFX Maestro™ thermo cycler with the following parameters: One cycle of 25°C (primer annealing) for 10 minutes, one cycle of 42°C (reverse transcription) for 15 minutes, one cycle of 48°C (additional reverse transcription for highly structured RNA) for 15 minutes and one cycle of 85°C (inactivation) for 5 minutes. The resultant samples were stored at -20°C for mRNA quantification.

**mRNA expression level quantification - Quantitative polymerase chain reaction (qPCR)**

In order to quantify mRNA expression levels, qPCR was performed using the SensiFAST SYBR™ No-ROX kit as per the manufacturers protocol whereby mastermix was prepared containing: 2X SensiFAST SYBR™ No-ROX mix, 10 µM of the forward primer (refer to the Supplementary data section), 10 µM of the reverse primer (refer to the Supplementary data section), nuclease-free water and lastly, 1 µg of cDNA. This mixture was pipetted into the necessary wells of a 96-well qPCR plate, ensuring that the mixture was thoroughly combined. Thereafter, the samples were placed in the CFX Maestro™ thermo cycler with the following parameters: One cycle at 95°C for 3 minutes, 45 cycles at 95°C for 15 seconds, one cycle T_m of gene for 30 seconds, melt curve: one cycle at 95°C for 10 seconds, 65°C for 1 minute and one cycle at 72°C for 30 seconds. Refer to the the Supplementary data section for the melt peaks of each gene.

Thereafter, the average C_q value from technical repeats was used for calculations. In addition, to acquire reliable comparisons of gene expression levels between samples, corresponding qPCR reactions must be performed for reference genes known have invariant expression. The reference genes used in this study included *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) and *ACTB* (β-actin). These reference
genes were used since these genes are present in all cells. Moreover, the reference genes were used because when tested, the mRNA levels of these genes were not affected between each treatment (refer to supplementary data section). Normalisation with stably expressed reference genes as internal controls, known as the comparative C\textsubscript{q} or the \(\Delta\Delta\text{C}_{q}\) method, was used for the normalisation of mRNA data. In addition, reverse transcriptase controls (RTC’s) were subtracted from all samples for further normalisation. The comparative C\textsubscript{q} method normalises the C\textsubscript{q} value of a target gene to internal reference genes before comparisons are made between samples. First, the difference between C\textsubscript{q} values ( \(\Delta\text{C}_{q}\) ) of the target gene and the mean of two reference genes was calculated for each sample, and then the difference in the \(\Delta\text{C}_{q}\) ( \(\Delta\Delta\text{C}_{q}\) ) was calculated between samples. The fold-change in expression of the two samples was calculated as \(2^{-\Delta\Delta\text{C}_{q}}\), where 2 derives from 1 + efficiency and efficiency is assumed to be 1 (i.e., 100% efficiency) [45]. Refer to the Supplementary data section for primer sequences, qPCR melt curves and peaks for each gene.

Sequential Window Acquisition of All Theoretical Mass Spectra (SWATH MS)

Sample preparation

A total of 750 000 DLD-1 cells were seeded per well in 6-well tissue culture plates prior to transfection for 72 hours. The cells were then harvested and centrifuged at 1500 x g for 10 minutes. Cell pellets were resuspended in 200 ul of lysis buffer [1% SDS, 100 mM Tris-HCl, pH 8.0, MS grade H\textsubscript{2}O] per pellet. Thereafter, cells were sonicated on ice for 9 pulses (10 sec per pulse) followed by centrifugation at 15 000 x g for 10 minutes to clear cell debris. Cell lysates were then incubated with 25 units, (1ul of stock –2500 units in 100ul) of benzonase, per 0.5 million cells and at 2mM MgCl\textsubscript{2} at 37°C for 30 minutes. This was followed by another centrifugation at 15 000 x g for 10 minutes. The supernatant was collected, and the concentration determined using a BCA assay. Protein solutions were then reduced using 10 mM DTT for 30 minutes at 37°C and alkylated using 40 mM IAA for 30 minutes in the dark.

Sample clean-up and digestion

All experiments were performed with a KingFisher™ Flex magnetic particle processing robot. The Fisher™ Flex system was configured for automated HILIC-protein clean-up and on-bead trypsin digest. Deep-well 96-plates were loaded in each carousel position with each plate filled as follows: 1) 96 well tip heads; 2) 10 µl, 20mg/ml hyper porous magnetic HILIC micro spheres in 20% ethanol and 180 µl equilibration buffer (100 mM NH\textsubscript{4}Ac, 15% ACN pH 4.5); 3) equilibration Buffer (500 µl); 4) Protein extract mixed 1:1 with bind buffer (200 mM NH\textsubscript{4}Ac, 30% ACN pH 4.5), final volume of 100 µl; 5) 500 µl 95% ACN (wash 1); 6) 500 ul 95% ACN (wash 2); 8) 200 µl 50 mM ammonium formate pH 8.2 and Promega sequencing grade trypsin for an enzyme:protein ratio of 1:10. The Bindit programme was then run with the magnetic pins transferring the magnetic HILIC beads from position 2 to 8 and in the process binding proteins,
washing off SDS and other contaminants and finally generating peptides ready for LC-MSMS analysis post the on-bead trypsin digest.

**Spectral library building (Data-dependent analysis)**

Post HILIC peptide samples were vacuum dried, resuspended in 2% ACN/0.2%FA and spiked with iRT peptide standards. Three injections were then performed per sample for each of the conditions. Analysis was performed using a Dionex Ultimate 3000 RSLC system coupled to an AB Sciex 6600 TipleTOF mass spectrometer. Peptides were first de-salted on an Acclaim PepMap C18 trap column (75 µm × 2 cm) for 5.5 min at 5 µl/min using 2% acetonitrile/0.2% formic acid, than separated on Acclain PepMap C18 nanoRSLC column (75 µm × 15 cm, 2 µm particle size) using a 60 min linear gradient at a flow-rate of 0.5 µl/min (A: 0.1% formic acid; B: 80% acetonitrile/0.1% formic acid). An electrospray voltage of 2.5 kV was applied to the fused silica emitter (New Objective: 20 µm ID x 5 cm, 10 µm tip). The 6600 TipleTOF mass spectrometer was operated in Data Dependant Acquisition mode. Precursor MS scans were acquired from m/z 400-1500 using an accumulation time of 250 ms followed by 80 MSMS scans, acquired from m/z 100-1800 at 25 ms each, for a total scan time of 2.3 sec. Multiply charge ions (2+ - 5+, 400 -1500 m/z) were automatically fragmented in Q2 collision cells using nitrogen as the collision gas.

**SWATH-MS analysis**

Three injections, representing each of the technical replicates, were performed per sample for each of the conditions. For SWATH-MS, the LC gradient used for spectral library building was applied. The SWATH-MS method consisted of the acquisition of 100 MS2 scans of overlapping sequential precursor isolation windows (variable m/z isolation width, 1 m/z overlap, high sensitivity mode) covering the 400 to 900 m/z mass range, as well as a single MS1 scan. The accumulation time was 300 ms for the MS1 scan and 25 ms for the MS2 scans for a total of 2.3 s total cycle time.

**Protein identification and spectral library building**

Raw data were searched against the human UNIPROT sequence database (reviewed entries, downloaded on 2 June 2017) supplemented with a list of common contaminating proteins as well as the sequences of the Biognosys iRT peptide retention time standards. Thereafter, data processing was performed using Protein Pilot (v 5.0.1). The following search settings were applied: trypsin as the proteolytic enzyme, IAA based alkylation, thorough search effort. False discovery rate (FDR) analysis was then performed with 1% global FDR cut-off applied at PSM, peptide and protein levels. A spectral library was constructed by importing the .group Protein Pilot output into the Skyline (v 4.1.1.18179) spectral library builder. A cut-off of 0.995, corresponding to 1% peptide FDR, was applied during import. The Biognosys iRT peptides were appended to the library in order to normalize the peptide retention time. The following filters were applied for peptide and protein import into Skyline: Tryptic peptide, size 5-36 amino acids, with up-to 1miss-cleavage and one matched cleavage site allowed; Structural modifications: Carbomethoxymethyl (Cys), Oxidation (Met), Acetylation (N-terminal); Precursor charge states 2-4, product charge states 1-2, product
ions: b and y; Ion match tolerance of 0.1 m/z. Post protein, peptide and transition import into Skyline a
decoy peptide list was generated by shuffling the sequences of all imported peptides.

**SWATH-MS processing**

SWATH data files were converted to mzML format as well as centroided using the Proteo Wizard MS Convert tool. The converted SWATH mzML files were imported into Skyline (v 4.1.1.18179). The following filters were applied for SWATH mzML import: Precursor charge states 2-4, product charge states 1-2, product ions: b and y; 3-6 transitions per peptide; Product m/z range 100-1800; MS1 filtering: Isotope peaks included (Count), Precursor mass analyzer (Centroid), Peaks (3), Mass accuracy (20 ppm); MS2 filtering: Acquisition method (DIA), Product mass analyzer (Centroid), Mass Accuracy (20 ppm) and an isolation scheme of 80 variable windows as per the SWATH method run in Analyst; For retention time filtering only scans within 10 min of the predicted iRT retention times were selected. Once all SWATH mzML files were imported all repeated peptides and proteins were removed and a peak scoring model was trained using mProphet and the decoy peptides generated during spectral library building. All peaks were then re-integrated using this model and only peptides with q-values of less than or equal to 0.01 were used for further processing.

**Selection of differentially expressed proteins**

Differentially expressed proteins were detected via the Skyline external tool, MS Stats. Thereafter, the MS Stats output list of differentially expressed proteins was further filtered so that only entries fitting the following criteria remained: Minimum fold change ≥ 2 and Maximum adjusted p-value ≤ 0.01.

**Statistical evaluation**

A one-way ANOVA followed by a two-tailed student’s t-test with a confidence interval of 95% was used as a means of analysing and confirming the data. To further validate the data, the Bonferroni post-hoc test was applied, with p-values of less than 0.05 considered to be significant. The statistical analysis was performed using the Microsoft® Excel 365 statistical programme.

**Results**

Successful knock-down of LRP/LR was performed as per Vania et al (2018) and confirmed before subsequent experiments (see figs. S1-S3) [46]. Moreover, due to LRP/LR's role in cell viability maintenance, cell cycle analysis was performed in order to obtain insight into the effect of LRP/LR knock-down as well as to confirm the occurrence of apoptosis. Upon Human-RPSA transfection of DLD-1 cells for 3-days, there was a significant 15% increase in the number of dead cells in the sub G0/G1 stage (apoptotic stage) and 17.4% decrease in G0/G1 phase, indicating the occurrence of apoptosis (Fig.S4).

Although this result was significant, it was not a substantial increase. It is known that apoptosis is a time-dependent process where the occurrence of apoptotic biochemical alterations to cells may depend on several internal cellular factors, where some alterations may take longer than others to show an effect.
Thus, it was decided to extend the transfection period by 48 hours, for a total of 5 days to ensure that the period of apoptotic activity was not missed. Upon transfection of DLD-1 cells with Human-RPSA for 5 days, there was a significant 41% increase in the number of dead cells in the sub G0/G1 stage with a 43% decrease in G0/G1 phase, indicating a higher percentage of cells undergoing apoptosis (Fig. S4b). It is noteworthy that there was no cell cycle arrest in any other particular phase of the cell cycle. esiRNA-RLUC and PCA acted as the negative and positive controls, respectively (see supplementary data section).

**siRNA-mediated knock-down significantly affects telomere-related proteins in late stage colorectal cancer cells.**

As mentioned previously, a study performed by Shay and Bacchetti (1997) showed that there is increased telomerase activity in approximately 90% of malignant human tumours [33]. Moreover, an increased level of hTERT, is found to increase telomerase activity and as a result, enhance tumourigenesis [30]. Additionally, it has also been shown that telomere dysfunction and elevated levels of telomerase activity are associated with cancer recurrence and chemotherapeutic resistance [48], outlining the important role that telomerase has in cancer progression. More particularly to this study, it has been found that LRP/LR and telomerase co-localize in the perinuclear compartment of breast (MDA-MB-231) cancer cells and that down-regulation of LRP/LR through siRNA technology, significantly reduced telomerase activity in these cells [30]. In addition, the phosphorylation of TERT at Serine residue 824 via Akt kinase is a pre-requisite for telomerase activity to occur [49] and has been found to correlate to increased telomerase activity, thereby aiding in cancer progression [50]. Taken together, these factors incited the question of whether siRNA-mediated knock-down of LRP/LR will influence hTERT and pTERT protein expression levels as well as telomerase activity in DLD-1 cells.

It was found that once the DLD-1 cells were transfected with the Human-RPSA siRNA, hTERT mRNA and protein expression levels were significantly decreased by 0.5-fold (Fig. 1a) and 0.4-fold (Figs. 1b and 1c), respectively, when compared to non-transfected cells. Moreover, siRNA-mediated knock-down of LRP/LR resulted in a significant decrease of 0.8-fold in telomerase activity, (Fig. 1d) and a significant 0.6-fold decrease in pTERT protein expression levels, when compared to non-transfected cells (Figs. 1e and1f).

As mentioned previously, TRF-1 and TRF-2, together with four other proteins, make up the complex known as “shelterin” which has a high specificity for telomeres [41]. Moreover, telomere sequences are found to be directly bound to TRF-1 and TRF-2 where both proteins are found to protect and regulate telomere length [42]. Since these proteins play a role in telomerase and cancer, this prompted investigations on how siRNA-mediated knock-down of LRP/LR would affect these telomere-related proteins. When DLD-1 cells were transfected with the Human-RPSA siRNA, it was found that the protein expression levels of TRF-1 was unaffected (Figs. 1g and 1h), while TRF-2 levels were significantly decreased by 0.5-fold (Figs. 1i and1j), in comparison to non-transfected cells.
siRNA-mediated knock-down of LRP/LR results in the significant increase of several proteins of the MAPK and apoptotic pathways in late stage colorectal cancer cells.

The mitogen-activated protein kinase (MAPK) pathway consists of serine/threonine kinases which link several extracellular signals to cellular processes involved in gene expression, apoptosis, differentiation, proliferation and migration [51]. Thus, it comes as no surprise that mutations in the signalling of the MAPK pathway may play key roles in developing cancer. In addition, a study performed by Sun et al (2014) showed that the LRP/LR-laminin-1 interaction allows for LRP/LR’s interaction with FAK, leading to the activation of the MAPK/ERK 1/2 cellular survival pathway as well as an increased expression of the anti-apoptotic Bcl-2 protein [52]. Thus, investigations on whether LRP/LR influences proteins involved in the MAPK signalling pathway as well as apoptotic pathways were prompted. Proteome Profiler Antibody Arrays™ were used to analyse these pathways.

A Human phospho-MAPK array kit containing 43 kinase antibody capture sites was first used to determine the role of LRP/LR in the MAPK pathway. Upon siRNA-mediated knock-down of LRP/LR, densitometric analysis revealed that 9 proteins of the phospho-MAPK pathway were affected however, only 3 of these proteins, Chk-2, β-catenin and AMPKα2, were found to be significant. The remaining 6 proteins including FAK, CREB, AMPKα1 as well as three phosphorylation sites of p53 (Fig. 2a) revealed changes, however upon statistical analysis, these changes were found to be non-significant. In order to confirm the results from this profiler array, a second MAPK array kit was performed, containing 26 MAPK antibody capture sites. It displayed consistent results with the phospho-MAPK array in that CREB and p53 were once again found to be affected (Fig. 2a and 2b). However, the change in these proteins was also found to be non-significant.

To confirm that LRP/LR plays a role in cell survival through evasion of apoptosis, Human Apoptosis Proteome Profiler Antibody Arrays™ were performed which contained 35 capture antibody sites. It was found that upon siRNA-mediated knock-down of LRP/LR, 11 proteins were affected. Several pro-apoptotic proteins including Bax, cleaved caspase-3 and p53 phosphorylation sites were significantly up-regulated, while anti-apoptotic proteins such as claspin, cIAP1, XIAP and Survivin were significantly down-regulated (Fig. 2c). Cytochrome C and HIF-1α proteins were also affected by siRNA-mediated knock-down of LRP/LR however these changes were found to be non-significant upon densitometric analysis. Several of the proteins found confirmed the results of the MAPK Proteome Profiler Arrays™. Moreover, it was found that majority of the proteins affected in the array play roles in the intrinsic mitochondrial pathway of apoptosis. The membrane arrays as well as the identity and the respective coordinates of all the antibodies on the arrays can be found in the Supplementary Data section (Figs. S11-S14).

siRNA-mediated LRP/LR reveals significant changes in relative mRNA expression levels of specific genes in late stage colorectal carcinoma cells.
The Proteome Profiler Antibody Arrays™ showed favourable results in illustrating that LRP/LR influences proteins involved in the MAPK pathway as well as the apoptotic pathways. However, similar to western blotting, Proteome Profiler Antibody Arrays™ only provide semi-quantitative data. Thus, to confirm the results obtained from the arrays, specific proteins namely: p53, Bcl-2, Bax, and CREB, were chosen for relative mRNA expression level analysis upon siRNA-mediated knock-down of LRP/LR.

It was found that upon siRNA-mediated knock-down of LRP/LR, relative mRNA expression levels of the tumour suppressor gene, p53 and the pro-apoptotic protein, Bax significantly increased by 1.7-fold and 4-fold, respectively, when compared to non-transfected cells (Fig. 3a and b), confirming both the MAPK and Apoptotic Proteome Profiler Antibody Arrays™ (Fig. 2). In addition, siRNA-mediated down-regulation resulted in a decrease in relative mRNA expression levels of the anti-apoptotic protein Bcl-2 in comparison to non-transfected cells (Fig. 3c). Interestingly, the CREB protein was found to be over-expressed in cells transfected with the Human-RPSA siRNA in the Apoptotic Proteome Profiler Array™, thus investigations on whether its mRNA expression level was similarly affected were incited. Upon siRNA-mediated knock-down of LRP/LR, it was found that the relative mRNA expression level of CREB was significantly increased by 4-fold compared to non-transfected cells (Fig. 3d) thus, confirming the results from the array (Figs. 2a and 2b). Refer to the Supplementary data section for primer sequences, qPCR melt curves and peaks for each gene.

siRNA-mediated knock-down of LRP expression significantly changes the proteome of late stage colorectal cancer cells.

Although a significant change in various proteins was observed in the Proteome Profiler Antibody Arrays™ upon siRNA-mediated knock-down of LRP/LR, the data obtained provided only semi-quantitative results. Sequential Window Acquisition of all Theoretical fragment ion spectra Mass Spectrometry (SWATH-MS) was used to quantify proteins associated with LRP/LR. SWATH-MS is a modified method of data-independent analysis (DDA) MS which integrates quantitative accuracy and consistency together with the ability for deep proteome coverage on a large scale [53]. As a result, this allowed for novel screening of proteins affected by siRNA-mediated down-regulation of LRP/LR, enabling insight into the mechanism by which LRP/LR exerts its effects. In order to exclude the effects of the transfection reagent, the negative control esiRNA-RLUC-transfected cells (rather than only the non-transfected) and the Human-RPSA siRNA-transfected samples were compared to one another. Additionally, the non-transfected samples were compared to Human-RPSA siRNA-transfected samples as well as mock-transfected to obtain more insight into the DLD-1 cells (Refer to Fig. S9).

Several proteins were found to be differentially expressed (Fig. 4a and 4b) and from these proteins, only those that had a 2-fold increase or greater together with a p-value < than 0.05 were considered significant. It has been found that statistical significance is increased for proteins with larger fold changes (i.e. 2 and above) and relatively larger sample variances, thus eliminating false positives [54]. In saying that, several future experiments must be performed to investigate and confirm the proteins found to be significantly
affected upon LRP/LR down-regulation in DLD-1 cells. As shown in the graphs below, there is a significant change in the proteome of late stage colorectal cancer cells when transfected with Human-RPSA siRNA. Upon analysis between the Human-RPSA siRNA transfected samples and the esiRNA-RLUC-transfected samples, it was found that 40 proteins were down-regulated while 44 proteins were up-regulated (Fig. 4a). Moreover, a similar result was found when the Human-RPSA siRNA-transfected samples were compared to the non-transfected samples (Fig. S9), confirming the data. Additionally, when analysing the functions of each of these proteins, most were found to play roles such as: ribosomal processing and protein synthesis [3]; nucleus and chromatin maintenance [4]; cell proliferation and differentiation [55]; cell anchorage and cytoskeletal involvement [5] and lastly, apoptotic regulation [30] (See Tables S4-12 for grouped proteins). In addition, other proteins identified were found to be involved in vesicle transportation and regulation as well as autophagy. Moreover, novel stand-alone proteins have been found to be influenced by LRP/LR, which could provide additional information on how the receptor works. Observations from this technique suggest that several proteins involved in suppressing tumourigenesis were up-regulated, while proteins involved in promoting tumourigenesis were down-regulated proposing that knock-down of LRP/LR could be a potential anti-cancer therapeutic tool.

Discussion

Tumourigenic cells are characteristically found to overexpress the 37kDa/67kDa laminin receptor (LRP/LR) compared to their normal cell counterparts. This receptor is involved in promoting several tumourigenic processes such as cell migration and adhesion, cellular viability maintenance as well as apoptotic evasion. We have previously shown that the knock-down of LRP/LR via siRNA technology significantly reduced the viability of early (SW-480) and late (DLD-1) stage colorectal cancer cells through the induction of apoptosis, evident by decreased membrane integrity and caspase-3 activation [46]. The current study further reveals that the knock-down of LRP/LR results in the significant decrease in LRP/LR mRNA expression levels (Fig. S3) as well as increases sub G0/G1 apoptotic levels, further confirming successful down-regulation and the occurrence of apoptosis via siRNA technology (Fig. S4). The apoptotic induction observed is suggested to be due to the reduced interaction of LRP/LR with focal adhesion kinase (FAK) [52, 56]. In particular, a study performed by Lu et al (2016) demonstrated that siRNA-mediated knock-down of LRP/LR reduced FAK phosphorylation, leading to the knock-down of the anti-apoptotic protein Bcl-2 and an up-regulation of the pro-apoptotic Bax protein, reiterating that the knock-down of LRP/LR induces apoptosis [56]. Moreover, another study performed by Sun et al (2014) showed that the LRP/LR-laminin-1 interaction may allow for LRP/LR's interaction with FAK, leading to the activation of the PI3-kinase/AKT and MEK/ERK 1/2 cellular survival pathways as well as an increased expression of the Bcl-2 protein [52]. Both the MEK/ERK 1/2 and PI3-kinase/AKT cell signalling cascades are found to inhibit the effector caspase-8 (extrinsic) and -9 (intrinsic) apoptotic pathways.

Thus, to understand the mechanistic role of LRP/LR in proliferation and apoptosis, pathway analysis was performed on the DLD-1 cells after knock-down of LRP/LR. Firstly, LRP/LR's involvement in telomerase and telomere biology was investigated. When telomeres shorten to a critical length, the dysfunctional telomere is recognised as damaged DNA and cellular senescence is triggered [57]. Cells can remain in this
senescent state for years, provided no other changes occur and as such, this is considered to be a tumour suppressor mechanism in humans. However, cancerous cells are found to overcome inhibitory pathways induced by DNA damage, as well as senescence, through the upregulation of telomerase activity [58]. This leads to the maintenance of these unstable telomeres, resulting in the formation of cancer [58]. Upon siRNA-mediated knock-down of LRP/LR, not only was telomerase activity inhibited but hTERT, phospho-TERT and TRF-2 protein expression levels were also decreased (Fig. 1). In addition, a high and positive correlation of $R=0.91$ was found between pTERT protein expression levels and telomerase activity post knock-down with the Human-RPSA siRNA. These findings suggest that LRP/LR does indeed influence both hTERT and pTERT expression levels, and subsequently telomerase activity.

In addition, LRP/LR could be linked to TRF-2 through protein interactions with TERT and p53. In this regard, p53 has been found to influence the expression of both TERT and LRP via transcriptional repression in order to limit proliferation [59, 60]. Additionally, p53 has also been found to affect TERT, telomerase activity as well as TRF-2 for apoptotic induction or cell cycle arrest [61]. Consequently, a decrease in LRP/LR, TERT and TRF-2 (which all share an antagonistic relationship with p53) may allow the elevation of p53 and subsequent induction of apoptosis in the cancerous cells. Furthermore, as both LRP and TERT have been intricately linked to proliferation pathways like the MAPK pathway [62, 63], knock-down of LRP and subsequently TERT and TRF-2, could severely impact the aggressive and rapid proliferation observed in cancer cells. Thus, by targeting LRP/LR with siRNA technology, its consequent effects on TERT/telomerase and TRF-2, will compromise multiple key cancer hallmarks including invasion, apoptotic and growth suppressor evasion as well as replicative immortality.

The proteomics data obtained upon knock-down of LRP/LR in DLD-1 cells via siRNA technology identified several proteins that have novel interactions with LRP/LR linking LRP/LR to other processes thereby, exposing novel roles for the receptor (Figs. 2 and 4). In addition, several identified proteins also confirmed the receptor’s role in the various processes it is known to be involved in including ribosomal biogenesis and translation; cytoskeletal maintenance and cell anchorage; as well as nuclear and chromatin maintenance. The data also revealed that LRP/LR does indeed play a role in the MAPK and apoptotic pathways (Fig. 4). However, there have also been several proteins identified where their interaction with LRP/LR is not easily explained, highlighting the need for further experiments to confirm many of these interactions.

The MAPK pathway regulates various downstream molecules including transcription and translation factors, kinases and cell cycle molecules [51]. In particular, one such protein is focal adhesion kinase (FAK) where upon densitometric analysis, was shown to be down-regulated compared to non-transfected cells (Fig. 2a). FAK is a non-receptor tyrosine kinase that associates with several receptors including growth factor receptors and G-protein coupled receptors as well as integrins, ultimately leading to its activation [64]. FAK is found to play crucial roles in regulating cell proliferation, adhesion and survival in several cell types thus, it is said that FAK is the connection of several signalling pathways. As mentioned previously, it was found that the LRP/LR-laminin-1 interaction allowed for FAK and LRP/LR to interact, thereby activating the MAPK signalling pathway and the anti-apoptotic Bcl-2 protein, ultimately leading to
apoptotic inhibition [52]. Since FAK was down-regulated upon siRNA-mediated knock-down of LRP/LR, this shows that these results are consistent with findings of Sun et al (2014) and confirms the relationship between FAK and LRP/LR [52]. In addition, it was also found that the knock-down of LRP/LR decreased the mRNA expression levels of Bcl-2, further confirming the results of the previous study (Fig. 3c) [52].

Another interesting protein linking LRP/LR to the MAPK pathway was the CREB (cyclic AMP-response element binding protein) that was found to be up-regulated (Figs. 2 a and 2b). To confirm this result, qPCR was performed, and it was found that upon siRNA-mediated knock-down of LRP, there was a significant 4-fold increase in mRNA expression levels when compared to non-transfected cells (Fig. 3d). This protein normally functions as a transcription factor that regulates various processes including cell growth and survival by binding to cAMP response elements (CRE) sequences [65]. CREB has also been implicated in genotoxic stressed-induced transcription as a result of DNA damage and oxidative stress [65]. These stressors activate the phosphorylation of CREB which result in its further phosphorylation by ATM kinase. ATM-mediated phosphorylation of CREB leads to the recruitment of its cofactor CREB-binding protein (CBP)/p300 to initiate transcription [66]. For full transcriptional activity of the p53 tumour suppressor, the coactivator CBP/p300 is required to bind to p53 [67]. The binding of these proteins is facilitated by phosphorylated CREB [66]. Moreover, Chk-2-mediated phosphorylation (a protein also found to be significantly up-regulated upon LRP/LR knock-down) of p53 stabilizes the binding of CBP/p300 to p53 [68]. This results in p53 transactivating a number of proteins that may be used for apoptotic induction. The findings of the current study suggest that LRP/LR may link CREB, CBP/p300, Chk-2 and p53 since a decrease in LRP/LR expression levels results in each of these proteins aiding in apoptotic induction.

Furthermore, analysis of the SWATH-MS data revealed that upon siRNA-mediated knock-down of LRP/LR, the AMP-activated protein kinase catalytic subunits α1 and 2 (AMPKα1 and 2) were both up-regulated, when compared to esiRNA-RLUC-transfected cells (Fig. 4). AMPK is known to primarily function in regulating energy and metabolism homeostasis [69]. The protein is activated upon stressors such as nutrient depletion and hypoxia, resulting in it also being a regulator of autophagy. Moreover, the loss of AMPK has been shown to promote cell proliferation through the increased signalling of the MAPK pathway. Interestingly, a study performed by Choi et al (2015) revealed that CREB binds to AMPKα in order for the transactivation of p53 in response to energetic stress [69]. Furthermore, it was found that by down-regulating CREB via siRNA technology, p53 was significantly down-regulated together with a substantial decrease in AMPKα [69]. The authors therefore proposed that the interaction between AMPKα and CREB facilitates the regulation of transcriptional p53 and also contributes to the activation of p53-mediated apoptosis. Taken together with the results from the current study where CREB was found to be up-regulated (Figs. 2 and 3d) upon knock-down of LRP/LR, it is suggested that the CREB-AMPKα association additionally facilitates p53-mediated apoptosis induced by the Human-RPSA siRNA.

SWATH-MS also demonstrated how the DLD-1 colorectal cancer cells responded to a decrease in the levels of the receptor, in that several enzymatic proteins involved in metabolism were also affected by
LRP/LR down-regulation. This could most likely be due to the tumour cells adapting in response to stress by undergoing the above-mentioned process known as autophagy. Cancer cells have been found to undergo autophagy or type II programmed cell death (PCD) to degrade damaged or unessential cell constituents. This degradation serves as an alternative energy source to sustain survival [70]. The activation of autophagy is associated with stress such as low nutrient levels, DNA damage, oxidative stress and inflammation [71]. The products from the tumour cells undergoing autophagy include macromolecules and fatty acids that are able to be redirected to new metabolic pathways so that tumour cell viability can be sustained [72]. Studies have shown that autophagy and apoptosis can take place in the same cell in which autophagy precedes apoptosis [71].

Additionally, it must be noted that the protein with the largest fold-change of 8.2 was the Heterogeneous nuclear ribonucleoprotein A1 (HnRNPA1) and was found to be down-regulated after LRP knock-down, when compared to the esiRNA-RLUC-transfected cells from the SWATH-MS data (Table S4). HnRNPA1 is a ribonucleoprotein present in the nucleus and has been reported to play key roles in pre-mRNA processing which not only includes splicing and stabilizing the mRNA but also transporting and metabolising it, thus it is deemed a nucleocytoplasmic shuttling protein [73]. HnRNPA1 has also been interestingly found to play a role in telomerase and telomere biology [74]. Moreover, although HnRNPA1 normally functions in splicing and RNA processing, it is also able to be phosphorylated and cleaved upon stress, resulting apoptosis. In particular, Hermann et al. (2001) found that apoptotic induction resulted in the cleavage of HnRNPA1 and increased expression of caspase activity [75].

It is known that p53 primarily functions in the intrinsic apoptotic pathway however, there have been several studies showing that p53 is also able to play a central role in the extrinsic pathway of apoptosis [76]. This is due to p53 being able to activate the transcription of key proteins involved in both pathways including Bax of the intrinsic pathway and the Fas death receptor of the extrinsic pathway [76]. Moreover, cytochrome c release, which is normally an important incident in the intrinsic pathway, has also been found to occur in the extrinsic pathway [76]. From this study, the Apoptotic array showed that cytochrome c was increased in the DLD-1 cells upon siRNA-mediated knock-down of LRP/LR, when compared to non-transfected cells (Fig. 2c). Moreover, as mentioned previously, DLD-1 cells were also found to have significant increases in both caspase-8 and -9 upon siRNA-mediated knock-down of LRP/LR, when compared to non-transfected cells [27]. In the intrinsic pathway, cytochrome c release is a result of increased activation of Bax by stressed-induced p53, ultimately leading to the activation of caspase-9-mediated apoptosis. On the other hand, the extrinsic pathway releases cytochrome c by cleaving the pro-apoptotic Bid protein through caspase-8 [76]. The results from this study however show that upon siRNA-mediated knock-down of LRP/LR, Bax mRNA and protein expression levels are significantly increased (Figs. 2c and 3b), correlating to the increased expression levels of p53 on account of genotoxic stress. The large increases of p53 and Bax may therefore have stimulated the increased release of cytochrome c, resulting in caspase-9 cleaving caspase-3 – also shown to be significantly up-regulated in the array (Fig. 2c) – ultimately, activating mitochondrial intrinsic apoptosis. Moreover, the fact that caspase-8 was found to be increased but none of its death receptors were differentially expressed in the arrays (Fig. 2c), could be due to an amplification loop, where caspase-9 cleaves caspase-3, directly leading to caspase-8
activation [77]. On the other hand, activated caspase-8 is also found to cleave Bid which results in the release and activation of cytochrome c and caspase-9, respectively, ultimately leading to apoptotic induction through the intrinsic pathway [76].

Another protein that confirms that siRNA-mediated knock-down of LRP/LR results in apoptosis is Histone H2AX that was found to be up-regulated by a significant 5-fold, when compared to esiRNA-RLUC-transfected cells (Table S5). H2AX is a protein marker that becomes phosphorylated to γH2AX in response to DNA damage and double stranded breaks which mostly accumulate due to the dysfunction of telomeres [78]. γH2AX aids in recruiting proteins that contain phospho-specific interaction domains which as a result, help to obtain other proteins involved in DNA damage/repair. As previously mentioned, telomere dysfunction together with elevated levels of telomerase activity have been associated with cancer recurrence and chemotherapeutic resistance [48]. However, studies have shown that dysfunctional telomeres are able to activate a DNA damage response pathway involving γH2AX, ATM and p53 proteins, ultimately leading to cell cycle arrest and apoptosis of cancer cells [79]. In the current study, it has been found that siRNA-mediated knock-down of LRP/LR not only significantly decreases hTERT expression levels and telomerase activity but also increases the expression of pro-apoptotic protein Bax and the p53 tumour suppressor. The fact that the expression of the DNA damage marker H2AX is also increased further reiterates that DLD-1 cells undergo apoptosis upon LRP/LR knock-down. Moreover, this finding is also in accordance with Otgaar et al (2017) which found that an increase in LRP/LR expression significantly decreases γH2AX expression and increases in telomere length [14].

**Conclusions**

Thus, from the current study, it would seem that siRNA-mediated knock-down of LRP/LR induces cell death in the DLD-1 cells in the following way: The up-regulation of p53, CREB, AMPKα, H2AX, PDCD4, RS26 and Hif-1α proteins suggests that the Human-RPSA siRNA was able to induce DNA damage in the DLD-1 cells, possibly as a result of diminished expression levels of telomerase activity as well as hTERT and TRF-2. This led to p53 stimulating the significant up-regulation of Bax as well as BH3 pro-apoptotic protein BNIP1, resulting in an imbalance of the Bax/Bcl-2 ratio and possible dysfunction of the mitochondria. In addition, FAK and β-catenin were down-regulated as well as several anti-apoptotic proteins including Bcl-2, c-IAP, XIAP and Survivin. Consequently, Bax promoted the release of cytochrome c, resulting in caspase-9 activation and apoptosis. Complementing this pathway, was the activation of caspase-8, which may have aided the caspase-9 pathway in activating apoptosis by cleaving Bid, leading to the release of cytochrome c. Together, these findings show the important role that LRP/LR plays in maintaining cell viability and evading apoptosis. Moreover, the results reveal that siRNAs targeting LRP/LR as well as other proteins related to the receptor could be used as potential therapeutic tools for the treatment of early and late stage colorectal cancer cells. Although interesting responses were observed in the colorectal cancer cells, the exact mechanism of action of LRP/LR in tumourigenesis is far from being fully understood. The expression of these proteins as well as their genes should be studied at various time points post treatment to determine the exact sequence of events. Figure 5 below shows a
graphical representation of the possible apoptotic pathway induced by the Human-RPSA siRNA in DLD-1 cells.

**Abbreviations**

AD, Alzheimer’s Disease; AMPK, AMP-activated protein kinase; APS, Ammonium persulfate; ATM, Ataxia-telangiectasia mutated; Bax, BCL2 associated X protein; BCA, Bicinchoninic acid; Bcl-2, B-cell lymphoma protein 2; BH3, Bcl-2 homology-3; BNIP, BCL2 adenovirus E1B 19kDa interacting protein; BSA, Bovine serum albumin; Caspases, Cysteine-dependent aspartate-specific proteases; CDK, Cyclin-dependent kinase; cDNA, Complementary DNA; CHAPS, 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulphonate; CHK-2, Checkpoint kinase 2; cIAP1, Cellular inhibitor of apoptosis 1; CO₂, Carbon dioxide; CREB, Cyclic AMP-response element binding protein; DDA, Data-independent analysis; DMSO, Dimethyl sulfoxide; DNA, Deoxyribonucleic acid; DTT, Dithiothreitol; EDTA, Ethylenediaminetetraacetic acid; ERK, Extracellular signal-regulated kinase; FACS, Fluorescence Activated Cell Scanning; FAK, Focal adhesion kinase; FCCC, Fox Chase Centre; FDR, False discovery rate; FITC, Fluorescein isothiocyanate; FBS, Fetal bovine serum; HCl, Hydrochloric acid; HRP, Horseradish peroxidase; HNRNPA1, Heterogeneous nuclear ribonucleoprotein A1; IAA, Imidazoleacetic acid; IAP, Inhibitor of apoptosis; LRP/LR, Laminin receptor precursor/high-affinity laminin receptor; MAP, Mitogen activated protein; mRNA, Messenger RNA; mtDNA, mitochondrial DNA; PAGE, Polyacrylamide gel electrophoresis; PBS, Phosphate buffered saline; PCA, Protocatechuic acid; PCD, Programmed cell death; PDCD4, Programmed Cell Death Protein 4; PI, Propidium iodide; PI3K, Phosphoinositide-3 kinase; POT1, Protection of telomeres-1; qPCR, Quantitative real-time PCR; RAP-1, Repressor activator protein-1; RNA, Ribonucleic acid; RNase, Ribonuclease; RPMI, Roswell Park Memorial Institute; RPSA, Ribosomal protein SA; RLUC, Renilla luciferase; SDS, Sodium dodecyl sulphate; siRNA, Small interfering RNA; SWATH-MS, Sequential Window Acquisition of All Theoretical Mass Spectra; TBE, Tris-Boric acid-EDTA; TEMED, Tetramethylethylenediamine; TERT, Telomerase reverse transcriptase; TRF-1/2, Telomeric repeat-binding factor-1/2; Tris, 2-Amino-2-(hydroxymethyl)-1,3-propanediol; TSE, Transmissible Spongiform Encephalopathies; XIAP, X-linked mammalian inhibitor of apoptosis protein

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and materials**
All data generated or analysed during this study are included in this published article [and its supplementary information files].

**Competing interests**

The authors declare that they have no competing interests.

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**Author contributions**

S.F.T.W. conceptualized and designed the study. L.V performed experiments, analyzed the data and wrote manuscript. G.M., E.F. and S.F.T.W. edited the manuscript. All authors have read and approved this version of the manuscript and confirm that this is the case.

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