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Novel LRP/LR induced stem-cell-like cells to aid wound healing and regeneration.

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DISSERTATION

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Declaration

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Abstract

The 37/67kDa Laminin Receptor (LRP/LR) LR is a multifunctional cell surface receptor that maintains several survival processes. It has recently been found that there is a direct relationship between LRP/LR and the stem cell marker telomerase. Studies have shown that overexpression of FLAG tagged LRP increases hTERT levels and telomerase activity. Telomerase is a reverse transcriptase enzyme found in actively dividing cells whose core function involves telomere maintenance and elongation. The rate limiting component of telomerase, hTERT, is also often up-regulated in rapidly dividing cells to aid stem cell renewal and cell survival and its ectopic expression can induce epithelial to mesenchymal transition (EMT) resulting in stem cell like characteristics. In adults, the primary role of stem cells is to repair and regenerate tissue. Hence, this study aims to determine whether overexpressing LRP::FLAG and the subsequent increase in hTERT levels induces stem-cell-like characteristics and promotes repair and regeneration in MRC5 lung fibroblasts and HEK293 embryonic kidney cells. Cells were stably transfected with the pCIneo-moLRP-FLAG plasmid in order to induce LRP::FLAG overexpression. Post-transfection, an increase in hTERT and phospho-TERT protein levels was observed in both cell lines which is crucial in maintaining the self-renewal capacity of stem cells. Additionally, an increase in the levels of pluripotency stem cell markers involved in cell reprogramming and alkaline phosphatase activity was also observed for HEK293 after transfection. However, in MRC5 cells there was an insufficient expression of reprogramming factors but, a Cadherin switch indicative of EMT. Moreover, HEK293 cells overexpressing LRP::FLAG showed no significant changes in the protein levels of the pro-inflammatory cytokine NF-κB1 and an increase in the anti-inflammatory cytokine TGFβ1 which modulates wound healing. In turn, it led to an increase in the adhesion and migratory capacity of HEK293 cells. This data suggests that overexpressing LRP::FLAG induces EMT similar to that observed during induced cell reprogramming and could possibly promote wound healing by upregulating TERT and TGFβ1 protein levels resulting in stem-cell-like characteristics.

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α	alpha
β	beta
γ	gamma
K	kappa
µ	micro
n	nano
°	degrees

List of abbreviations

Aβ	Amyloid Beta
AD	Alzheimer's disease
ASC	Adult Stem Cells
BCA	Bicinchoninic Acid Assay
BSA	Bovine serum albumin
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulphonate
CNS	Central nervous system
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
ds	double stranded
ECM	Extracellular Matrix
EGF	Epidermal growth factors
EMT	Epithelial to Mesenchymal Transition
ERK	Extracellular signal-regulated kinase
ESC	Embryonic Stem Cells
FAK	Focal adhesion kinase
FBS	Foetal Bovine Serum
FGF	Fibroblast growth factors
FITC	Fluorescein isothiocyanate
GSC	Goosecoid
HEK293	Human Embryonic Kidney Cells
HRP	Horseradish Peroxidase

HSPG	Heparin sulphate proteoglycan
hTERT	Human Telomerase Reverse Transcriptase
hTERC	Human Telomerase RNA Component
IgG	Immunoglobulin G
IFN	Interferon
IL	Interleukin
iPSC	Induced Pluripotent Stem Cells
JNK	c-Jun N-terminal protein kinase
kDa	Kilo Dalton
LRP/LR	37 kDa Laminin Receptor Precursor/67 kDa High Affinity Laminin Receptor
MAPK	Mitogen-activated Protein Kinases
MMP	Matrix metalloproteases
MRC-5	Human normal Lung Fibroblast cells
MSC	Mesenchymal stem cells
NF-κB	Nuclear Factor kappa B
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PDGF	Platelet-derived growth factor
PrP^{SC}	Scrapie Prion Protein
PVDF	Polyvinylidene Fluoride
RIPA	Radioimmunoprecipitation
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulphate
siRNA	Short interfering RNA
ss	single stranded
t-loop	Telomere loop
TGFβ	Transforming Growth Factor beta
TMB	Tetra-methylbenzidine
TNF-α	Tumour necrosis factor-alpha
VEGF	Vascular Endothelial Growth Factor

1 Introduction

1.1 Wound healing

Wound healing is the dynamic process by which body tissue repairs injured or diseased tissue (Stadelman et al., 1998). It is an interactive process involving a coordinated cascade of biochemical events to repair the damaged tissue (Stadelman et al., 1998). This process is divided into three overlapping phases: an inflammatory response, proliferation leading to tissue formation, and tissue remodelling (Rieger et al., 2014) (Figure 1.1). The inflammatory phase firstly involves vascular responses characterised by blood clotting to stop bleeding. In addition, it is also characterised by the removal of cell debris or pathogens by cytokine release and phagocytosis which initiates the proliferative stage of wound healing (Stadelman et al., 1998). During the proliferative phase; collagen deposition, angiogenesis, epithelialization, granulation tissue formation and wound contraction are observed. Epithelisation by proliferation occurs to overlay the wound surface where new granulation tissue fills the wound area below the new epithelial cells. Once new tissue is formed within the wound, the maturation/remodelling phase begins. During this phase, collagen realignment and apoptosis of unneeded cells occurs to restore structural integrity and produce functional tissue (Stadelman et al., 1998) (Figure 1.1). The process of wound healing is strictly regulated by a complex signalling network involving matrix metalloproteases (MMPs), numerous growth factors, cytokines and chemokines (Gill and Parks, 2008; Werner and Grose, 2003). These include: epidermal growth factors (EGF), fibroblast growth factors (FGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), interleukins (IL) family, tumour necrosis factor-alpha (TNF-α) and transforming growth factor beta family (TGFβ) (Barrientos et al., 2008; Werner and Grose, 2003). Of particular importance to this study is TGFβ1 which is considered the most important regulator of wound healing and wound healing fate (Sporn et al., 1987). TGFβ1 is a wound-healing promoting factor secreted by monocytes/macrophages, endothelial cells, platelets and fibroblasts. It functions to regulate cell proliferation, differentiation and growth, as well as regulation of the expression and activation of other cytokines (Roberts et al., 1986); Sporn et al., 1987). It is believed to play key roles such as inducing: angiogenesis, the inflammatory response, granulation tissue formation, extra-cellular matrix deposition and remodelling through its effect on mesenchymal cells (Klass et al., 2008; Sporn et al., 1987). Another protein that plays a major role in wound healing is the transcription factor NF-κB. Similar to TGFβ1, Nf-κB also regulates the secretion of cytokines and growth factors for wound healing in addition to activating innate immune responses and MMP expression (Ambrozova et al., 2017). Moreover, aberrant expression of both TGFβ1 and Nf-κB has been linked to the formation of

keloids, hypertrophic scaring fibrosis and chronic wounds, (Kuilman et al., 2010; Roberts, et al., 1986; Rodier and Campisi, 2011; Sporn et al., 1987). The process of wound healing is very fragile and susceptible to interruption of the complex process leading to the formation of non-healing chronic wounds. Chronic wounds are defined as wounds that lack structural integrity or have failed to progress through a timely sequence of repair (Lazarus et al., 1994). The main factors involved in the development of chronic wounds include arterial insufficiencies, diabetes, weak immune system or mechanical pressure (Lazarus et al., 1994). These risk factors mostly affect aged individuals given that wound repair slows down as the body ages, and incidences of cardiovascular disease and diabetes, which increase the incidence of chronic wounds, also increase with age (Jabrink et al., 2017). Therefore, as the human population becomes progressively older worldwide, there is an urgent need to advance therapies used for the repair or regeneration of damaged tissue (Isobe et al., 2014). This has led to the development of a research field known as regenerative medicine.

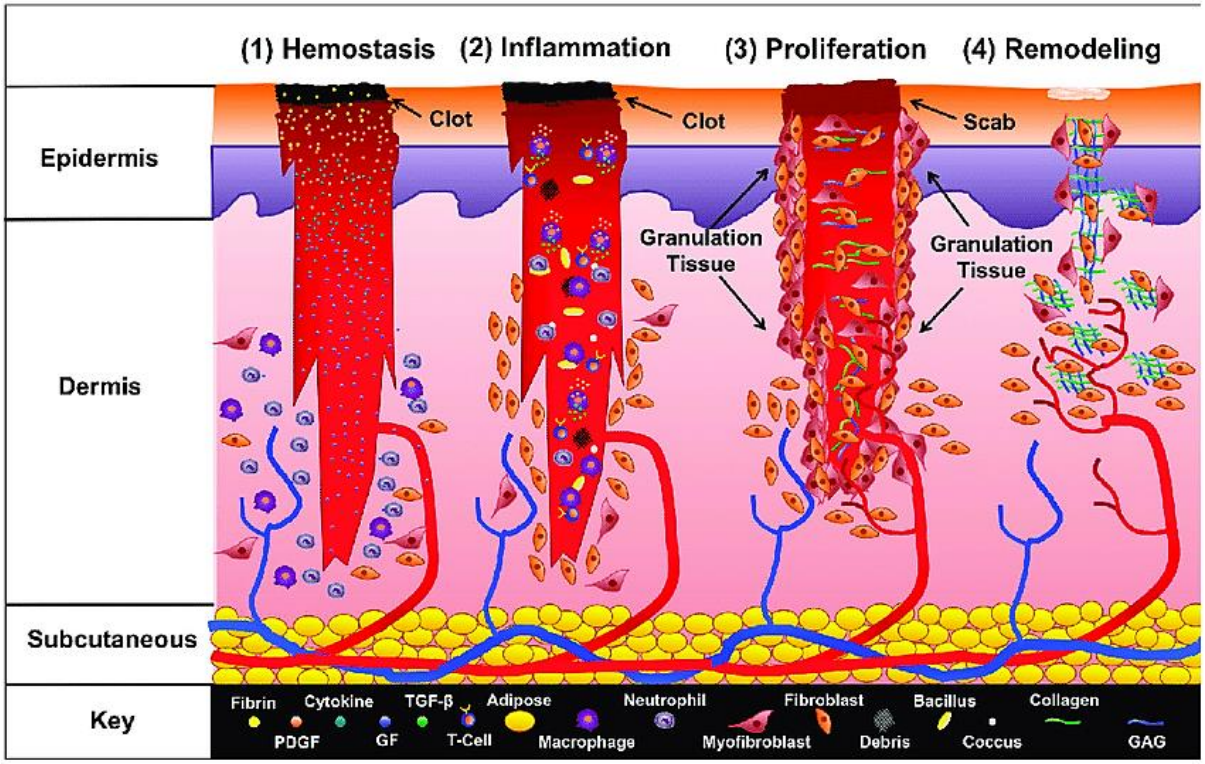


Figure 1.1: **Phases of cutaneous wound healing and key wound healing factors.** (1) During phase (1) haemostasis occurs in order to form a blood clot. Thereafter, the release of cytokines, growth factors and immune cells occurs in phase (2). In phase (3) proliferation occurs in order to form granulation tissue from fibroblasts and myofibroblasts. Lastly, phase (4) is characterised by tissue remodelling (Mellot et al., 2016).

1.2 Regenerative medicine

Regenerative medicine is focused on restoring normal physiological function by replacing or regenerating damaged tissues and cells in patients (Lysaght and Crager, 2009). Loss of physiological function has been mainly attributed to the dying off or malfunctioning of specialised cells which is usually observed in injured, diseased or aged individuals (López-Otín et al., 2013). Research in regenerative medicine presently focuses on trying to engineer damaged tissues and organs by stimulating the body's own repair mechanisms to functionally heal previously irreparable damage to tissues or organs (Riazi et al., 2009). As stem cells have the ability to not only repair but regenerate damaged tissue, stem cell-based therapies have emerged as a promising tool to improve methods of disease diagnosis, treatment and prevention in those cases where conventional treatments have failed (Bongso and Lee 2005; Chaudhary and Roninson, 1991; Clarke, 2000).

1.3 Stem cells

Stem cells are unspecialised cells with the capability to differentiate into specialised cells. These cells are usually found as tiny populations in multicellular organisms distinguishable from other cells by their ability to divide and either self-renew or differentiate into multiple cell lineages (Becker et al., 1963; Siminovitch et al., 1963). Commonly, there are three main types of stem cells: embryonic stem cells (ESC) derived from embryonic blastocysts (Thomson at al., 1998), induced pluripotent stem cells (iPSC) derived from somatic cell reprogramming (Takahashi et al., 2006) and Adult Stem Cells (ASC) derived from adult tissue (Bongso and Lee 2005). ESCs are pluripotent cells whose main function is to differentiate into cell lineages of all three germ layers during embryonic development (Evans and Kaufman, 1981; Martin, 1981; Thomson at al., 1998). ESCs are also defined by the expression of several transcription factors and cell surface antigens. The transcription factors (TF) Oct-4, NANOG, and Sox2 form the core regulatory network that suppresses the expression of genes that lead to cell differentiation and promotes the expression of genes that maintain pluripotency (Boyer et al., 2005). Even so, given their unique regenerative abilities and therapeutic potential, research is continuously conducted to find ways of deriving stem cells due to the ethical concerns that arise from harvesting embryonic stem cells. One major breakthrough was the development of iPSCs by Takahashi et al., (2006). iPSCs are stem cells derived by artificially activating specific genes in somatic cells. As they are pluripotent, these cells are similar to ESCs because they can differentiate into any cell type of any tissue origin (Takahashi et al., 2006; Yu et al., 2007). In addition, they are also derived by upregulating the expression of Oct-4, NANOG and Sox2 which are the primary factors involved in maintaining ESC pluripotency (Takahashi et al., 2006; Yu et al., 2007). Moreover, iPSCs have a useful advantage over ESC since

they can be produced from adults and overcome the ethical concerns and immunogenicity associated with ESCs. However, current methods of deriving iPSCs have low efficacy and are associated with tumourigenic onset (Takahashi et al., 2006; Yu et al., 2007). Apart from embryo-derived or cell reprogramming stem cells, stem cells are also found naturally in adult tissue. ASCs are multipotent cells found in clusters in various niches throughout the body. ASCs are usually maintained in a quiescent state and, when activated, they proliferate to replenish damaged tissues (Bongso and Lee 2005). Therefore, ASCs are a renewable source of specialized cells for tissue repair, maintenance and development. They also maintain host tissue through the regulation of inflammation and immune responses (Figure1.2). The ASC phenotype is highly regulated by signalling pathways such as the Notch, Wnt and TGFβ pathways that control stem cell proliferation and growth (Bongso and Lee 2005). However, ASC niches are rare and tend to be depleted with ageing (Flores and Blasco, 2010). Different types of ASCs are found in the body depending on their tissue of origin. Of interest are mesenchymal stem cells (MSC) that arise from stromal tissue and found in the connective tissue of most organs. They play a key role in wound healing by proliferating and differentiating to replace multiple cell types within the wound bed and are capable of modulating innate immune responses (Dekoninck and Blanpain, 2019; Hay, 1995; Phinney and Prockop, 2007). Furthermore, in response to injury, normal epithelial cells can be transformed to give rise to more MSCs a phenomenon known as Epithelial to Mesenchymal Transition (EMT) (Hay, 1990/Hay, 1995).

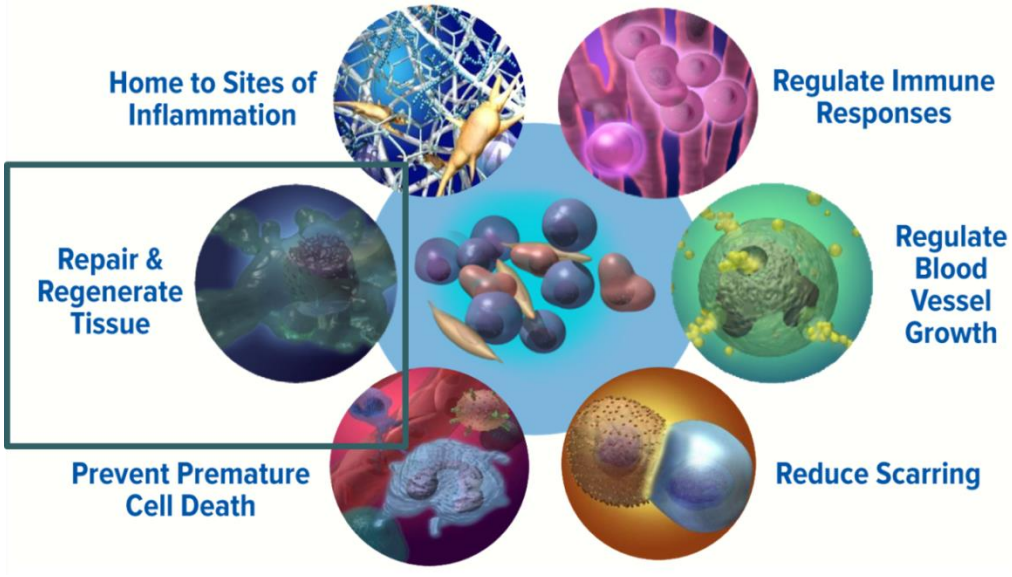


Figure 1.2: **The functions of adult stem cells in the body.** Adult stem cells play various roles in the body including regulating inflammation, immune responses, angiogenesis, scarring, apoptosis and repair and regeneration. (www.slideshare.net/esraa_tarek/stem-cells-abcs).

1.4 Epithelial to Mesenchymal Transition (EMT)

EMT is a process by which epithelial cells lose their polarity and cell-cell adhesion properties, and gain migratory and invasive properties to become mesenchymal stem cells. EMT is essential for various developmental processes, including neural tube and mesoderm formation in embryology (Hay, 1990/Hay, 1995). In adults, EMT facilitates regrowth during wound repair and tissue regeneration as well as organ morphogenesis (Hay, 1995). However, there is awareness that EMT associated processes are exploited during carcinogenesis in order to produce cancer stem cells as well as invasive and metastatic tumour growth (Boyer et al., 1989; Kong et al., 2011; van Staalduinen et al., 2018). EMT can be induced by several different pathways and transcription factors. These include activation of the Wnt/β catenin, Notch, Ras-MAPK, FGF, TGFβ pathways (De Craene et al., 2013; Thiery and Sleeman, 2006). Typically, in order to gain motility and increased proliferative capacity, there is a loss in expression of epithelial markers such as E-Cadherin and gain in expression of mesenchymal markers such as Vimentin or N-Cadherin among others is required (Figure 1.3) (Hay, 1990/95). The resultant MSCs play a primary role in wound repair.

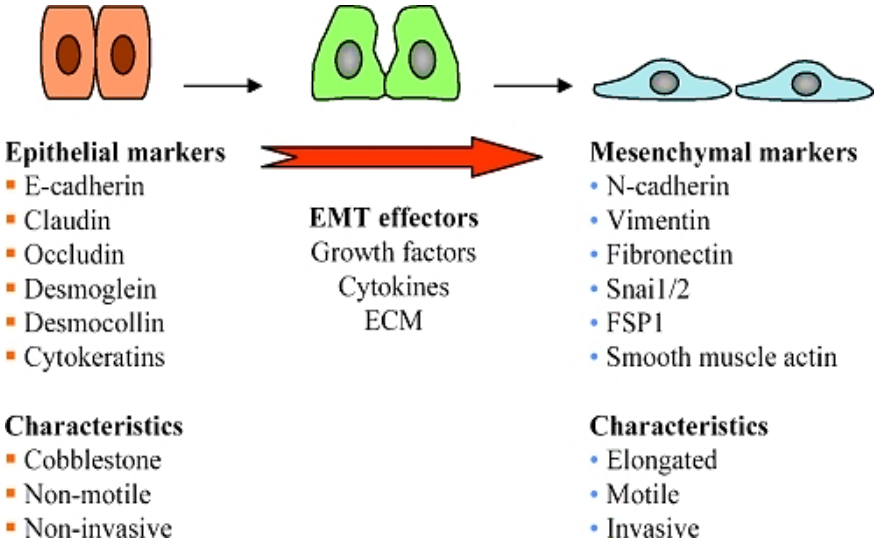


Figure 1.3: The process of Epithelial to Mesenchymal Transition (EMT). Epithelial cells are characterised by a cobblestone shape and a non-motive and non-invasive phenotype while mesenchymal cells exhibit an elongated, motile and invasive phenotype. The key effectors involved in the process include growth factors, cytokines and ECM leading to a change in the expression patterns of the two cell types (Gout et al., 2008).

1.5 MSCs in repair and regeneration

During normal wound healing MSCs are involved in all three phases of wound healing. MSCs migrate to sites of injury due to chemotactic signals, such as growth factors and cytokines, where they reduce inflammation, promote angiogenesis, and modulate immune responses. MSCs secrete various tissue repair factors such as growth factors, cytokines, and chemokines, including VEGF and TGFβ1 (Aggarwal and Pittenger, 2015). TGFβ1 is an anti-inflammatory cytokine that is responsible for initiating and stopping inflammatory phase of wound healing thereby allowing progression of wounds from the inflammatory phase to prevent the development of chronic wounds (Martinello et al., 2018). They can regulate immune responses by suppressing T and B lymphocyte proliferation in a non–major histocompatibility complex–restricted manner and promote the secretion of anti-inflammatory cytokines, TNF, IL4, IL10, and interferon (IFN) in immune cells (Aggarwal and Pittenger, 2015; Aguello et al., 2007). Martinello et al., (2018), showed that the application of MSCs to surgical wounds significantly improved wound healing by attenuating inflammation and suppression of the proliferation of immune cells thereby resulting in complete regeneration. Thus, making MSCs are an attractive option for regenerative medicine. However, as mentioned previously the self-renewal properties of adult stem cells including MSCs tend to decline with age, which is usually referred to as stem cell depletion or exhaustion (López-Otín et al., 2013). Stem cell depletion is the core contributor to the change in appearance from young to old and loss of functionality in an organism due to a failure to repair damaged tissue (Flores and Blasco, 2010; López-Otín et al., 2013). The exhaustion of stem cells is considered one of the consequences of ageing-related damages due to an accumulation of toxic metabolites (Oh et al., 2014; Rossi et al., 2007). Toxic metabolites such as reactive oxygen species (ROS) are elevated as a result of cellular damage and declining mitochondrial activity which leads to DNA damage (Oh et al., 2014). Stem cell DNA damage halts proliferation, induces apoptosis or causes dysregulated protein homeostasis, thereby affecting stem cell reserves and subsequently impeding regeneration (Harle-bachor and Boukamp., 1996; Shay et al., 1991). Furthermore, stem cell reserves can also be depleted as a result of excessive stem cell proliferation without efficient telomere maintenance resulting in decreased proliferative capacity (Aggarwal and Pittenger, 2015). Therefore, telomere length preservation is crucial for sustaining stem cells and in turn tissue repairs plus regeneration.

1.6 Telomere biology

1.6.1 Telomeres

Telomeres are nucleoprotein complexes found at the ends of eukaryotic chromosomes (Harley and Villeponteau, 1995). The main functions of telomeres are to prevent DNA erosion, fusion and degradation. Subsequently, this maintains chromosomal structure and function of linear DNA (Blackburn, 1978; Letsolo et al., 2010). In humans, telomeres are made up of double-stranded tandem repeats of TTAGGG and a single-stranded 3′ guanine-rich overhang (Harley and Villeponteau, 1995; Blackburn, 1992). It is believed that telomeres are an evolutionary solution to the 'end replication problem'. This problem is a result of the imperfect replication of DNA ends during lagging strand synthesis that occurs during DNA replication (Blackburn, 1992). Since DNA polymerase requires primers on the 3' end to initiate DNA synthesis, when this primer is removed it leaves a 3' end gap (Blackburn, 1992; Harley et al., 1990). The presence of telomeres enables 50-200bp of telomeric DNA instead of genomic DNA to be lost during each replicative cycle thereby conserving genomic DNA integrity and resulting in telomere shortening (Shay et al., 2001). Nonetheless, eukaryotic cells undergo a limited number of divisions before the telomeres become critically shortened, which is known as the 'Hayflick limit', after which the cells enter into replicative senescence (Hayflick, 1965). When a critical telomere length is reached, DNA damage checkpoints and apoptotic proteins such as p53 and p21 are activated (Shay and Wright, 2000). In turn, these cells then enter into a state of permanent cell cycle arrest in the G1 phase of the cell cycle; however, they remain metabolically active. In this manner, telomeres act as a 'mitotic clock' which essentially tracks ageing (Shayand Wright, 2019; Calado et al., 2008). In order to bypass this telomere-dependent senescence, telomeres have to be elongated by the enzyme telomerase (Greider and Blackburn, 1989).

1.6.2 Telomerase

Telomerase is a ribonucleoprotein reverse transcriptase enzyme found in actively dividing cells such as germline cells, embryonic stem cells and immortalised cells including cancer cells (Kilian, et al., 1997; Shay et al., 2001). The enzyme's core function involves telomere maintenance and elongation by the addition of TTAGGG repeats to 3′ ends of telomeres (Greider and Blackburn, 1989; Feng et al., 1995). Telomerase is a holoenzyme that comprises of two essential subunits among others (Figure 1.4) (Saretzki, 2009; Shay et al., 2001). In humans, the essential subunits are a catalytic reverse transcriptase component known as Human Telomerase Reverse Transcriptase (hTERT) and an RNA integral component termed Human Telomerase RNA Component (hTERC)

(Weinrich et al., 1997; Feng et al., 1995). When telomerase elongates telomeres in the leading strand in the 5′ → 3′ direction, hTERC acts as the template for hTERT which synthesises new single-stranded (ss) telomeric DNA. This newly synthesised telomeric DNA on the leading strand then acts as a template for the extension of the 5′ end of the lagging strand (Fig. 1.4) (Blackburn, 1992; Nakamura and Cech, 1998). Interestingly, TERT also plays other major roles independent of telomerase activity where it affects cell signalling, mitochondrial function, cell survival and influences gene expression (Cong and Shay, 2008; Lee et al., 2008; Li and Tergaonkar, 2014; Saretzki, 2014). It activates the transcription of genes involved in the Wnt signalling pathway that regulates the expression of pro-proliferation and cell survival genes. In addition, it is further capable of activating the proteins involved in the NF-κB signalling pathway, resulting in the expression of inflammatory and apoptotic resistance genes (Li and Tergaonkar, 2014). However, most somatic cells express low to non-detectable levels of TERT rendering them unable to elongate their telomeres by telomerase. However, studies have shown that overexpression of hTERT activates telomerase activity leading to telomere elongation (Bodnar et al., 1998); hence it is often regarded as the rate-limiting factor for telomerase activity (Counter et al., 1998). Therefore, regulation of hTERT may be used as a therapeutic strategy for pathologies characterised by dysregulated telomerase activity or excessive telomere shortening.

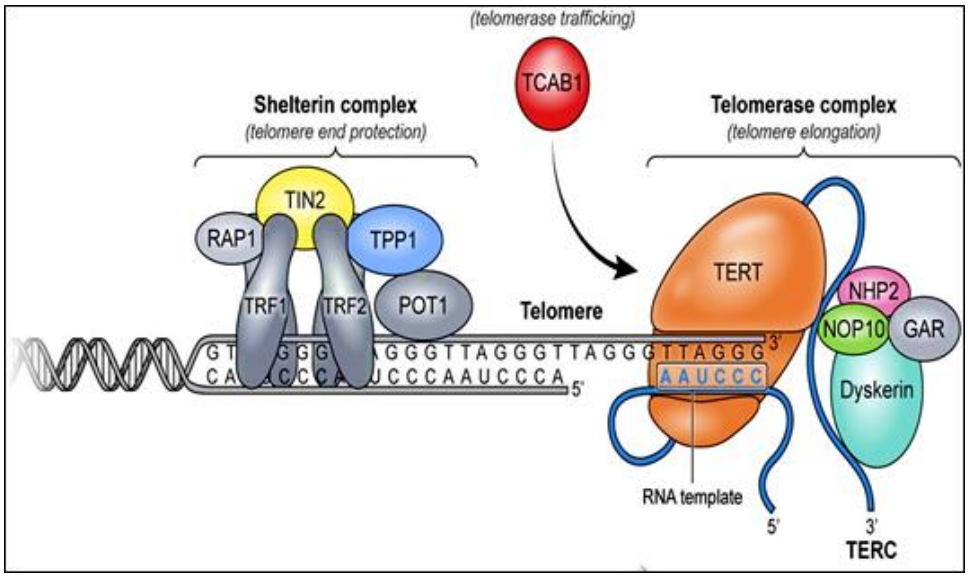


Figure 1.4: Extension of telomeres by telomerase. Telomeric DNA in the 5′ → 3′ direction is elongated by the telomerase complex. The RNA component, hTERC acts as the template for the reverse catalytic component, hTERT, which synthesises new single-stranded (ss) telomeric DNA. Proteins from the shelterin complex that protects telomeres are depicted (adapted from Townsley et al., 2015).

1.6.3 Telomeres and telomerase in disease

Telomere attrition is also considered a key primary hallmark of ageing and is believed to be the most significant contributor to the ageing phenotype (Lopez-Otin et al., 2013). Due to the lack of telomerase activity in most somatic cells, telomere attrition is inevitable as a result of the end replication problem (Counter et al., 1998). These shortened telomeric regions have been shown to increase the risk of developing age-related idiopathic organ fibrosis and are also linked with other age-related diseases, such as cardiovascular disease, diabetes and Alzheimer's disease (AD) (Alder et al., 2008; Rudolph 1999). Furthermore, defects or critical shortening of telomeres can lead to end-to-end chromosomal fusions and non-reciprocal translocations collectively known as telomere dysfunction (Letsolo et al., 2010). In turn, this dysfunction of the telomeres can lead to large scale genome rearrangements/ aberrations and consequently, genomic instability (Capper et al., 2007; Letsolo et al., 2010). Moreover, critical telomere shortening can also activate DNA damage response pathways, such as p53, which lead to cell senescence and/or apoptosis (Shay et al., 1991). Increased apoptosis of irreplaceable specialised cells and loss of tissue function plays a key role during the ageing of tissue or whole organs such as the heart, muscle and brain (Pollack et al., 2002).

A common cause of DNA damage and telomere shortening is prolonged Reactive Oxygen Species (ROS) production which normally occurs in chronic wounds or a diseased state (Coluzzi et al., 2014; Rieger et al., 2014). ROS' are typically produced during the inflammatory phase of wound healing to destroy all pathogens on the injured site. Due to the high prevalence of guanine residues in telomeric DNA which have high oxidation potential, telomeres are particularly susceptible to radiation or oxidative damage (Henle, et al., 1999). This DNA damage is usually in the form of double-stranded breaks that also affect telomeric DNA. In addition, telomere shortening is also observed in stem cells where it is responsible for stem cell depletion. This depletion in stem cell pools is one of the hallmarks of ageing that causes a decrease in repair and rejuvenation (Flores and Blasco, 2010; López-Otín et al., 2013).

1.6.4 Telomeres and telomerase in repair and regeneration

Telomerase activity has been implicated in perpetually regenerating human tissue such as proliferative basal cells, where, telomerase activity is activated in the epidermis by inflammation in order to promote cell proliferation to repair tissue damage (Haik et al, 2000). Previous studies have reported that hTERT can activate the transcription of genes involved in growth factor production and cell proliferation including WNt and NFκB (Li and Tergaonkar, 2014). Some of the factors that

disrupt wound healing include impaired or decreased angiogenic response, growth factor production, and fibroblast migration and proliferation (Baloui et al., 2009; Knighton, et al., 1981). Furthermore, with the shortening of telomeres, there is an accumulation of senescent cells. These cells tend to secrete more pro-inflammatory cytokines and matrix metalloproteinases which are deleterious and accelerate tissue damage (Kuilman et al., 2010; Rodier and Campisi, 2011). Therefore, lengthening of the telomeres using telomerase is believed to reduce the amount of cells that enter into senescence, thereby alleviating tissue damaging factors (Coluzzi et al., 2014; Haik et al, 2000).

Moreover, hTERT and telomerase activity play a major role in the maintenance of stem cell phenotype (Flores et al., 2006). hTERT is often up-regulated in rapidly dividing cells, including both ASCs and ESCs, where its high expression is often reported as a marker for pluripotency or multipotency (Flores and Blasco, 2010). The increase in hTERT coincides with increased telomerase activity to elongate and maintain the telomeres of stem cells. As a consequence, maintenance of telomeres increases the life expectancy of the stem cells by allowing for unlimited division thereby enabling replenishment of stem cell niches (Thompson, et al., 1998). Conversely, telomere attrition in stem cells limits their replicative potential leading to a gradual depletion of stem cell pools and decreased regenerative potential (Flores and Blasco, 2010; Sharpless and DePinho, 2007). A prime example of decreased repair and regeneration due to stem cell exhaustion is the ageing of the hematopoietic system due to exhaustion of hematopoietic stem cells (HSC) (Shaw et al., 2010). This age-related decline of the hematopoietic system is usually associated with anaemia, myeloid malignancies and immune dysfunction. The immune dysfunction brought about by stem cell exhaustion results in senescence of the immune system, which is the decreased production of adaptive immune cells (Shaw et al., 2010). While stem cell exhaustion can be harmful to the maintenance of an organism, excessive stem cell proliferation without efficient telomere maintenance can also cause the exhaustion of stem cell niches which can result in premature ageing (Rera et al., 2011). In addition, hTERT is responsible for maintaining the stem cell phenotype independently of telomerase activity. As previously mentioned, hTERT is involved in the transcriptional activation of genes involved in proliferative pathways such as the Wnt and NF-κB signalling pathways which aid in stem cell renewal ability, cell survival and resistance to apoptosis (Saretzki, 2014; Li and Tergaonkar, 2014).

Moreover, studies where hTERT was upregulated, illustrated its ability to directly mitigate ageing. Bodnar et al, (1998) ectopically expressed the catalytic component of telomerase, hTERT which is usually the limiting factor in differentiated cells. They found that telomerase activity was activated in normal somatic cells where these cells were able to evade senescence, causing cell

immortalization and further imparted stem cell properties (Bodnar et al, 1998). Since telomerase reactivation is usually a consequence of cancer, the study also demonstrated that hTERT immortalized normal human cells without inducing tumorigenesis (Nakayama et al., 1998; Bodnar et al, 1998). In addition, De Jesus et al., (2010) also demonstrated that telomerase gene therapy in adult and old mice had a positive effect on health and fitness including: an increased lifespan, and improvement in several ageing molecular biomarkers. Importantly, the experimental mice did not show increased formation of neoplasia in comparison to the control mice (De Jesus et al., 2010). These studies imply that upregulation of hTERT may induce stem cell like traits to aid wound healing without causing cancer. A recently discovered modulator of hTERT expression and telomerase activity is the 37 kDa Laminin Receptor Precursor/67 kDa High Affinity Laminin Receptor (LRP/LR).

1.7 The 37 kDa Laminin Receptor Precursor/67 kDa High Affinity Laminin Receptor (LRP/LR)

LRP/LR, also commonly known as LAMR1 or RPSA, is a multifunctional, non-integrin laminin binding (type II transmembrane) cell surface receptor protein, predominantly located in the plasma membrane (Gauczynski et al., 2006). Its C-terminus also has multiple binding sites for heparin sulphate proteoglycans, immunoglobulins and extracellular matrix (ECM) proteins, such as elastin and carbohydrates (Ardini et al., 1995; Jovanovic et al., 2015; Mbazima et al., 2010). LRP/LR was originally identified as a binding protein for an ECM glycoprotein called laminin-1(Figure 1.5 (2 and 3)) which provides cellular adhesion to the basement membrane (Lesot et al., 1983; Rao et al., 1983). This interaction maintains cell biological processes such as mediating cell polarity, migration, adhesion, viability, growth, apoptosis, proliferation and differentiation (Mercurio, 1995; Miner, 2008; Moodley & Weiss, 2013; Omar et al., 2012; Ryan et al., 1996; Satoh et al., 1999; Yurchenco, 2011). Additionally, LRP/LR is also localised in the cytoplasm where it associates with ribosomes in order to promote protein synthesis and implement pre-rRNA processing (Figure 1.5) (Ford et al., 1999; O'Donohue et al., 2010). Moreover, LRP/LR was also found to aid the tethering of ribosomes to microtubules for the promotion of protein synthesis by playing a role in cytoskeletal organisation (Venticinque et al., 2011). In fact, it has been shown that LRP/LR co-localizes and directly interacts with intracellular actin, focal adhesion complexes and cytoskeletal stress fibres in order to carry out this role (Kim et al., 2005; Keppel & Schaller, 1991; Yannariello-Brown et al., 1988). Furthermore, LRP/LR is found in the nucleus where it associates with histones to maintain nuclear structures (Kinoshita et al., 1998). This said, nuclear LRP/LR is required for the nucleolar exit of the pre-40S ribosomes and may also serve to recruit and anchor DNA damage repair proteins within the nucleolus through loosely binding to them (Guerra-Rebollo et al., 2012; O'Donohue et

al., 2010). Due to the involvement of LRP/LR in several key cellular processes this protein has been implicated in various pathologies (Jovanovic et al., 2015).

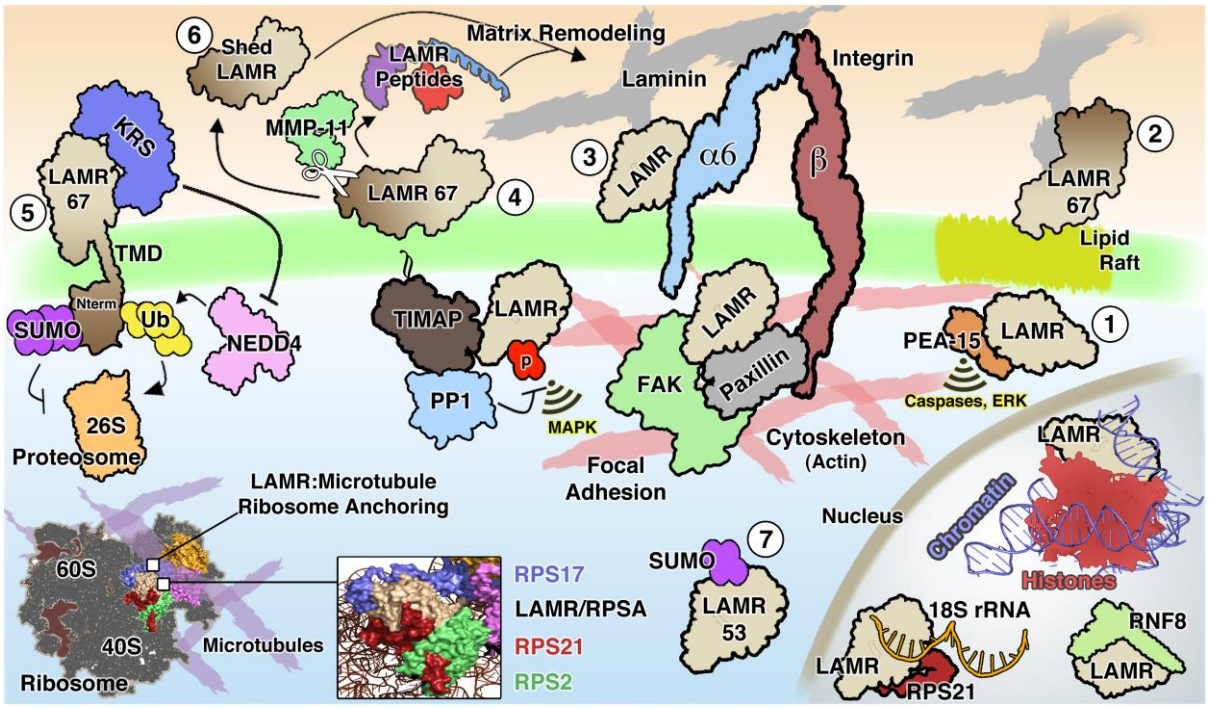


Figure 1.5: The localisation of LRP/LR or LAMR-1 and its theoretical binding partners. The localization of LRP/LR (denoted as LAMR) on the cell surface, cytoplasm and nucleus are shown. Several LRP/LR species are depicted as follows: (1) Intracellular 37kDa LRP, (2) Peripheral membrane 67kDa LR independently binding laminin in the lipid raft regions, (3) Peripheral membrane 67kDa LR supporting integrin-laminin binding, (4) 67kDa LR membrane association via the properties of a homo/ heteromer, (5) transmembrane 67kDa LR (6) shed 67kDaLR and (7) intracellular sumoylated 53 kDa LR. (DiGiacomo and Meruelo, 2015)

1.7.1 The role of LRP/LR in disease

LRP/LR overexpression has been observed in cancer where its binding capability confers susceptibility to pathological agents associated with various diseases (Jovanovic et al., 2015). Its receptor function is commonly associated with Sindbis and Dengue virus infections, prion disorders, neurodegenerative disorders such as AD and cancer (Ardini et al., 2002; Jovanovic et al., 2015; Mbazima et al., 2010; Otgaar et al 2017). In neurodegenerative disorders, LRP/LR mediates the endocytosis of PrP^{Sc}, which is an infectious prion protein associated with encephalopathies (Gauczynski et al., 2001). It also serves as a receptor for $A\beta_{42}$ which is responsible for AD pathophysiology through the facilitation of $A\beta_{42}$ uptake and aiding the cleavage of amyloid precursor protein (APP) to form cytotoxic $A\beta_{42}$ (Da Costa Dias et al., 2013). In cancer, LRP/LR

overexpression aids critical steps in cancer progression, where it has been implicated in adhesion and invasion (Chetty et al., 2014; Khumalo et al., 2013; Omar et al., 2012; Zuber et al., 2008) as well as apoptosis evasion (Moodley et al., 2013). Additionally, *in vitro* studies in non-tumourigenic cell lines suggests that LRP/LR plays a role in angiogenesis (Khusal et al., 2013) and ageing (Otgaar et al., 2017). Moreover, Otgaar et al., (2017) proposes that elevation of LRP levels decreases the accumulation of senescence related markers, possibly through upregulation of hTERT and activation of telomerase activity (Otgaar et al., 2017). Hence the implication of LRP/LR in various pathologies associated with increased cytotoxicity such as AD or increased inflammation such as cancer suggests that this protein may also play a role in the expression of proteins involved in modulating repair and regeneration.

1.7.2 The role of LRP/LR in repair and regeneration

Previous studies have shown that LRP/LR is usually upregulated in response to traumatic/ischemic injury (Baloui et al., 2009). This elevation of LRP/LR levels is due to signalling from hormones or cytokines such as those of the TGFβ and TNF families (Annabi et al., 2007; Raghunath et al., 1993; Wenzel et al., 2010). Furthermore, the upregulation of LRP/LR is usually in association with increased laminin-1 expression (Baloui et al., 2009). The interaction between the membrane-associated LRP/LR and ECM glycoprotein laminin-1 has been studied in several cell systems where it was shown to be crucial for angiogenesis and immune responses (Baloui et al., 2009; DiGiacomo and Meruelo, 2015; Stitt et al., 1998). LRP/LR facilitates angiogenesis by promoting the secretion of MMPs (MMP-2 and MMP-9) into the ECM that degrades the basal lamina in order to allow blood vessel formation (Khusal et al., 2013). In addition, the secreted MMPs also induce the release of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) and TGFβ in order to induce cell migration towards the angiogenic stimuli (Gill and Parks, 2008; Khusal et al., 2013; Wenzel et al., 2010). Moreover, LRP/LR may play a role in cellular stress response by inhibiting the de-phosphorylation of Mitogen-activated Protein Kinase (MAPK) pathways which are primary cell stress response pathways (Givant-Horwitz et al., 2004). Givant-Horwitz et al., (2004) illustrated that increased LRP/LR coincides with a decrease in the levels of the three major subgroups of MAPK signalling; c-Jun N-terminal protein kinase (JNK), extracellular signal-regulated kinase (ERK) and p38. Of note are the JNK and p38 signalling pathways that are activated by inflammatory cytokines, environmental stress, or chemical induced stress Givant-Horwitz et al., (2004). This suggests that LRP/LR may play a role in wound healing through modulation of cellular stress response signalling cascades.

1.8 The relationship between telomeres, telomerase and LRP/LR

Reportedly, LRP/LR interacts with telomerase and these proteins co-localise in the same subcellular locations: the cell surface, cytoplasm and perinuclear compartment (Bignoux et al., Revised; Naidoo et al., 2015; Otgaar, et al., 2017) (Figure 1.6). In addition, both proteins have been implicated in various similar pathologies were they play a role in promoting similar functions (Jovanovic et al., 2015). Moreover, increased levels of LRP/LR and telomerase have also been implicated in major cancer hallmarks which lead to immortalisation of cells and increased invasiveness of tumours (Naidoo et al., 2015; Otgaar et al., 2017). In fact, both proteins have parallel functions of increasing cell viability and aiding in apoptosis evasion (Jovanovic et al., 2015). Furthermore, a recent study by Otgaar et al., (2017), showed that LRP::FLAG overexpression in somatic cell lines resulted in a significant increase in hTERT levels, telomerase activity, and telomere length, while the inverse was observed after siRNA knockdown of LRP/LR (Naidoo et al., 2015). Therefore, the relationship between these two proteins can be exploited for the development of therapies aimed at pathologies where these proteins are dysregulated.

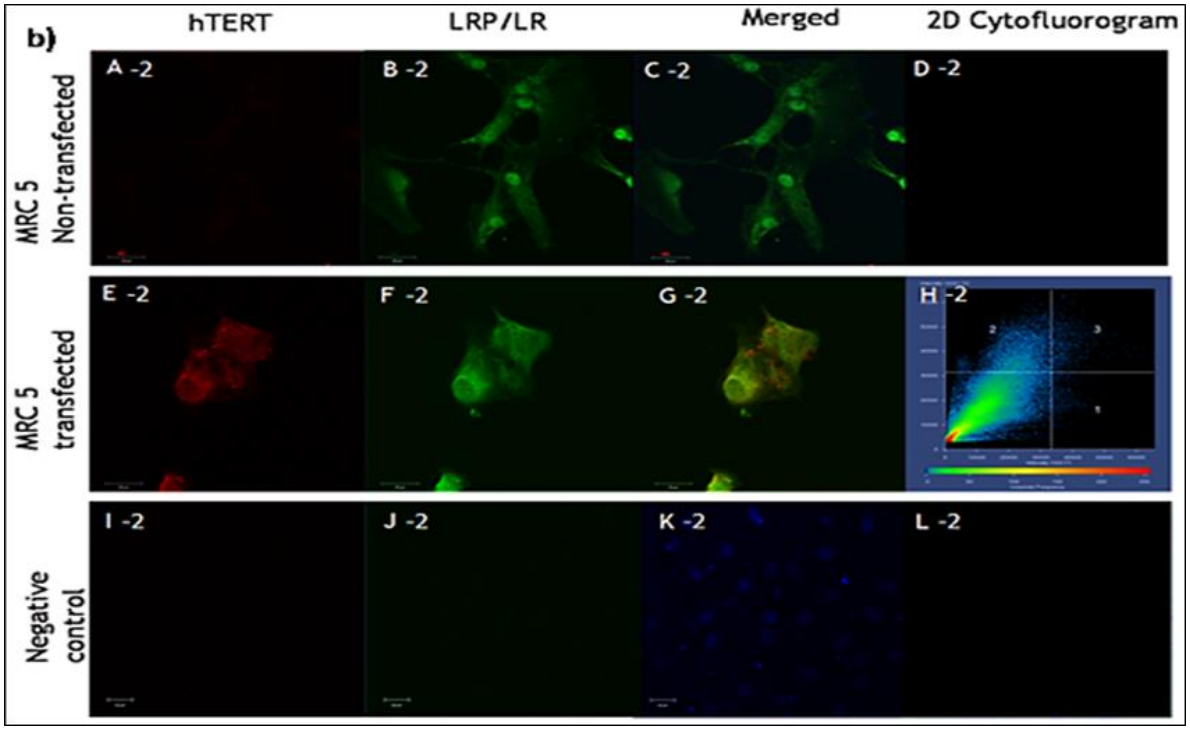


Figure 1.6: Co-localisation of LRP/LR and hTERT in MRC5 cells after overexpressing LRP::FLAG. Confocal microscopy showed a significant increase in hTERT expression and changes in the morphology of MRC5 cells overexpressing LRP::FLAG. Transfected MRC5 cells had increased nuclear to cytoplasmic ratio and a more rounded phenotype (Otgaar et al, 2017).

1.9 Rationale of the study

Currently, there is an enormous worldwide demand for therapies to promote efficient wound healing with minimal scarring. As the human population becomes progressively older worldwide due to improving living conditions and health care, there is an urgent need for curative therapies to combat the effects of stem cell depletion and impaired wound healing. Both stem cell depletion and impaired wound healing affect the process of regenerating body tissues, especially in aged organisms (Isobe et al., 2014). As such, stem cells have emerged as a potential therapeutic approach for regenerative medicine which aims to restore normal tissue function in patients (López-Otín et al., 2013). Recent *in vitro* studies with mesenchymal stem cells (MSCs) have identified numerous mechanisms by which these cells can promote the process of wound healing. Their therapeutic potential is largely due to their ability to secrete pro-regenerative factors, making them an attractive option for the treatment of chronic and acute wounds (Isobe et al., 2014). However, current methods of deriving stem cells are costly and have low efficiency (Skpvortsova et al., 2018; Stadhouders et al., 2018; Takahashi et al., 2018). Recently, studies have shown that LRP::FLAG overexpressed in somatic cell lines results in a significant increase in hTERT levels (Naidoo et al., 2015; Otgaar et al, 2017). In addition, the cells overexpressing LRP::FLAG showed stem cell like characteristics such as a rounded morphology, enlarged nucleus (Fig 1.8), increased population doublings and decreased adhesiveness (Becker et al., 1963; Siminovitch et al., 1963; Otgaar et al., 2017). Moreover, overexpression of hTERT is known to lead normal somatic cells to develop stem cell like properties (Bodnar et al, 1998). Since both LRP/LR and hTERT promote physiological processes that are characteristic of stem cells it was hypothesised that overexpressing LRP/LR and subsequently hTERT will result in stem-cell-like cells which will aid wound healing and regeneration *in vivo* and *in vitro*.

2 Aim and objectives

2.1 Aim

The first aim of the study was to characterise the stem-cell-like traits of human embryonic kidney cells (HEK293) and normal lung fibroblast (MRC5) cells transfected with LRP::FLAG.
The second aim of the study was to investigate the effect of LRP::FLAG overexpression on the wound healing process in human embryonic kidney cells (HEK293) and normal lung fibroblast (MRC5) cells.

2.2 Objectives

- To overexpress LRP::FLAG in HEK293 and MRC5 cells
- To investigate whether overexpression of LRP::FLAG has a significant effect on TERT and TERT-related proteins
- To investigate whether LRP::FLAG overexpression induces Epithelial to Mesenchymal Transition
- To determine the protein levels of stem cell markers in cells overexpressing LRP::FLAG and investigate whether overexpressing LRP::FLAG results in pluripotent cells
- To determine the protein levels of inflammatory markers in cells overexpressing LRP::FLAG and investigate whether overexpressing LRP::FLAG has an effect of wound healing characteristics

3 Experimental Procedure

3.1 Cell Culture

3.1.1 Cell lines

The experimental cell lines used were human embryonic kidney cells (HEK293) and normal lung fibroblast (MRC5). HEK293 cells were used as a control for telomerase as they are an epithelial cell line that exhibits high hTERT levels and telomerase activity (Cohen, S.B. et al., 2007). MRC5 cells were used because they are a somatic fibroblast cell line that has undetectable/ low levels of hTERT as well as telomerase activity with finite population doublings not exceeding 60 (Jacobs et al., 1970). MRC5 cells are therefore an ideal *in vitro* model for investigating whether overexpression of LRP induces stem-cell-like traits in normal somatic cells. All cell lines were obtained from Fox Chase Cancer Research Centre and their characteristics and growth media are described in Table 2.1.

Table 3.1: List of cell lines used with their description and growth requirements.

Cell Line	Description	Growth media
HEK293	Human embryonic kidney cells- derived from foetus (Graham et al., 1977)	Dulbecco's Modified Eagle Medium (DMEM) high glucose containing 4mM L-Glutamine (Gibco)
MRC5	Human normal lung fibroblast- derived from normal lung tissue of a 14-week-old male foetus (Jacobs et al., 1970)	Dulbecco's Modified Eagle Medium (DMEM) high glucose containing 4mM L-Glutamine (Gibco)
*All media was supplemented with: • 10 % Foetal bovine serum (FBS) (Biowest) • 1 % Penicillin-Streptomycin (Sigma-Aldrich)		

3.1.2 Cell culture method

All cell lines were maintained in their appropriate media (Table 2.1) which is optimal for growth of the cells. The cells were cultured at 37 °C, 5 % carbon dioxide (CO_2) and pH 7.4 in a humidified incubator which mimics *in vivo* conditions. Cells were also sub-cultured when they reached a confluency of approximately 80 % to maintain cellular growth potential. Firstly, the cells were

washed with 5 ml of 1X phosphate buffered saline (PBS) (buffer composition is in appendix 8.1) and thereafter detached by incubating with 1ml of 1X trypsin-EDTA (ethylene diaminetetra acetic acid) (Sigma-Aldrich) at 37 °C for 5 minutes. Afterwards, 9 ml of media was added to inactivate the trypsin. HEK293 cells were subcultured in a 1:10 ratio whereas MRC5 cells were subcultured in a 1:2 ratio with fresh appropriate media (Table 2.1) to make a total of 10 ml of culture media per 75 cm^3 flasks.

3.1.3 Cryopreservation and storage of cells

Cells were cryopreserved to avoid loss by contamination and to avoid ageing and transformation in finite cell lines. Since MRC5 cells are a finite cell line, stocks were made between population doublings of 20-40 passages. Detached cells were re-suspended in their growth media and centrifuged at 1200 rpm for 10 minutes. Thereafter, the supernatant was discarded and the cells were re-suspended in 1 ml of cryopreservation media. All cell lines were preserved in appropriate fresh media, 10 % FBS (Biowest) and 10 % (Dimethyl sulfoxide) DMSO (Lab Supply). Following re-suspension, cells stocks were stored at -80 °C.

3.1.4 Cell counting

Cell counting was performed in order to ensure that there was an equivalent number of cells for experiments. This allowed for an effective comparison of the magnitude of effects observed in this study. To perform a cell count, cells were washed with 1X PBS and detached using 1X trypsin/EDTA (Sigma-Aldrich). Cells were re-suspended in the appropriate media and a 10 µl aliquot of the cell suspension was combined with 10 µl of 0.4 % Trypan blue solution (Sigma-Aldrich) for staining. Trypan blue dye stains dead cells blue because the chromophore can enter through the compromised membranes of dead cells and viable cells appear clear because they have intact membranes that exclude the dye. Post-staining, cells were then counted using a Bio-Rad TC20™ Automated Cell Counter.

3.2 LRP::FLAG overexpression in cells using the pCIneo–moLRP-FLAG plasmid

In order to investigate the effect of elevating LRP protein levels on EMT, stem cells and wound healing markers, cells were transfected with the pCIneo–moLRP-FLAG plasmid to induce an overexpression of LRP with the FLAG™ tag (peptide sequence DYKDDDDK) (Hopp et al., 1988; Vana and Weiss, 2006). The FLAG epitope is an artificial antigen which allows for differentiating

recombinant overexpressed LRP, from endogenous LRP (Hopp et al., 1988). The procedure involved culturing cells until they reached approximately 60 % confluency in a 25 cm^2 flask. Thereafter, cells were transfected using Clontech Xfect™ reagents, according to the manufacturer's protocol. Briefly, a mixture of 200 µl Xfect reaction buffer, 3 µl Xfect Polymer and 10 ng of the pCIneo-moLRP-FLAG plasmid were used. After vortexing thoroughly, the mixture was incubated at room temperature for 10 minutes to allow the formation of nanoparticle complexes with the DNA construct. Cells were then incubated overnight with the Xfect nanoparticle complexes at 37 ^{0}C and 5 % CO_2 in a humidified incubator. Thereafter, a media change was performed to remove the nanoparticle complexes and cells were allowed to proliferate for 48 hours to allow sufficient production of the LRP::FLAG protein and selectable marker. Selective treatment commenced using Geneticin® (Gibco) to ensure that only cells containing the plasmid survived. Initial treatment of 8000 ng/µl was performed for one week and subsequently, cells were maintained in 3000 ng/µl of Geneticin® in order to ensure a stable overexpression of LRP::FLAG.

3.3 Protein extraction and quantification

3.3.1 Protein extraction

To determine whether overexpression of LRP::FLAG had an effect on the protein levels of EMT, stem cells and wound healing associated markers, protein was extracted from both transfected and non-transfected HEK293 and MRC5 cells. Firstly, the culture media was removed and then the cells were washed with 1X PBS. The cells were then detached using 1ml of trypsin/EDTA and centrifuged at 4000 g for 5 minutes to pellet the cells. The pellet was re-suspended in 200 µl of 1X Radioimmunoprecipitation Assay (RIPA) lysis buffer (buffer composition is in appendix 8.1) with protease and phosphatase inhibitors and incubated on ice for 20 minutes. Thereafter, a Bicinchoninic Acid (BCA) assay was performed to quantify the protein concentration.

3.4 Protein quantification using a Bicinchoninic Acid ™ (BCA) assay

A BCA assay was utilized to quantify protein concentration of the cell lysate to ensure equal protein loading for subsequent experiments. This assay is a colourimetric test that relies primarily on two reactions. Firstly, Cu^{2+} ions in the assay are reduced to Cu^{+} ions and then the Cu^{+} ions react with two molecules of bicinchoninic acid in the solution to produce a purple substrate that can be measured at 562 nm using an ELISA plate reader (Smith et al., 1985). A standard curve was constructed using Bovine Serum Albumin (BSA) of a known concentration serially diluted from 1

mg/ml to 0 mg/ml in 0.2 mg/ml increments. Thereafter, 25 µl of each standard was pipetted into a 96 well plate and 5 µl of cell lysate was added into separate wells containing 20 µl of ddH_2O (5 times dilution). A solution of 97 % BCA (Sigma-Aldrich) and 3 % Copper (II) Sulphate (Sigma-Aldrich) was then prepared and 200 µl of this solution was added to each well. All reactions were performed in triplicate. The plate was then incubated at 37 ^{0}C for 30 minutes. The absorbance was measured at 562 nm using the Multiskan Go ELISA plate reader (Thermo) and the standard curve was used to extrapolate the total protein concentration of the cell lysates. Stocks of a 2 mg/ml concentration were then prepared using 3X loading dye (Labocare^{TM}) and 1X RIPA buffer (Appendix 8.1).

3.5 SDS-PAGE and western blotting

Western blotting was performed to confirm successful transfection of cells with the pCIneo–moLRP-FLAG plasmid. In addition, western blotting was also used for the relative quantification of hTERT, phospho-TERT (pS824), EMT markers: α-E-catenin, N-Cadherin and Vimentin and wound healing markers: NF-κB and TGFβ1 in both transfected and non-transfected HEK293 and MRC5 cells. Furthermore, β-actin was used as the loading control since there is uniform and high expression levels of it in different cell types. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and western blotting were employed as they allowed the separation of whole protein lysates by size. It is also possible to semi-quantify the target protein using densitometric analysis and to determine its size by electrophoresing a molecular weight standard in parallel (Laemmli, 1970/71).

SDS- PAGE was employed to separate and resolve protein lysates according to size in an electric field (Laemmli, 1970/71). A 10 % or 12 % (w/v) polyacrylamide gel was used to electrophorese between 10-60 µg/ml of protein lysate depending on the size and abundance of the protein to be probed for. All gels were electrophoresed at 150V in 1X Running Buffer until dye front was about 5 mm from the bottom of the gel. The separated proteins were semi-dry transferred from the gel onto a PVDF membrane in 1X Transfer Buffer for 30 minutes at 25V using the Bio-Rad® Trans-Blot Turbo. In order to prevent non-specific binding of antibodies, the membrane was then blocked in 3 % BSA in 1X PBS/0.1 % Tween (PBS/T) solution for an hour. Thereafter, the PVDF membranes were incubated with the relevant primary antibody (Table 2.2) overnight at 4°C with gentle shaking. The next day, three washes of 10 minutes each with 1X PBS/T were performed in order to remove any unbound primary antibody. The membranes were then incubated for an hour at room temperature with an appropriate secondary antibody coupled to a horseradish peroxidase enzyme

(HRP) (Table 2.2), followed by 3 more washes with 1X PBS/T of 10 minutes each. Once chemiluminescent substrate (Clarity™ Western ECL, Thermo Scientific) was added to membranes, the precipitate formed was detected by radiation exposure using Chemi-doc™ (BioRad). Thereafter, densitometric analysis (Image Lab 4.0) was employed for relative quantification of the target proteins by measuring the intensity of the bands. All experiments were performed in triplicates, buffer compositions are given in appendix 8.1 and all antibodies used are shown in Table 2.2.

Table 3.2: List of primary and secondary antibodies with concentrations used for western blotting.

Target Protein	Primary antibody	Secondary antibody
β-actin	Mouse anti-β-actin-peroxidase. (Sigma A3854) *Dilution factor -1:10 000*	N/A
LRP::FLAG	Mouse anti-FLAG (Sigma F-3165) *Dilution factor -1:2 500*	anti-mouse IgG-HRP (Cell signalling 7076S) *Dilution factor -1:10 000*
LRP/LR	Rabbit anti-W3 (Affimed therapeutics) *Dilution factor -1:2 500*	anti-rabbit IgG-HRP (Abcam 6858) *Dilution factor -1:10 000*
hTERT	Rabbit anti-hTERT (abcam 183105) *Dilution factor -1:1 000*	anti-rabbit IgG-HRP (Cell signalling 7074S) *Dilution factor -1:5 000*
phospho-TERT (pS824)	Rabbit anti- phospho-TERT(pS824) (Sigma-Aldrich 111183) *Dilution factor -1:1 000*	anti-rabbit IgG-HRP (Cell signalling 7074S) *Dilution factor -1:5 000*
α-E-catenin	Mouse anti-α-E-catenin (Biolegend 844802) *Dilution factor -1:1 000*	anti-mouse IgG-HRP (Cell signalling 7076S) *Dilution factor -1:10 000*
N-Cadherin	Mouse anti-N-Cadherin (BDTransduction Laboratories™ 610920) *Dilution factor -1:1 000*	anti-mouse IgG-HRP (Cell signalling 7076S) *Dilution factor -1:10 000*

Vimentin	Chicken anti-Vimentin (Biolegend 919101) *Dilution factor -1:5 000*	anti- chicken IgG-HRP (Abcam ab6877) *Dilution factor -1:10 000*
TGFβ1	Mouse anti-LAP (TGF-β1), clone TW4-6H10 (Merk 21848) *Dilution factor -1:1 000*	anti-mouse IgG-HRP (Cell signalling 7076S) *Dilution factor -1:10 000*
NFκB	Rabbit anti-NFκB1 (Abcam SAB430301) *Dilution factor -1:2 500*	anti-rabbit IgG-HRP (Cell signalling 7074S) *Dilution factor -1:5 000*

3.6 Confocal Microscopy

Confocal microscopy is a qualitative technique that was performed in order to visualise specifically labelled nucleic acids or proteins within the cells (Miller and Shakes, 1995). This technique was utilised to determine the localisation and expression patterns of LRP, hTERT, EMT markers: α-E-catenin, N-Cadherin and Vimentin as well as wound healing markers: NF-κB and TGFβ1. Both transfected and non-transfected cells were cultured on coverslips (Labocare – 18X18 mm; 0.19 mm thick) until they reached 60 % confluency in a 6 well plate. Upon reaching the correct confluency, the cover slips were washed twice using 2 ml of 1X PBS. Thereafter, the cells were fixed with 4 % paraformaldehyde for 20 minutes followed by three 5 minute washes in 1X PBS. In order to visualise the intracellular localization of the proteins of interest, cells were permeabilised using 0.25 % Triton-X in 1X PBS for 15 minutes. After performing three 5 minute washes in 1XPBS, the cells were then blocked using 0.5 % BSA for 30 minutes to prevent non-specific binding of antibodies. Next, the coverslips were incubated with the relevant primary antibodies overnight (Table 2.3). After washing off unbound primary antibodies using by performing three by 5 minute washes in 1X PBS), the coverslips were then incubated with the appropriate Fluorescein isothiocyanate (FITC)-coupled or IgG-CF^{TM}647-coupled secondary antibody in the dark for an hour (Table 2.3). Thereafter, coverslips were washed three times and incubated for 5 minutes with 1 ml of 0.05µg/ml 4', 6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) diluted in 1X PBS for nuclear staining. The coverslips were then washed five times with 1X PBS and Gelmount (Sigma-Aldrich) was used to mount the coverslips containing the cells onto a slide. The slides were allowed to set for 2 hours in the dark then stored at 4º C until viewing. The Zeiss LSM 780 Confocal microscope was used to view the slides at 630x magnification. FITC was excited at 490 nm and emitted at 525 nm, whereas CF^{TM}647 was excited at 650 nm and emitted 665 nm.

Table 3.3: List of primary and secondary antibodies with concentrations used for Confocal microscopy.

Target Protein	Primary antibody	Secondary antibody
LRP/LR	Human anti-LRP/LR IgG1-iS18 (Affimed therapeutics) *Dilution factor -1:250*	anti-human IgG-FITC (Abcam 6854) *Dilution factor -1:500*
hTERT	Mouse anti-hTERT (abcam 183105) *Dilution factor -1:100*	anti-mouse IgG-CF™647 (Sigma-Aldrich 7074S) *Dilution factor -1:500*
α-E-catenin	Mouse anti-α-E-catenin (Biolegend 844802) *Dilution factor -1:250*	anti-mouse IgG-CF™647 (Sigma-Aldrich 7074S) *Dilution factor -1:500*
N-Cadherin	Mouse anti-N-Cadherin (BD Transduction Laboratories™ 610920) *Dilution factor -1:200*	anti-mouse IgG-FITC (Sigma F0257) *Dilution factor -1:500*
Vimentin	Chicken anti-Vimentin (Biolegend 919101) *Dilution factor -1:500*	anti-chicken IgG-FITC (Biolegend 410802) *Dilution factor -1:500*
TGFβ1	Mouse anti-LAP (TGF-β1), clone TW4-6H10 (Merk 21848) *Dilution factor -1:250*	anti-mouse IgG-CF™647 (Sigma-Aldrich 7074S) *Dilution factor -1:500*
NFκB1	Rabbit anti-NFκB1 (Abcam SAB430301) *Dilution factor -1:250*	anti-rabbit IgG-FITC (Sigma F0382) *Dilution factor -1:500*

3.7 Fluorescence-activated cell scanning (FACS)

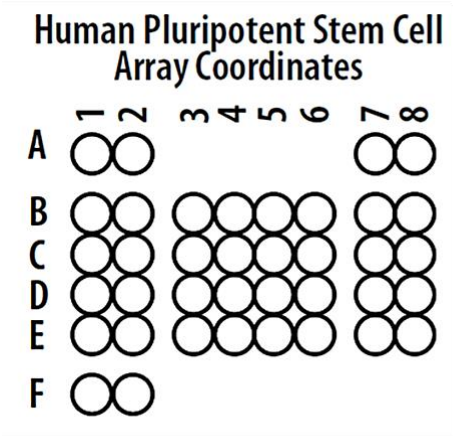
Flow cytometry was utilised for the quantification of total protein levels of TGFβ1 in order to confirm the results shown by western blotting. This technique uses immunodetection whereby specific fluorochrome-coupled secondary antibodies recognise primary antibodies attached to the target protein. The flow cytometer comprises of an argon laser beam which excites fluorochromes attached to cellular proteins to emit light at a particular wavelength. This emission is measured by

an electronic detection apparatus as light scatter and fluorescence intensity, thus allowing for the quantification of target proteins on the cell surface or intracellularly (Hullet et al., 1969). Since the technique uses live cells in suspension, 1X Trypsin/EDTA was used to detach cells as previously mentioned in section 2.1.2. Cells were then centrifuged at 1200 rpm for 10 minutes. Thereafter, the cells were fixed by re-suspension in 2 ml 4 % paraformaldehyde and incubation for 15 minutes at 4 °C. The cells were then washed by centrifuging in 1X PBS at 5000 rpm for 5 minutes. Next, cells were permeabilised using 1 ml 0.1% Triton-X and incubated for 15 minutes at 4 °C. Thereafter, cells were washed by centrifuging in 1X PBS at 5000 rpm for 5 minutes. Afterwards, the cells were incubated with Mouse Anti-LAP (TGF-β1), clone TW4-6H10 (Merk 21848) (dilution factor 1:100) primary antibody overnight at 4 °C. The next day, following three centrifugation wash steps with 1X PBS, the cells were incubated with an anti-mouse IgG-FITC (Sigma F0257) (dilution factor 1:300) coupled secondary antibody at room temperature for an hour in the dark. Furthermore, three more centrifugation wash steps were performed as previously described and cell suspensions were analysed using the BD Accuri C6 flow cytometer. Unlabelled cells or secondary antibody only labelled cells were used as negative controls. All experiments were performed in triplicates, with all antibodies and concentrations used shown in Table 2.4.

3.8 The Proteome Profiler Human Pluripotent Stem Cell Array ™ Identification Kit

The relative expression levels of 15 stem cell markers were determined using the Human Pluripotent Stem Cell Array™ Kit. This kit is a rapid and sensitive method to simultaneously detect the relative levels of 15 stem cell markers (Figure 3.1) without performing numerous individual western blots. The assay was performed according to the manufacturer's protocol. Firstly, the culture media was removed and then the cells were washed with 1XPBS. The cells were then detached using trypsin/EDTA and centrifuged at 4000g to pellet the cells. The pellet was re-suspended at 1 x 10^7 cells/ml in lysis buffer containing 10 µg/ml of Aprotinin (Sigma-Aldrich), 10 µg/ml Leupeptin (Sigma-Aldrich) and 10 µg/ml Pepstatin (Thermofisher) for 30 minutes at 4 ºC. Thereafter the samples were centrifuged at 14 000g for 5 minutes and the supernatant was removed to be used for further experiments. A BCA assay (see section 2.3.2) was then used to quantify protein concentration of the cell lysate to ensure equal loading for subsequent experiments. After activating the nitrocellulose membranes using Methanol (Sigma-Aldrich), that were spotted with capture and control antibodies, for an hour, the membranes were incubated with 300 µg of protein overnight at 4 ºC. The membranes were then washed three times using wash buffer and then incubated for two hours with the detection antibody cocktail. After washing the membranes three times for 10 minutes, they were incubated with Streptavidin-HRP for 30 minutes and then washed

again. Next, a chemiluminescent substrate was added to membranes to form a precipitate that was detected by radiation exposure using the Chemi-doc™ (Biorad). Reference spots were included to align the transparency overlay template and to identify and quantify positive signals. Thereafter, densitometric analysis (Image Lab 4.0) was employed for relative quantification of the target proteins by measuring intensity of the spots. All experiments were performed in duplicates and the Array coordinates are illustrated in figure 3.1. Furthermore, Adipose-derived Mesenchymal Stem Cell protein lysate (ScienCell) was used as a positive control when quantifying protein levels using the Proteome Profiler Human Pluripotent Stem Cell Array ™ Identification Kit. Mesenchymal stem cells were used as a positive control since they are the outcome of EMT, and also due to their critical role in wound healing, where they migrate to the site of injury and proliferate to overlay the wound (Hay, 1990/Hay, 1995).



Coordinates	Target/Control	Role
A1, A2	Reference Spots	Positive Control
A7,A8	Reference Spots	Positive Control
B1,B2	Oct-3/4	Transcription factor
B3, B4	Nanog	Transcription factor
B5, B6,	SOX2	Transcription factor
B7, B8	E-Cadherin	Junction protein-E-Cadherin
C1, C2	Alpha-Fetoprotein	Plasma glycoprotein
C3, C4	GATA-4	Transcription factor
C5, C6	HNF-3 beta/FoxA2	Transcription factor
C7, C8	PDX-1/IPF1	Transcription factor
D1, D2	SOX17	Transcription factor
D3, D4	Otx2	Transcription factor
D5, D6	TP63/TP73L	Transcription factor
D7, D8	Goosecoid (Gsc)	Transcription factor
E1, E2	VEGF R2/KDR/Flk-1	Receptor
E3, E4	HCG	Hormone
E5, E6	Snail	Transcription factor
E7, E8	PBS only	Negative Control
F1, F2	Reference Spots	Positive Control

Figure 3.1: List of stem cell markers in the Proteome Profiler Human Pluripotent Stem Cell Array ™ Identification Kit and their coordinates.

3.9 Alkaline Phosphatase Activity Assay (StemTAG™)

The expression of alkaline phosphatases appear to be dependent on the cellular microenvironment, as well as the differentiation status of cells and tissues which makes alkaline phosphatase (ALP) activity a good differentiation marker (Stefkova et al., 2015). In addition, high levels of ALP activity is considered a marker for pluripotent stem cells as it is highly expressed in embryonic germ cells, ESCs and iPSCs (Stefkova et al., 2015). Hence, the Alkaline Phosphatase Activity Assay (StemTAG™) was performed in order to determine whether cells overexpressing LRP::FLAG expressed this pluripotency marker. Briefly, cells were washed twice with cold PBS and then lysed by incubating cells for 10 minutes with 500 µl of Cell Lysis Buffer (StemTAG). Afterwards, the lysate was centrifuged at 10 500 rpm for 10 minutes then the supernatant was collected. Thereafter, a BCA assay was performed in order to determine the protein concentration of the cell lysate (see section 3.4). Following protein quantification, 50 µl of cell lysate was added to a 96-well plate and cell lysis buffer was used as the blank. The reaction of the alkaline phosphatase enzyme was initiated by adding 50 µl of StemTAG™ AP Activity Assay Substrate and then incubated for 10-30 minutes at 37°C. To stop the reaction, 50 µl of 1X Stop Solution was added and then the plate was placed on an orbital plate shaker for 30 seconds. The absorbance was read using the Multiskan Go ELISA plate reader (Thermo) at 405nm.

3.10 Migration assay

Cell migration is central to achieving functions such as wound repair, cell differentiation and embryonic development. To quantify cell migration, a simple transwell® migration assay was performed (Figure 3.2). Cells were harvested and washed with serum free media. Following this, 1×10^6 cells/ml were then loaded onto the upper chamber of a 24-well transwell® plate (BD Falcon, 8 µm pore size the transwell® inserts) and the lower chamber was filled with 500 µl culture medium containing 20% FBS, which acted as the chemoattractant, for the experimental cell lines. After incubation at 37°C, 5 % CO_2 and in a humidified incubator for 16 hours, both the upper and lower media were removed. The non-migratory cells were then removed with a cotton swab and the migrated cells were fixed with 100 µl of 4 % PFA and rinsed with 100 µl of PBS, followed by staining with 30 µl of 0.5% Toluidine blue for 2 min. The dye was then extracted with 100 µl of 1% SDS for 30–45 minutes. The absorbance of the resultant solutions was then obtained at 620 nm using the Multiskan Go ELISA plate reader (Thermo). Three biological repeats were performed. Images were taken at 0 hours and 24 hours using the Xiaomi Redmi 4A, 13 MP, AF, f/2.2 camera with the ID03 Invertoscope Inverted Microscope (Zeiss).

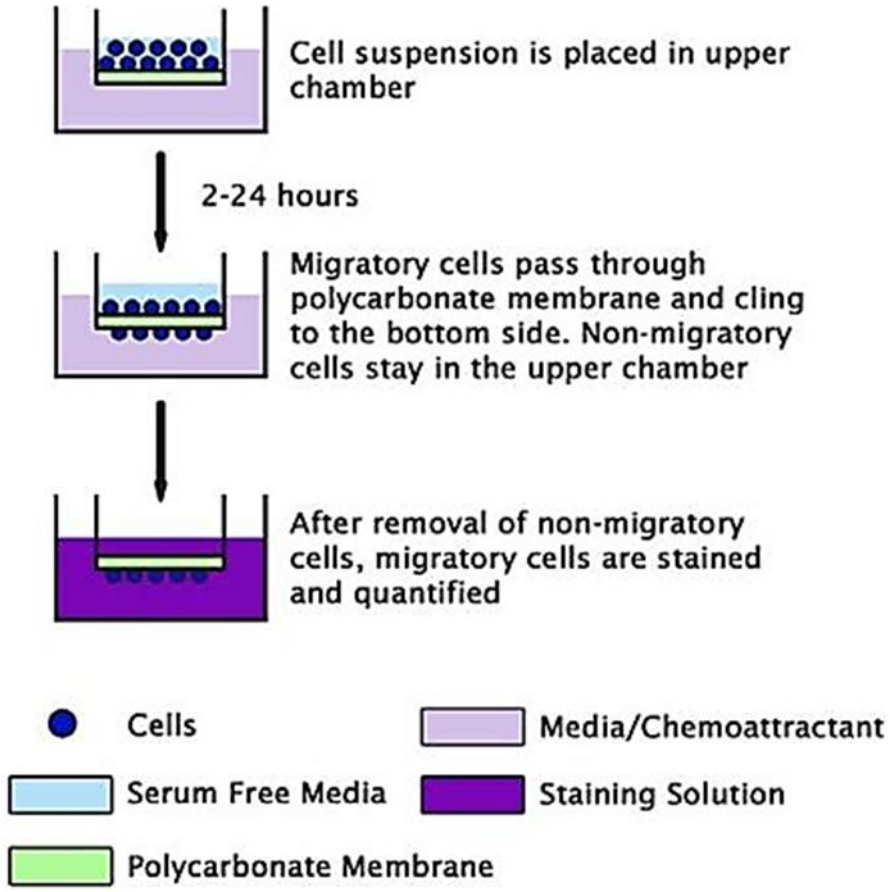


Figure 3.2: Principle of migration assay in order to mimic cell migration during wound healing. Migrating cells are able to move through the pores of the transwell® insert membrane (8μm pore size) as they are drawn toward the chemo-attractant (20% FBS). The migrated cells are then stained. The amount of dye extracted is representative of the number of cells that migrated. (https://d1stxoxfcl3pdw.cloudfront.net/tds/protocol_images/000000499999/MBS168617_TD.jpg)

3.11 Adhesion assay

Cell adhesion is the process by which cells interact and attach to neighbouring cells through specialised molecules of the cell surface. This process can occur either through direct contact between cell surfaces or indirect interaction, where cells attach to the surrounding ECM. Since LRP/LR is a transmembrane protein that has been heavily linked to cell adhesion by binding to the ECM protein laminin-1, changes in the ability of cells overexpressing LRP::FLAG to adhere to lamin-1 were determined. To evaluate the adherence of cells overexpressing LRP::FLAG to the basement membrane, an *in vitro* adhesion assay (Figure 3.3) was performed in 96-well plates coated with laminin-1 (10 μg/mL), with uncoated wells as negative controls. After coating the wells with laminin-1 for 1 hour at 37 °C, the 96-well plate was incubated with 100 μl blocking solution containing 0.5% BSA in DMEM for 1 hour at 37 °C. Then 100 μl of cells were then added to wells

at a cell density of 4×10^5 cells/ml and incubated for 1 hour at 37 °C. Following incubation, cells that did not attach were washed and removed with 1X PBS, while attached cells were fixed in 50 µl of 4% PFA for 10 minutes, prior to staining with 100 µl of 0.1% crystal violet for 10 minutes. The dye was then extracted with 100 µl of 2% SDS and the absorbance was measured at 550 nm using Multiskan Go ELISA plate reader (Thermo). This absorbance was an indicator of cells that were attached to laminin-1. Biological repeats were performed in triplicate.

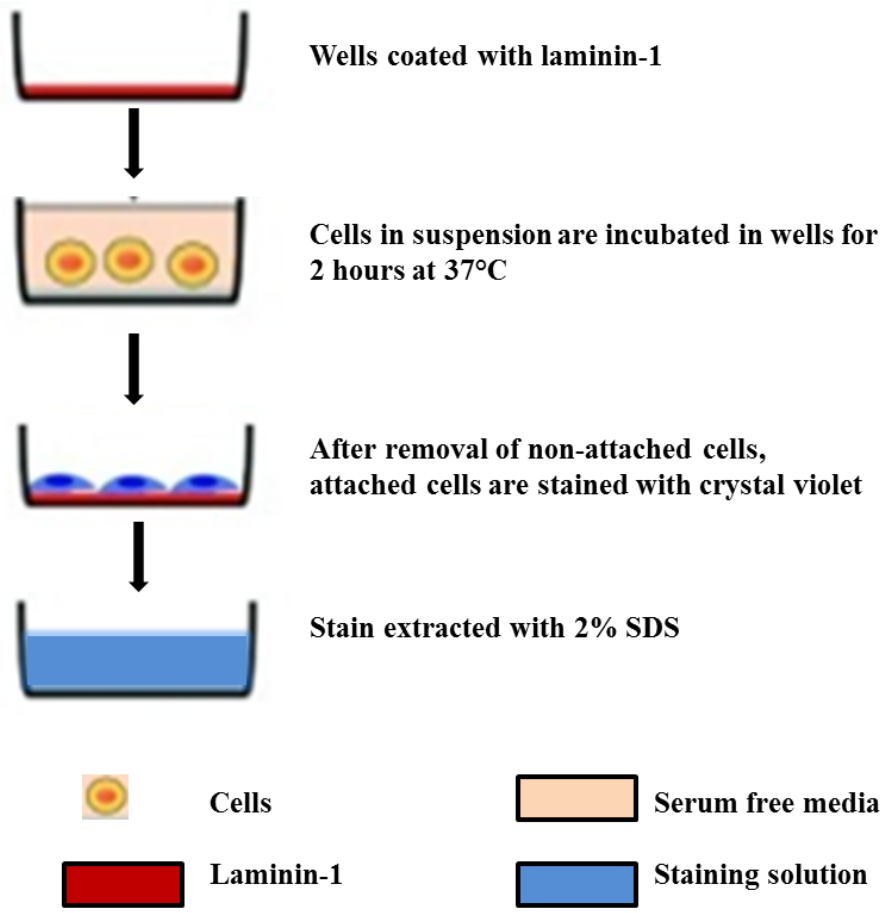


Figure 3.3: Principle of the Adhesion assay. The assay quantifies cell adhesive potential using Laminin-1 as the substrate. Attached cells are then stained and the absorbance is measured. The amount of dye extracted is a representation of the number of cells that had attached.

3.12 Scratch assay/*in vitro* wound healing assay

The *in vitro* scratch assay is a simple economic method to study cell migration. To some extent, this assay mimics the migration of cells that occurs during wound healing *in vivo*. This method is based on the observation that, upon creation of a new artificial gap from scratching off cells on a confluent cell monolayer leads the cells on the edge of the newly created gap to move toward the opening to close the "scratch" until new cell-cell contacts are established again. This technique was utilised to

determine the effect of overexpressing LRP::FLAG on wound healing. In a 6well plate (Corning), 500 µl of cells were seeded at a density of 1 x 10^6 cells/ml then cultured till confluency at 37 °C in a humidified atmosphere containing 5 % CO_2. Thereafter, a linear "wound" was subsequently generated in the monolayer using a sterile 200 µl plastic pipette tip to produce an approximately 1 mm wide scratch. Any cellular debris was removed by washing the wells with 1X PBS. Thereafter the plates were incubated for 24 hours at 37 °C, 5 % CO_2 and in a humidified incubator. Images were taken at 0 hours and 24 hours using the ID03 Invertoscope Inverted Microscope (Zeiss) with the Xiaomi Redmi 4A, 13 MP, AF, f/2.2 camera.

3.13 Data analysis and statistical evaluations

The experimental data obtained were statistically analysed using Graph Prism Version 5 (Graphpad Software, Inc). A two-tailed student's *t*-test with a confidence interval of 95 % was used for analysis, where values *$p<0.05$ are considered significant, values **$p<0.01$ are considered more significant and ***$p<0.001$ are considered very significant. All experiments were carried out in biological triplicates unless otherwise mentioned, to ensure data validity.

4 Results

4.1 Confirmation of LRP::FLAG overexpression in HEK293 and MRC5 cells

In order to investigate whether LRP/LR plays a role in maintaining stem cells and wound healing, HEK293 and MRC5 cells were stably transfected with the pCIneo-moLRP-FLAG plasmid (Vana and Weiss, 2006). Western blotting was employed in order to determine total LRP/LR protein levels within the cells and to confirm whether the cells were expressing LRP::FLAG. β-actin was used as the loading control (Figure. 4.1). Figure 4.1 shows that the HEK293 (Figure. 4.1A) and MRC 5 (Figure. 4.1B) cells were successfully transfected as seen by the detection of LRP::FLAG protein (Figure. 4.1 lanes 4-6). Moreover, by overexpressing LRP::FLAG a significant increase in the 37kDa LRP protein levels was observed in both cell lines. Although the 37kDa LRP is a precursor for the 67kDa LR there was no significant change in the 67kDa LR protein levels for both cell lines which is similar to what has been previously reported by Rao (1989) and Vana & Weiss, (2006).

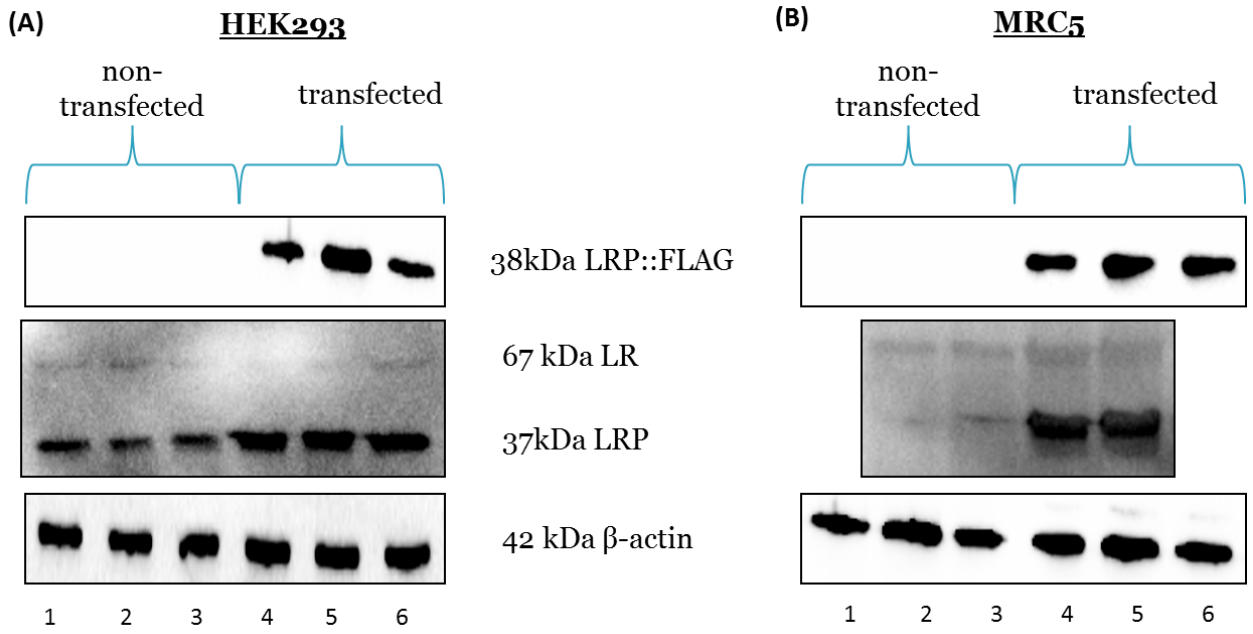


Figure 4.1: Overexpression of LRP::FLAG in HEK293 and MRC5 cells transfected with the pCIneo-moLRP-FLAG plasmid. Western blotting shows non-transfected cells (lanes 1-3) and cells transfected with the pCIneo-moLRP-FLAG plasmid (lanes 4-6) for both HEK293 (A) and MRC5 (B) cells. (A and B) LRP::FLAG is detected in transfected HEK293cells and MRC5 cells and not in non-transfected HEK293 and MRC5 cells. An increase in 37kDa LRP and not 67kDa LR is observed in both cell lines. Anti-FLAG and anti-mouse IgG-HRP was used to detect LRP::FLAG, W3 and anti-rabbit IgG-HRP detected LRP and β-actin (HRP) was used as the loading control.

4.2 Overexpression of LRP::FLAG increases hTERT expression

According to literature, hTERT, which is the limiting factor for telomerase activity, is often up-regulated in rapidly dividing cells (Bodnar et al., 1998; Kilian, et al., 1997). This includes both adult and embryonic stem cells where its high expression is often used as a trait for pluripotency or multipotency (Flores and Blasco, 2010). Western blotting was performed to determine whether overexpression of LRP::FLAG would affect levels of hTERT and its active form phospho-TERT (pS824). Western blotting quantified by densitometric analysis revealed a significant increase in hTERT and phospho-TERT (pS824) protein levels in transfected HEK293 and MRC5 cells when compared to non-transfected cells (Figure. 4.2). The telomerase positive HEK293 cells showed a significant increase in protein levels of the 90 kDa, 120 kDa and phosphorylated 126 kDa hTERT isoforms of 442%, 247% and 125% respectively post transfection (Figure. 4.2A2). MRC5 cells with negligible telomerase also showed a significant increase in hTERT protein levels of the 90 kDa, 120 kDa and phosphorylated 126kDa hTERT isoforms post transfection of 199%, 165% and 127% respectively (Figure. 4.2B2).

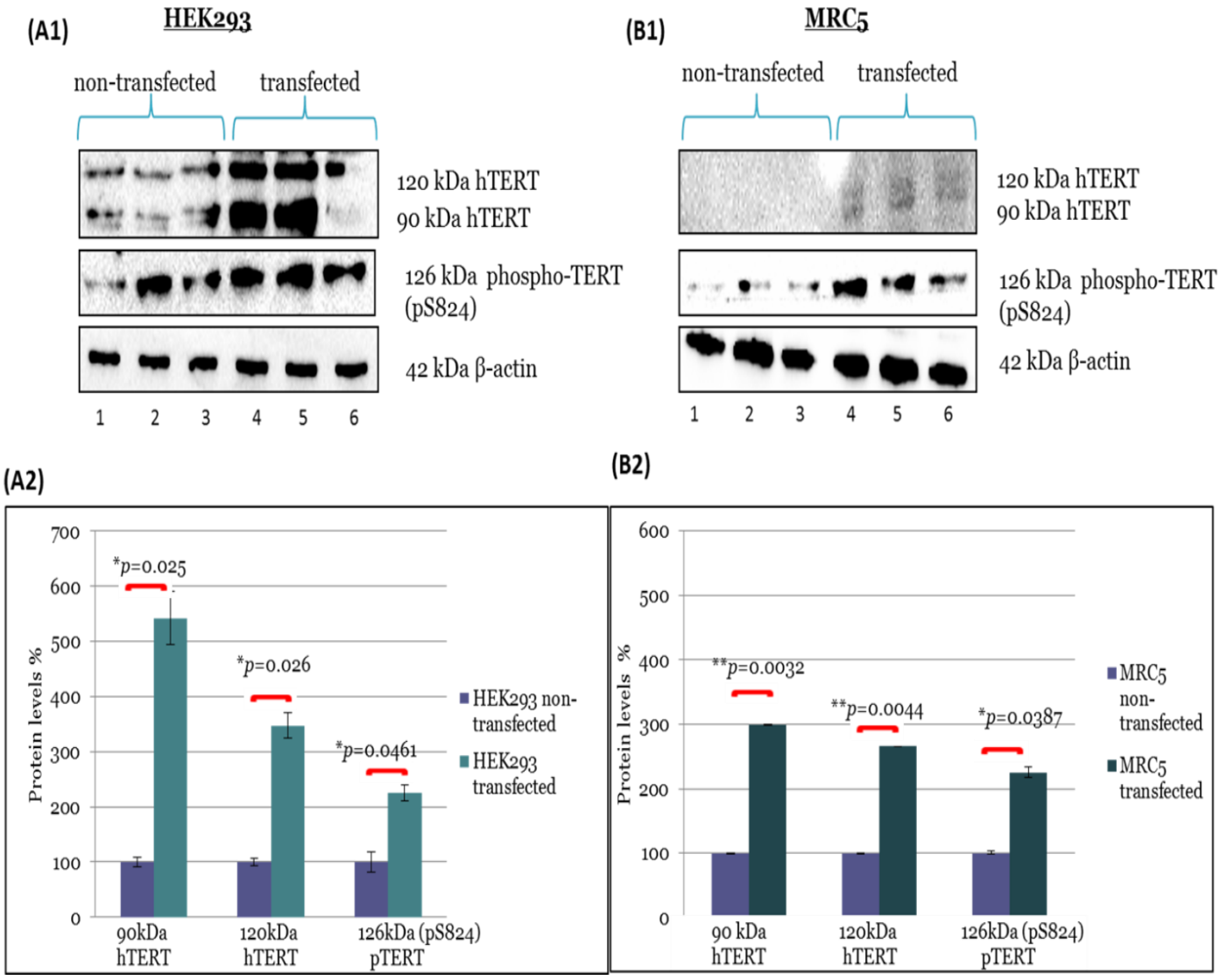


Figure 4.2: Overexpression of LRP::FLAG increases hTERT and phospho-TERT levels in HEK293 and MRC5 cells. Western blot analysis was performed and lanes 1-3 show non-transfected samples and lanes 4-6 samples transfected with the pCIneo-moLRP::FLAG plasmid for both HEK293(A1) and MRC5 (B1) cells. Anti-hTERT and anti-rabbit IgG-HRP was used to detect hTERT, anti- phospho-TERT (pS824) and anti-rabbit IgG-HRP detected phospho-TERT (pS824) and β-actin (HRP) was used as the loading control. Densitometric analysis performed on the western blots was relative to the non-transfected HEK293 (A2) and MRC5 (B2) cells set to 100 %. Error bars represent standard deviation, n=3 biological repeats. *p: 0.05, **p: 0.01, ***p: 0.001 N.S.: non-significant; Student's t-test.

Immunofluorescence microscopy was employed to confirm the expression of LRP and hTERT in the cells. In figure 4.3, red fluorescence represents hTERT, green fluorescence represents LRP and

blue fluorescence represents DAPI nuclear staining. The merged panels are a combination of DAPI staining and either LRP or hTERT. In comparison to the non-transfected HEK293 cells (Figure. 4.3A1), an increase in both LRP and hTERT expression in transfected HEK293 cells (Figure. 4.3A2) was observed. Likewise, transfected MRC5 cells (Figure. 4.3B2) showed an increase in the expression of LRP in comparison to non-transfected MRC5 cells (Figure. 4.3B1). In addition, non-transfected MRC5 cells (Figure. 4.3B1) showed no-detectable expression of hTERT as has previously been shown by Otgaar et al., (2017) and there was a detectable expression of hTERT observed in MRC5 cells post-transfection with the pCIneo-moLRP-FLAG plasmid, (Figure. 4.3B2) consistent with previously seen results.

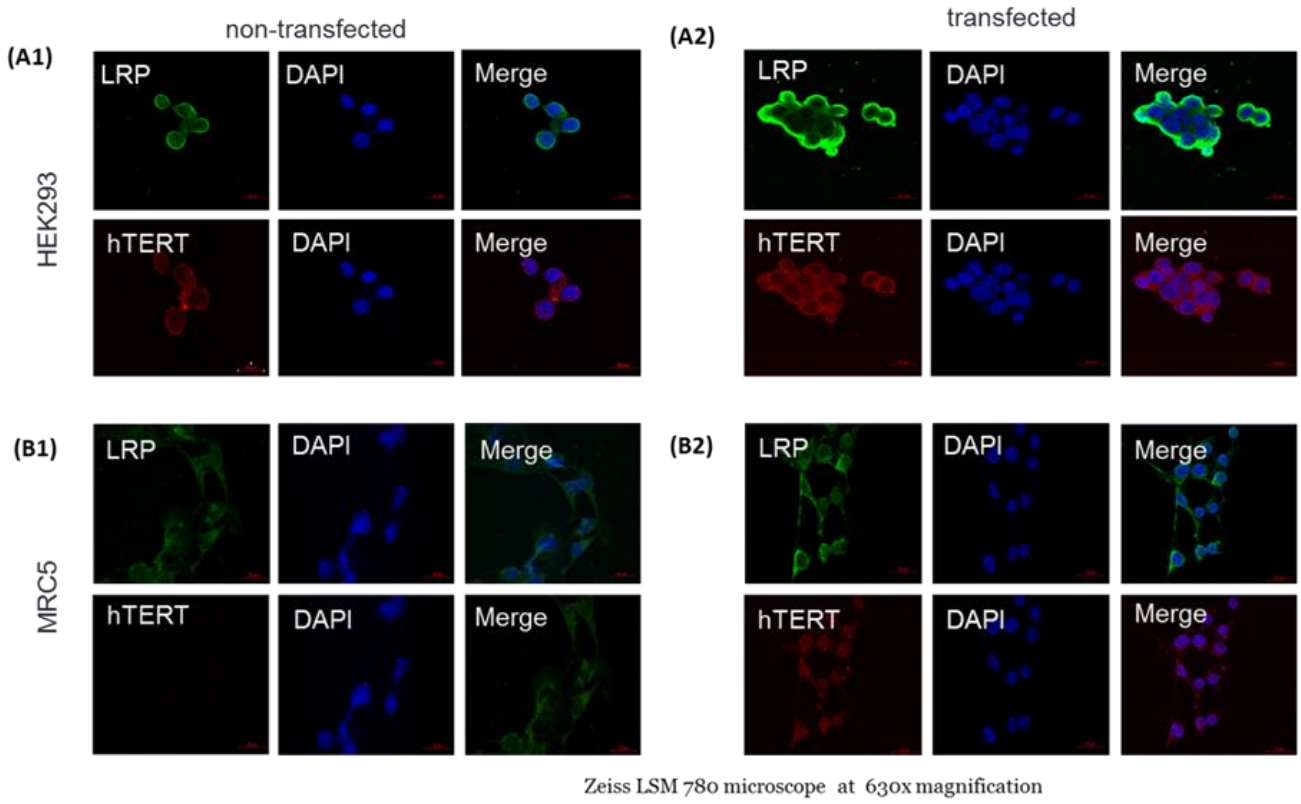


Figure 4.3: Intracellular expression of LRP and hTERT in transfected and non-transfected HEK293 and MRC5 cells. (A) Expression of total LRP and hTERT increases in transfected HEK293 cells (A2) in comparison to non-transfected HEK293 cells (A1). (B) Expression of total LRP and hTERT increases in transfected MRC5 cells (B2) in comparison to non-transfected MRC5 cells (B1). Anti-LRP/LR IgG1-iS18 and anti-human IgG-FITC was used to detect LRP, anti-hTERT and anti-mouse IgG-CFTM647 detected hTERT and nuclei are stained with DAPI. All images were taken with the Zeiss LSM 780 microscope at 630x magnification. Scale bars represent 20 μm.

4.3 LRP::FLAG overexpression promotes Epithelial to Mesenchymal Transition (EMT)

EMT is essential for various developmental processes and for the facilitation of regrowth during wound repair as well as tissue regeneration (Hay, 1995). For somatic cells to dedifferentiate into mesenchymal stem cells, they have to undergo EMT characterised by loss of expression of epithelial markers, such as E-Cadherin or α-E-catenin and gain in expression of mesenchymal markers, such as Vimentin or N-Cadherin among others (Hay, 1990/95). The protein levels of α-E-catenin, N-Cadherin and Vimentin were evaluated using western blotting illustrated in Figure. 4.4 and immunofluorescence microscopy illustrated in Figure 4.5. Figure 4.4A and 4.5A confirm that HEK293 cells have a neural phenotype (Lin et al., 2014) as seen by the lack of expression of the fibroblast marker, α-E-catenin, In addition, MRC5 cells were confirmed as fibroblasts cells (Jacobs et al., 1970) (Figure. 4.4B and 4.5B) as they have detectable levels of α-E-catenin at 102 kDa. In HEK293 cells, overexpression of LRP::FLAG significantly increased total N-Cadherin and Vimentin protein levels by 120 % and 330 % respectively (Figure. 4.4A2) when compared to non-transfected cells. Moreover, there was an increase in the cytosolic truncated 45kDa N-Cadherin and no detectable levels of the extracellular truncated 90kDa N-Cadherin and full-length 130kDa N-Cadherin. This implies that N-Cadherin shedding, which is the cleavage of full-length N-Cadherin into a cytosolic 45kDa N-Cadherin fragment and extracellular 90kDa N-Cadherin fragment, had occurred. Since no detectable levels of the 90kDa N-Cadherin were observed in the transfected HEK293 cells, this implies that the extracellular 90kDa fragment was released from the cell membranes. The 90kDa N-Cadherin fragment retains biological function similar to the full-length N-Cadherin. However, the functional importance of the cytosolic fragment is unknown (Reiss et al., 2005). In addition, overexpression of LRP::FLAG in MRC5 cells also significantly increased total N-Cadherin and Vimentin protein levels by 340 % and 37 % respectively but, decreased α-E-catenin expression by 94 % (Figure. 4.4B2). The increase in mesenchymal markers indicates that overexpression of LRP::FLAG promotes the mesenchymal phenotype which has similar markers to those of mesenchymal cells. Moreover, transfected MRC5 cells also exhibit N-Cadherin shedding; where they did not express the truncated 45kDa isoform of N-Cadherin and conversely, they expressed the 90kDa N-terminal variants of N-Cadherin strongly in association with the 130kDa full-length N-Cadherin (Figure. 4.4A1). Therefore, the suppression of epithelial markers and an increase in the mesenchymal markers suggests that overexpression of LRP::FLAG promotes N-Cadherin shedding and induces EMT.

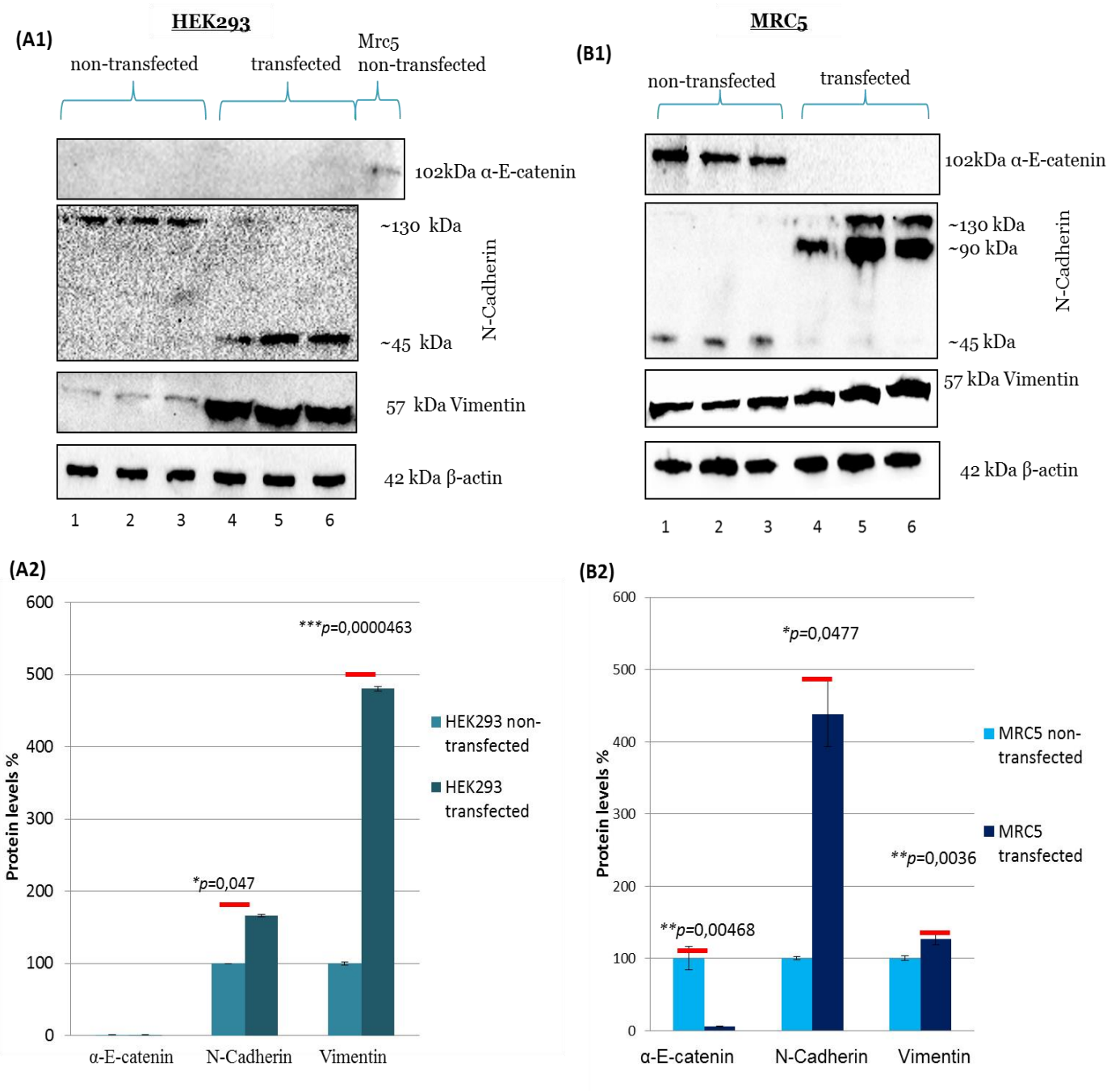


Figure 4.4: Overexpression of LRP::FLAG increases α-E-catenin, N-Cadherin, and Vimentin protein levels in HEK293 and MRC5 cells. . Lanes 1-3 show non-transfected samples and lanes 4-6 shows samples transfected with the pCIneo-moLRP-FLAG plasmid for both HEK293 (A1) and MRC5 (B1) cells. Anti-α-E-catenin and anti-mouse IgG-HRP was used to detect α-E-catenin, anti-N-Cadherin and anti-mouse IgG-HRP detected N-Cadherin, anti-Vimentin and anti- chicken IgG-HRP detected Vimentin and β-actin (HRP) was used as a loading control. Densitometric analysis performed on the western blots was relative to the non-transfected HEK293 (A2) and MRC5 (B2) cells set to 100 % (with the exception of α -E-catenin levels in HEK293 cells where no protein expression was detected). The quantity of the three variants of N-Cadherin were combined and

represented in one bar. Error bars represent standard deviation, n=3 biological repeats. *p: 0.05, **p: 0.01, ***p: 0.001 N.S.: non-significant; Student's t-test.

To confirm the results shown using western blotting, immunofluorescence microscopy was performed (Figure. 4.5). α-E-catenin (red) fluorescence was only observed in the non-transfected MRC5 cells (Figure. 4.5B1). There was also an increase in the expression of N-Cadherin (green fluorescence) and Vimentin (green fluorescence) in both HEK293 and MRC5 cells post-transfection with the pCIneo-moLRP-FLAG plasmid (Figure. 4.5B1 and 2).

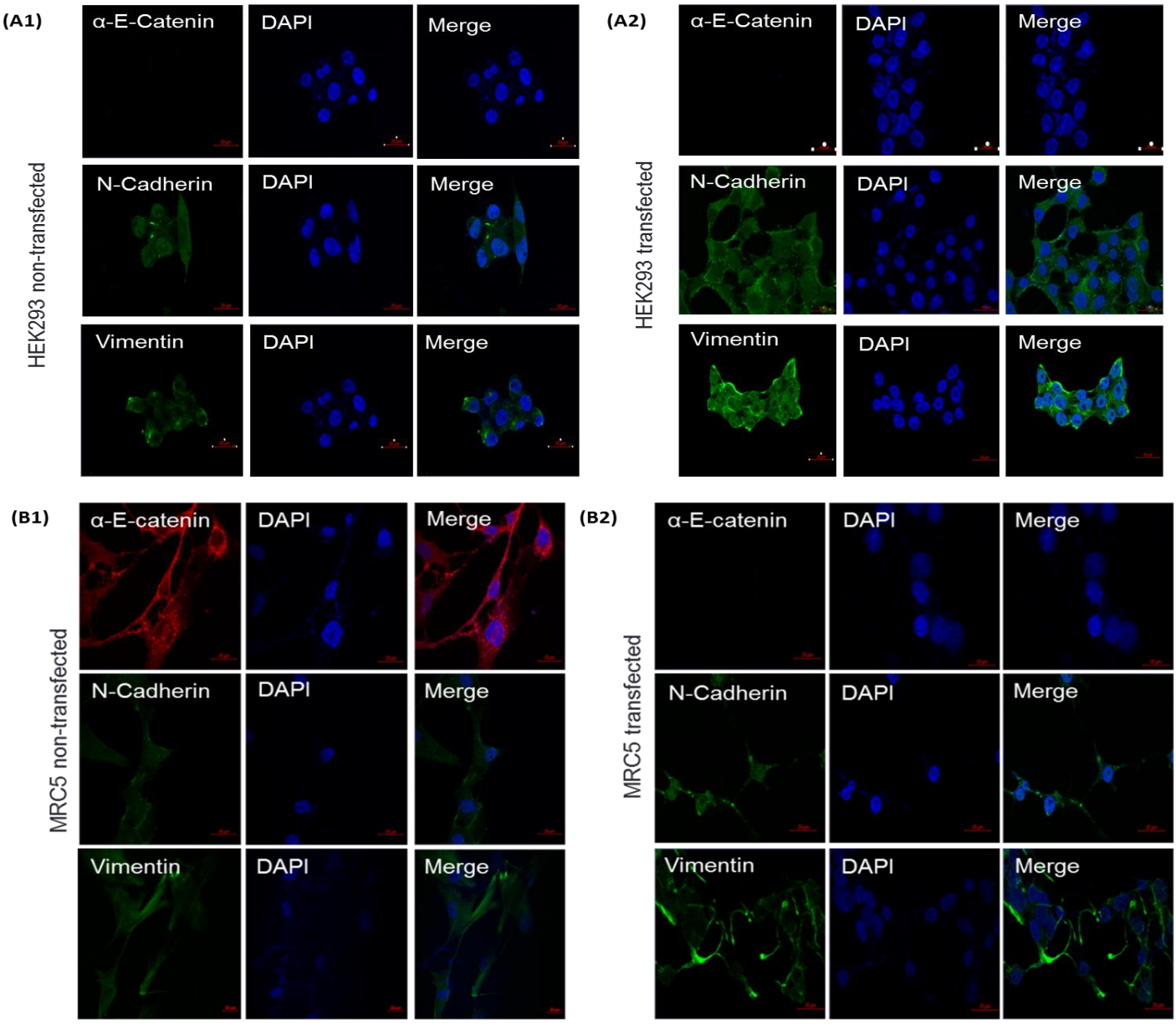


Figure 4.5: Intracellular expression of α-E-catenin, N-Cadherin, and Vimentin in HEK293 and MRC5 cells as determined by confocal microscopy. (A) No α-E-catenin expression in both non-transfected (A1) and transfected HEK293 cells (A2). Expression of total N-Cadherin and

Vimentin increases in transfected HEK293 cells (A2) in comparison to non-transfected HEK293 cells (A1). (B) Expression of total α-E-catenin decreases and total N-Cadherin and Vimentin increases in transfected MRC5 cells (B2) in comparison to non-transfected MRC5 cells (B1). Anti-α-E-catenin and anti-mouse IgG-CFTM647was used to detect α-E-catenin, anti-N-Cadherin and anti-mouse IgG-FITC detected N-Cadherin and anti-Vimentin and anti-chicken IgG-FITC detected Vimentin and the nuclei are stained with DAPI. All images were taken with the Zeiss LSM 780 microscope at 630x magnification. Scale bars represent 20 μm.

4.4 Overexpression of LRP::FLAG significantly increases total levels of stem cell markers

We investigated the levels of pluripotency-associated proteins using the Human Pluripotent Stem Cell Array kit which measures the protein levels of 15 pluripotent stem cell markers. After transfection, most of the proteins were upregulated in HEK293 cells and mostly downregulated in MRC5 cells (Figure.4.6). Of note, Oct3/4, NANOG and SOX2, which are the main transcription factors (TF) for induced cell reprogramming were upregulated in HEK293 cells (Figure. 4.6A) (Takahashi and Yamanaka, 2006). The highest increase in protein levels was observed for the TF GATA4 and the hormone HCG, while the lowest increase observed in this cell line was for the EMT associated TF, Snail (Gout et al., 2008). For MRC5 cells, an increase in protein levels was observed for the reprogramming factors Oct3/4 and SOX2. However, NANOG; which is one of the key transcription factors involved in cell reprogramming (Takahashi and Yamanaka, 2006), was downregulated in cells overexpressing LRP::FLAG. The highest increase in protein levels was observed for the TFs Oct3/4 and GATA4 and the lowest decrease observed in this cell line was also the TF Snail (Figure 4.6B) similar to HEK293 cells. Of interest is the decrease observed in E-Cadherin which is known to be downregulated during EMT (Gout et al., 2008). The list of stem cell markers and their coordinates is referred to in Figure 3.1.

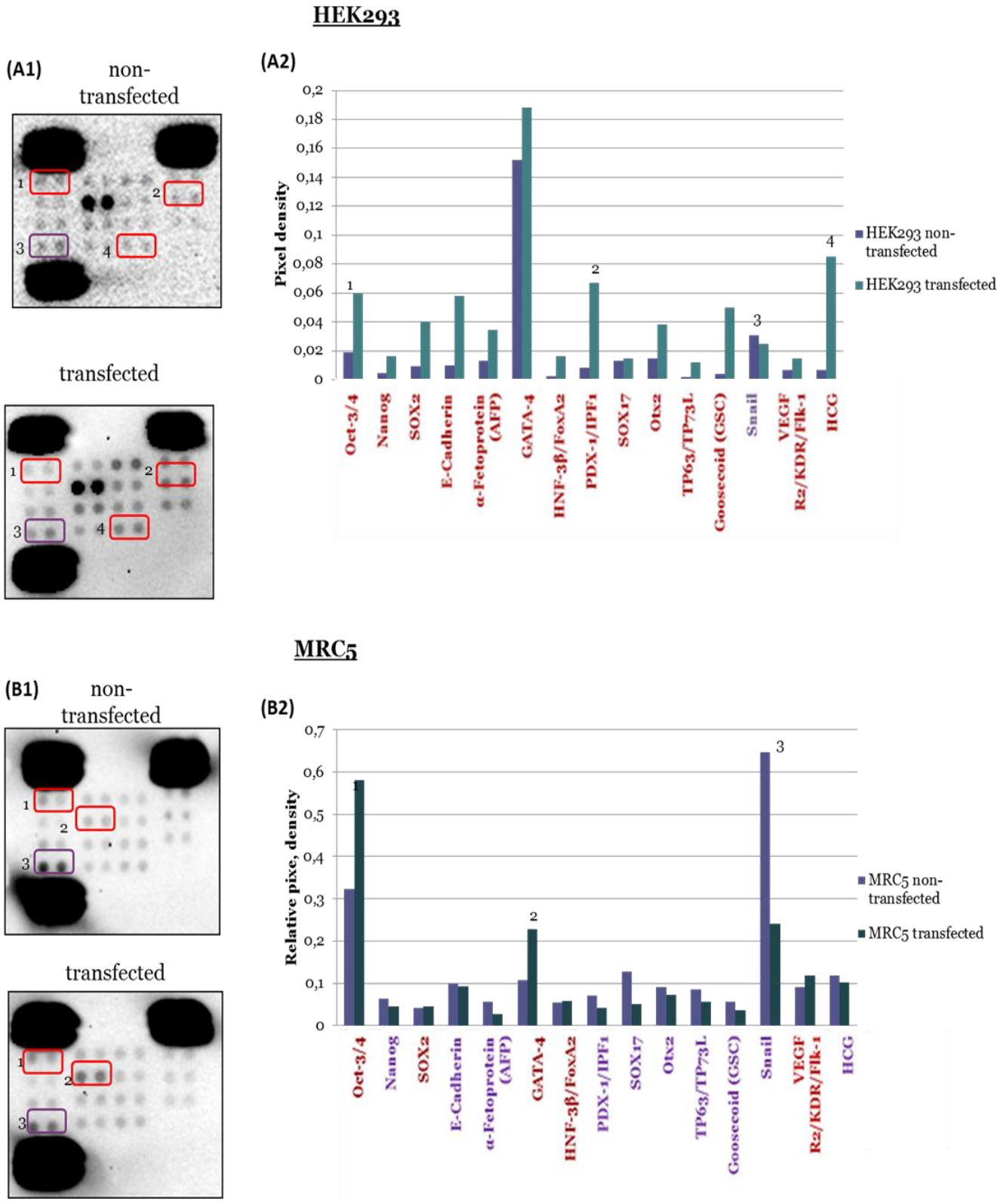


Figure 4.6: Analysis of 15 pluripotency markers in non-transfected and transfected HEK293 and MRC5 cells using the Human Pluripotent Stem Cell Array kit. (A1) Array of pluripotency markers expressed in HEK293 cells. (A2) Densitometric analysis performed for HEK293 cells was relative to the positive control reference spots. Proteins that were most significantly upregulated (1),(2) and (4) plus most significantly downregulated proteins (3) in HEK293 cells. (B1) Array of

pluripotency markers expressed in MRC5 cells. (B2) Densitometric analysis performed for MRC5 cells was relative to the positive control reference spots. Proteins that were most significantly upregulated (1) and (2) plus most significantly downregulated proteins (3) in MRC5 cells. Purple boxes/text denotes proteins that are downregulated and the red boxes/text indicate proteins that are upregulated.

In order to determine whether the expression profiles of cells overexpressing LRP::FLAG was similar to that of stem cells, we compared the protein levels of both cell lines to the protein levels of AMScs using the Human Pluripotent Stem Cell Array kit. The transfected HEK293 and MRC5 cells mostly exhibited lower protein levels of the 15 pluripotency markers when compared to AMScs (Figure. 4.7; Table 4.1). Although the levels most pluripotent markers was increased in transfected HEK293cells compared to non-transfected HEK293 cells, only GATA4 and HCG levels were higher than those in AMSc. However, MRC5 cells overexpressing LRP::FLAG showed increased expression of more markers which include: Oct-3/4, GATA4, Otx2, Snail, VEGF R2 and HCG regardless of whether they were downregulated or upregulated in comparison to non-transfected MRC5 cells (Figure. 4.7; Table 4.1). This implies that the levels of pluripotency markers may vary between adult multipotent stem cells and foetal cell lines however; this does not determine their cell phenotype or function.

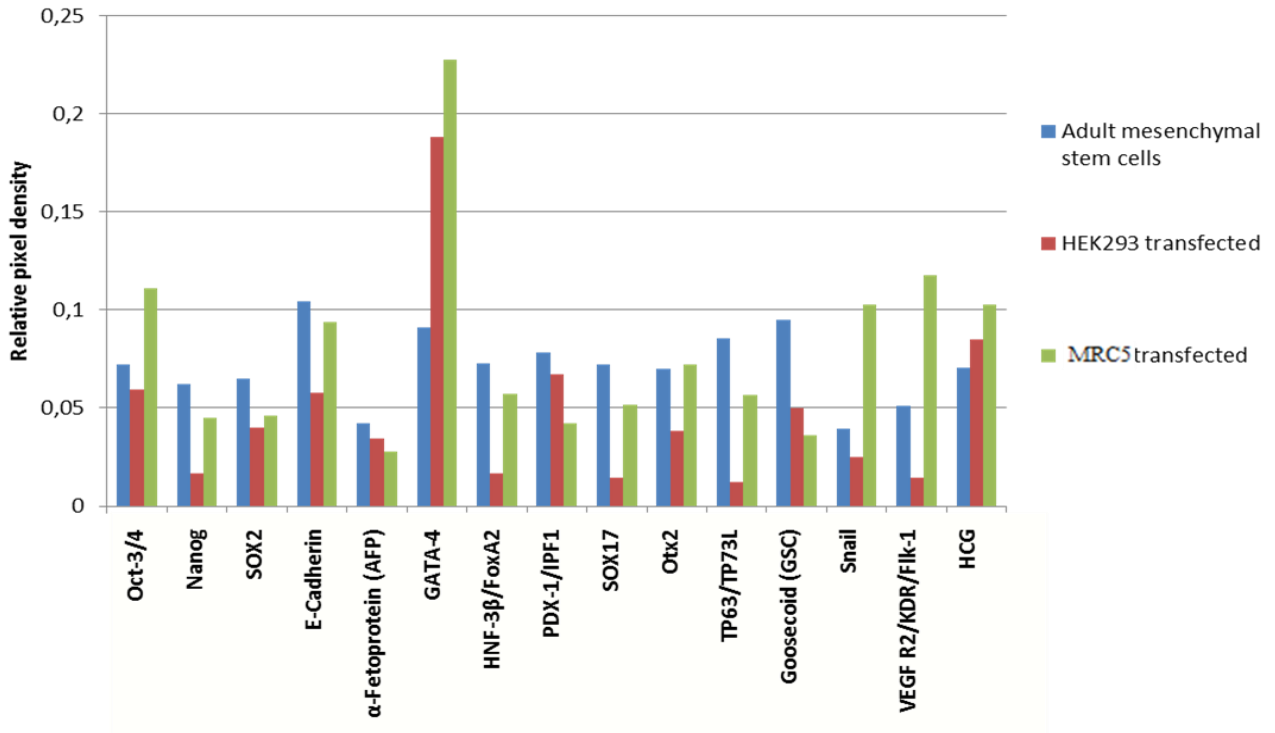


Figure 4.7: Comparison of protein levels of various pluripotent stem cell markers in Adult Mesenchymal Stem cells to protein levels of transfected HEK293 and MRC5 cells using the

Human Pluripotent Stem Cell Array kit. Adult mesenchymal stem cells are compared to HEK293 cells and MRC5 cells, both transfected with the pCIneo-moLRP-FLAG plasmid. Densitometric analysis performed on the blots was relative to the positive control reference spots.

Table 4:1: Illustrating list of pluripotent stem cell markers that are upregulated or downregulated in transfected HEK293 and MRC5 cells compared to AMScs. The protein levels of 15 pluripotency markers in AMScs, transfected HEK293 and transfected MRC5 cells were compared using the Human Pluripotent Stem Cell Array kit. List of upregulated/downregulated markers are given in comparison to the relative pixel density of AMScs.

Cell Line	Upregulated Markers	Downregulated Markers
HEK293	GATA-4 HCG	Oct-3/4 NANOG SOX2 E-Cadherin α-Fetoprotein (AFP) HNF-3β/FoxA2 PDX-1/IPF1 SOX17 Otx2 TP63/TP73L Goosecoid (GSC) Snail VEGF R2/KDR/Flk-1
MRC5	Oct-3/4 GATA-4 Otx2 Snail VEGF R2/KDR/Flk-1 HCG	NANOG SOX2 E-Cadherin α-Fetoprotein (AFP) HNF-3β/FoxA2 PDX-1/IPF1 SOX17 TP63/TP73L Goosecoid (GSC)

4.5 Overexpression of LRP::FLAG increases alkaline phosphatase activity in HEK293 cells.

It has previously been suggested that high levels of ALP activity may be considered a marker for pluripotency as it is highly expressed in embryonic germ cells, ESCs and iPSCs (Stefkova et al., 2015). Hence, the Alkaline Phosphatase Activity Assay (StemTAG™) was performed in order to determine whether overexpressing LRP::FLAG would affect the activity of alkaline phosphatase in HEK293 cells since they showed an increase in most of the pluripotency markers. A significant increase in ALP activity was observed in cells overexpressing LRP::FLAG (Figure 4.8), which indicates that transfected HEK293 cells may have been transformed to pluripotent stem cells.

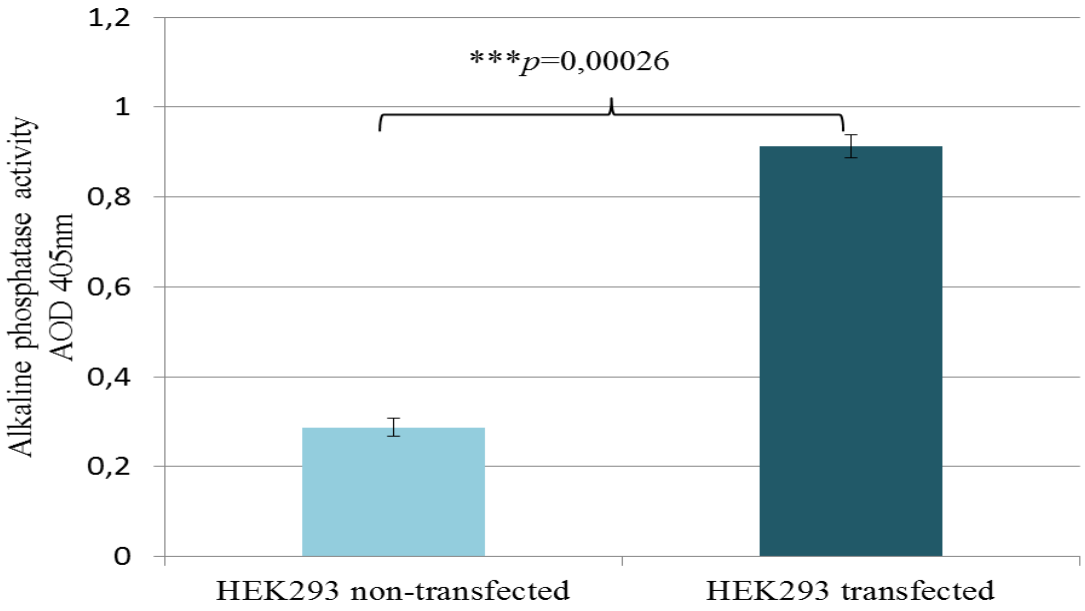


Figure 4.8: Overexpression of LRP::FLAG increases alkaline phosphatase activity in HEK293 cells. Alkaline phosphatase activity increases in HEK293 cells transfected with the pCIneo-moLRP-FLAG plasmid compared to the non- transfected HEK293 cells. Error bars represent standard deviation, n=3 biological repeats. *p <0.05, **p < 0.01, ***p < 0.001, Student's t-test.

4.6 Overexpression of LRP::FLAG promotes wound healing by decreasing the adhesive potential and increasing cell migration of HEK293 cell.

Wound healing involves the migration of cells into the wound bed. In particular, stem cells need to detach from the stem cell niche and migrate to the site of injury where they proliferate to fill the wound bed. Therefore, the migratory and adhesive potential of cells overexpressing LRP::FLAG was investigated. A modified Boyden's chamber migratory assay showed a significant increase in the migration of HEK293 cells towards the chemoattractant. After staining the migrated cells with crystal violet, light microscopy showed more migrated cells on the transwell® membrane of

transfected HEK293 cells in comparison to the transwell® membrane of non-transfected HEK293 cells (Figure 4.9 A1). Extraction of the dye and quantification using an ELISA reader showed that there was 68% more cell migration in transfected HEK293 cells compared to non-transfected HEK293 cells (Figure 4.9 A2). In addition, an adhesion assay with laminin-1 as the adhesive substrate revealed a significant decrease in adhesion to laminin-1 by 42% in transfected HEK293 cells (Figure 4.9 C). Moreover, a scratch assay, also known as the *in vitro* wound healing assay, showed an increased closing of the scratch after 24hours (Figure 4.9 B). This suggests that the increase in cell migration and decrease in adhesiveness of transfected HEK293 cells may promote the proliferative phase of wound healing and the recruitment of immune cells during the inflammatory phase.

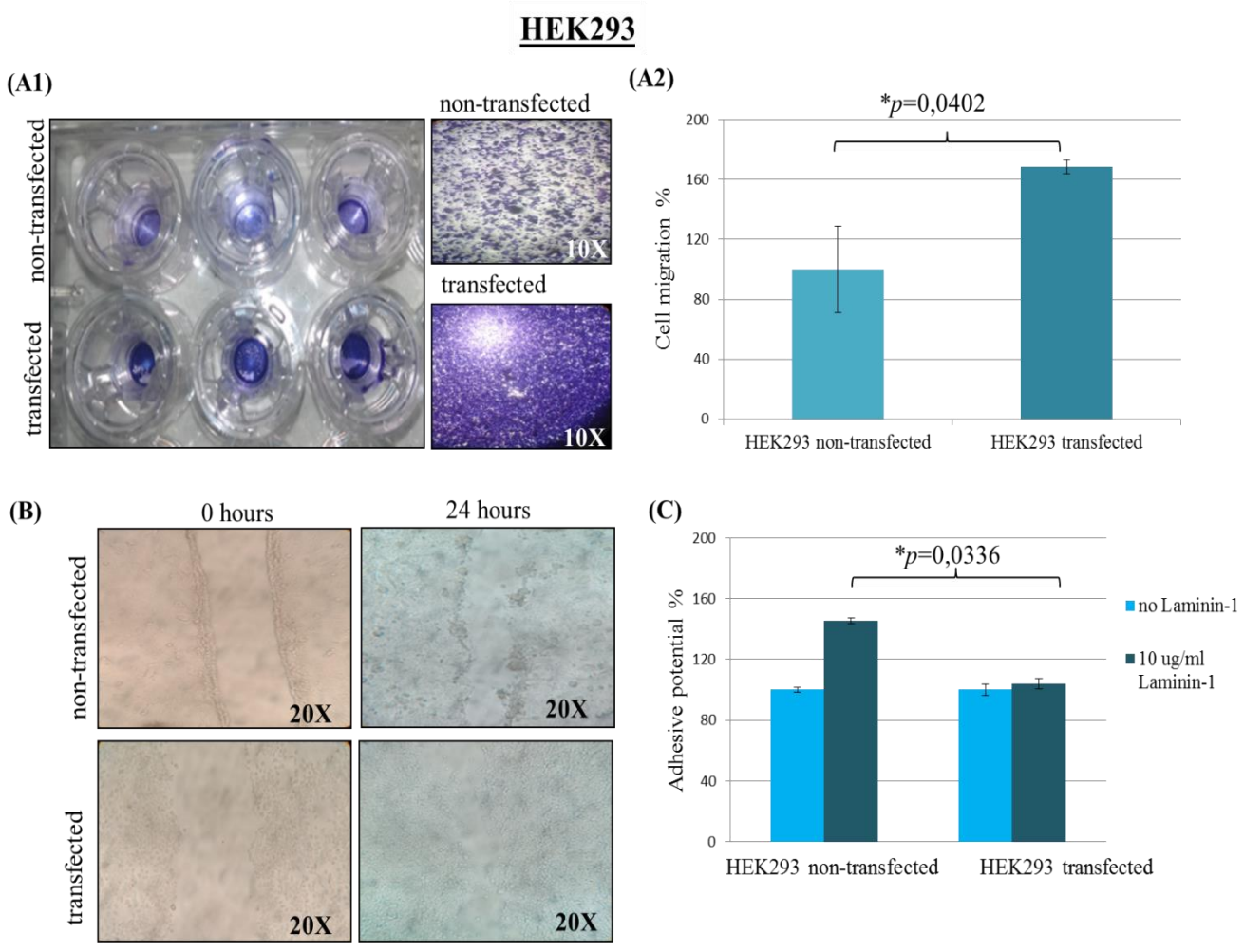


Figure 4.9: Overexpression of LRP::FLAG increases the migratory potential and decreases the adhesive potential of HEK293 cells. (A) An increase in cell migration was observed in transfected HEK293 cells compared to non-transfected HEK293 cells. (A1) Cells are stained with 0.5% Toluidine blue and images were taken at 10x magnification. (A2) Absorbance of dye was measured at 550nm and analysis performed on the wells was relative to the non-transfected

HEK293 cells set to 100 %. (B) Increased wound gap closure was observed after 24 hours in transfected HEK293 cells compared to non-transfected HEK293 cells and images were taken at 20x magnification. (C) A decrease in cell adhesion was observed in transfected HEK293 cells compared to non-transfected HEK293 cells. Analysis performed was relative to the no laminin-1 non-transfected HEK293 and transfected HEK293cells set to 100 %. All images were taken with Xiaomi Redmi 4A, 13 MP, AF, f/2.2 camera with the ID03 Invertoscope Inverted Microscope (Zeiss). Error bars represent standard deviation, n=3 biological repeats. *p< 0.05, **p<0.01, ***p< 0.001, Student's t-test.

4.7 LRP::FLAG overexpression promotes expression of anti-inflammatory cytokines

We investigated changes in the levels of TGFβ1 and NF-κB in order to determine whether the overexpression of LRP::FLAG had an effect of inflammatory markers. TGFβ1 and NF-κB are important cytokines that regulate inflammatory responses (Li and Tergaonkar, 2014). TGFβ1 and NF-κB signalling are important as they recruit other cytokines to the site of injury. Therefore, we analysed the levels of these proteins using western blotting (Figure. 4.10), and expression, using immunofluorescence microscopy (Figure.4.11). Western blotting quantified by densitometric analysis revealed no significant difference in the pro-inflammatory cytokine, NF-κB for HEK293 cells (Figure. 4.10A) post-transfection. However, there was a significant 456% increase in the total levels of the pro-inflammatory cytokine TGFβ1. Moreover, both the monomeric 12.5 kDa and dimeric 25 kDa variants of activated TGFβ1 were observed (Figure. 4.10A1). This result was confirmed using flow cytometry whereby an increase of 416% in TGFβ1 was observed (Figure 4.10C) in HEK293 cells. Furthermore, the presence of a double peak in the transfected HEK293 cells possibly denotes the presence of both the dimeric and monomeric isoforms of TGFβ1. Likewise, Figure 4.8B also suggests that a similar trend is observed for TGFβ1 and NFκB expression in MRC5 cells. No significant change in NF-κB was observed and there was a 146% increase in monomeric TGFβ1 levels for MRC5 cells overexpressing LRP::FLAG (Figure. 4.10B2). These results imply that overexpression of LRP::FLAG promotes expression of the anti-inflammatory marker, TGFβ1 and has no significant effect on NF-κB expression.

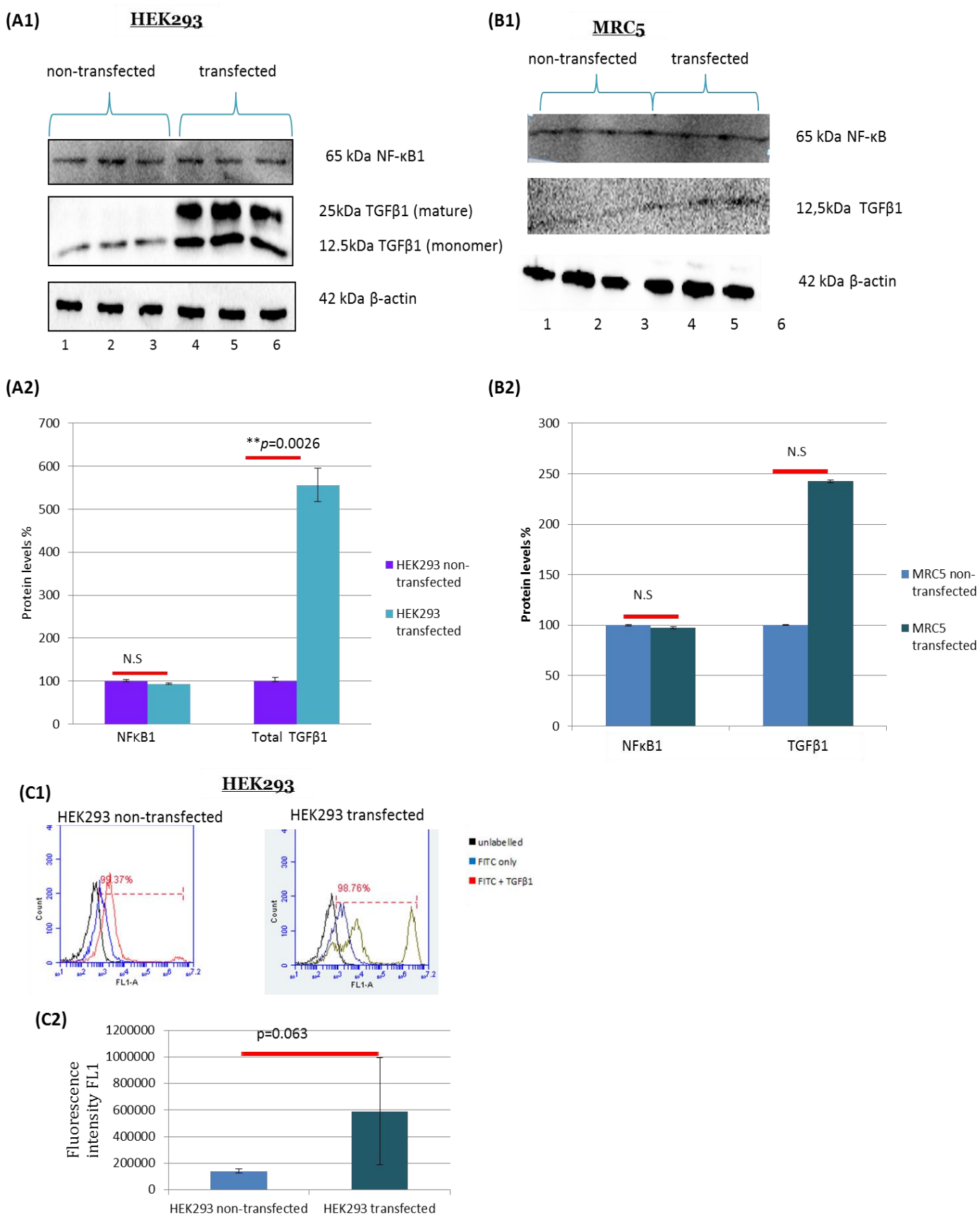


Figure 4.10: Effect of LRP::FLAG overexpression on NF-κB and TGFβ1 levels in HEK293 and MRC5 cells. (A and B) Western blotting shows non-transfected samples (lanes 1-3) and

samples transfected with the pCIneo-moLRP-FLAG plasmid (lanes 4-6) for both HEK293 (A1) and MRC5 (B1) cells. Anti-LAP TGF-β1and anti-mouse IgG-HRP was used to detect TGF-β1, anti-NFκB1 and anti-rabbit IgG-HRP detected NFκB1 and β-actin (HRP) was used as a loading control. Densitometric analysis performed on the western blots was relative to the non-transfected HEK293 (A2) and MRC5 (B2) cells set to 100 % (C) Flow cytometric analysis of TGFβ1 protein levels in HEK293 cells. The first plot represents non-transfected cells and the second plot transfected cells. Peaks are represented as follows: black- unlabelled cells, blue - secondary antibody only (FITC-coupled anti-mouse antibody, and red - TGFβ1 levels (labelled with anti- LAP-TGFβ1 primary and FITC secondary antibodies). The shift between the black and red peaks indicates the presence of TGFβ1 in the HEK293 cells. Median fluorescence intensity (MFI) values were used to assess differential expression of TGFβ1 and 20 000 cells were analysed per sample (C2). Error bars represent standard deviation, n=3 biological repeats. *p: 0.05, **p: 0.01, ***p: 0.001 N.S.: non-significant; Student's t-test.

Immunofluorescence microscopy was further employed to determine the expression and localisation of TGFβ1 and NF-κB in both cell lines (Figure. 4.11). Although there was no significant change in total NF-κB (green fluorescence) post-transfection in HEK293 cells (Figure. 4.11A), expression was strongly localised in the nucleus where it plays a role as a transcription factor. In contrast, active TGFβ1 (red fluorescence) expression switched from a stronger nuclear localisation to a higher signal in the cytoplasm and on the cell surface where it activates signalling pathways by binding to TGFβ receptors after transfection in HEK293 cells (Figure. 4.11A). Figure 4.11B shows the localisation of TGFβ1 and NF-κB in both the cytoplasm and nucleus of non-transfected MRC5 cells. In addition, TGFβ1 was also more strongly observed in the nucleus of MRC5 cells. However, this experiment is still to be performed in transfected MRC5 cells.

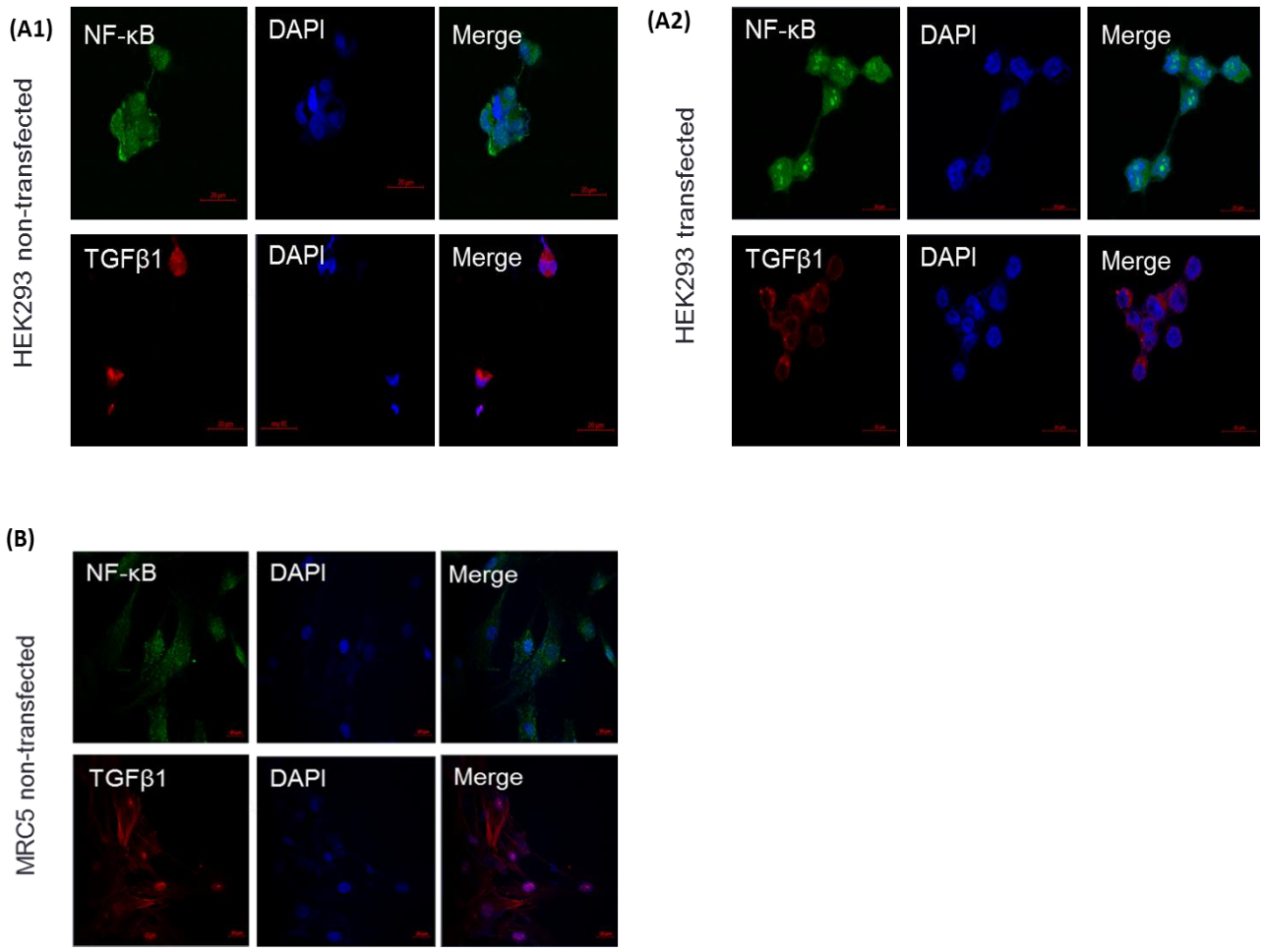


Figure 4.11: Intracellular expression of NF-κB and TGFβ1 in transfected and non-transfected HEK293 and MRC5 cells. (A) Expression of total NF-κB localises to the cytosol in non-transfected HEK293 cells (A1) and nucleus in transfected HEK293 cells (A2). Expression of total TGFβ1 localises to the nucleus in non-transfected HEK293 cells (A1) and cytosol and cell surface in transfected HEK293 cells (A2). (B) Expression of total NF-κB localises to the cytosol in non-transfected MRC5 cells (B1) and expression of total TGFβ1 localises to the cytosol, cell surface and nucleus in non-transfected MRC5 cells (A1). Anti-LAP (TGF-β1) and anti-mouse IgG-CFTM647was used to detect TGF-β1, anti-NFκB1 and anti-rabbit IgG-FITC detected NFκB1 and nuclei are stained with DAPI. All images were taken with the Zeiss LSM 780 microscope at 630x magnification. Scale bars represent 20 μm

5 Discussion

Currently, LRP/LR has been shown to have an effect on the expression of numerous proteins associated with normal physiology or various pathologies (Jovanovic et al., 2015). The role of LRP/LR in several cellular functions, such as translation, growth, migration survival and adhesion to the ECM is highlighted by their attenuation upon LRP/LR knockdown (Chetty et al., 2014; Khumalo et al., 2013; Jovanovic et al., 2015; Omar et al., 2012; Zuber et al., 2008). In contrast, the natural physiological upregulation of LRP/LR observed in microglia has been shown to direct the wound healing capability of microglia without resulting in carcinogenesis (Baloui et al., 2009). This suggests that the inverse is true and that by overexpressing LRP/LR it may be possible to promote cell growth, protein synthesis and cellular migration without carcinogenesis. Since LRP/LR exists in various isoforms (DiGiacomo and Meruelo, 2015), precisely knowing which aspects of LRP/LR contribute to its involvement in disease is crucial for the development of therapeutic strategies. Recent studies where FLAG-tagged LRP was overexpressed indicate that it is a potential therapeutic for ageing and age-related disorders such as AD, Parkinson's disease, cardiovascular disorders and diabetes (Bignoux et al., Revised); Otgaar et al., 2017: Weiss et al.,2018). Moreover, these disorders are also risk factors for the incidence of chronic wounds (Jabrink et al., 2017). Therefore, the successful development of LRP as a widespread treatment for normal physiological wound healing and age-related disorders largely depends on increasing our understanding of the proteins that are affected by LRP/LR.

5.1 LRP::FLAG overexpression increases the protein levels of 37 kDa LRP and different TERT isoforms.

In order to assess whether LRP/LR might play a role in wound healing and regeneration processes through its interaction with telomerase, HEK293 and MRC5 cells were transfected with the pCIneo–moLRP-FLAG plasmid to induce overexpression of LRP::FLAG. Western blotting was performed to confirm the overexpression of LRP::FLAG and to assess the levels of LRP (Figure 4.1). Since previous studies have shown that transfection with the 37kDa LRP cDNA results in overexpression of the immature 37 kDa LRP and not the mature 67 kDa LR (Rao et al., 1989), the levels of 67kDa LR and 37kDa were assessed using western blotting. Our results confirmed that an increase in 37kDa LRP did not result in increased 67kDa LR levels (Figure 4.1). Although the 67kDa LR and 37kDa are encoded by the same mRNA, the 67kDa LR is believed to be a chimeric protein composed of the 37kDa LRP and another protein that is the rate-limiting factor for the synthesis of the mature 67 kDa LR (Rao1989). Currently, the mechanism by which the 37kDa LRP matures into the 67kDa LR is not known however the more widely accepted theories propose that

the maturation may possibly be a result of fatty acylation by fatty acids or SUMOylation by small ubiquitin-like modifier (SUMO) proteins (DiGiacomo and Meruelo, 2015). The lack of an increase in 67kDa LR levels is important since, in diseased states, the 67kDa LR is upregulated where it acts as a receptor for viruses, prions and promotes cancer metastasis (Jovanovic et al., 2015). These functions of the 67 kDa LR are highlighted when the receptor is blocked. Antibody blockage of surface LRP/LR led to a decrease in angiogenesis in normal cells as well as adhesion and invasion in tumorigenic cells (Chetty et al., 2014; Khumalo et al., 2013; Omar et al., 2012; Zuber et al., 2008). In fact, blockage of the receptor has been shown to result in decreased total 37 kDa LRP levels in cancer cells but not in normal cells (Chigumba et al., unpublished).

Although LRP/LR is predominantly located on the cell surface, it has also found in the cytoplasm and nucleus. Therefore, the expression of LRP was determined using immunofluorescence. Confocal microscopy revealed an increase in LRP levels on the cell surface, in the cytoplasm and nucleus of cells overexpressing LRP::FLAG (Figure 4.3) as previously seen (Bignoux et al., Revised; Otgaar et al., 2017). An increase in cell surface LRP/LR, where it is predominantly located, has previously been shown to coincide with increased cell adhesion, growth and migration (Omar et al., 2012). Additionally, LRP/LR presence has also been reported in the cytosol where it plays a role in protein synthesis and pre-rRNA processing through its association with ribosomes, (Ford et al., 1999; O'Donohue et al., 2010). Furthermore, LRP/LR found in the nucleus has been shown to associate with histones to maintain nuclear structures and summon DNA damage repair proteins within the nucleolus (Guerra-Rebollo et al., 2012; Kinoshita et al., 1998; O'Donohue et al., 2010). Therefore, the increase in LRP observed may also lead to the promotion of one or more of these functions of LRP/LR although further investigation will be required to confirm this theory.

Moreover, the upregulation of the 37kDa LRP is also associated with upregulation of hTERT and telomerase activity in cancer, Alzheimer's disease and ageing (Bignoux et al., Revised; Otgaar et al., 2017; Naidoo et al., 2015). As mentioned previously, telomerase is responsible for maintaining the self-renewal capacity of stem cells and the catalytic subunit, hTERT, plays a role in regulating gene expression to maintain stem cells (Shay et al., 2001). Thus, the limiting factor to telomerase activity, hTERT and the telomerase active phospho-TERT (pS824) were assessed through western blotting and confocal microscopy. It was found that HEK293 and MRC5 cells overexpressing LRP::FLAG displayed elevated levels of hTERT (Figure 4.2), which is consistent with previous findings (Bignoux et al., revised; Otgaar et al., 2017). In the current study, transfected HEK293 cells showed increased levels of the 120kDa and 90kDa hTERT isoforms (Figure 4.2A), while

transfected MRC5 cells showed detectable levels of both the 120kDa and 90kDa hTERT isoforms (Figure 4.2B). Although an increase in the full-length 120kDa hTERT in response to LRP::FLAG overexpression has been previously shown, the upregulation of the 90kD hTERT isoform due to LRP::FLAG overexpression has not been previously reported. The 90kDa hTERT isoform is a truncated hTERT variant usually upregulated in embryonic stem cells (Brambilla et al., 2004). This non-telomerase active variant maintains pluripotency by increasing the pool of tRNAs associated with the expression of proliferation genes and inhibiting apoptosis by reducing caspase 3/7 activity (Listerman 2013). Notably, expression of this variant is usually antagonistic to the expression of the full-length 120kDa hTERT isoform as a result of highly controlled splicing patterns (Killian et al., 1997). Two factors that affect the hTERT-splicing patterns are hypoxia which favours expression of the 120kDa hTERT variant and high TGFβ1 levels that favour the 90kDa variant (Brambilla et al., 2004). This is believed to be because during hypoxia translocation of the 120kDa hTERT variant to the mitochondria mitigates ROS production while the increase in TGFβ1 promotes increased gene expression as seen with the 90kDa hTERT isoform (Brambilla et al., 2004; Listerman 2013). Thus the increase in the 90kDa hTERT observed here could be as a result of the increase in TGFβ1 observed in figure 4.8. However, an increase in the full-length 120kDa was also reported in this study. This isoform has canonical functions where it acts as the catalytic substrate for telomerase in order to increase telomere length and non-canonical functions such as regulation of gene expression, enhancing mitochondrial function and maintaining mitochondria DNA integrity (Ding et al., 2013. This suggests that apart from LRP interacting with hTERT, it is involved in regulating the expression patterns of hTERT variants by upregulating expression (Figure 4.8).

Furthermore, previous studies have shown that LRP::FLAG overexpression increases hTERT expression that also corresponds to an increase in telomerase activity and telomere length (Bignoux et al., revised; Otgaar et al., 2017). Otgaar et al., (2017) illustrated such an increase in telomerase activity and telomere length in HEK293 and MRC5 cells along with decreased senescent markers. However, for nuclear localisation of the full-length 120kDa hTERT variant to occur, it firstly undergoes phosphorylation at the serine residues 227 and/or 824 by PI3-kinase/AKT signalling (Chung et al., 2012). Therefore we investigated whether overexpression of LRP::FLAG increased the levels of TERT phosphorylated at the serine 824 residue. This study showed a significant increase in 126kDa phospho-TERT (pS824) levels (Figure 4.2). This suggests that the increase in telomerase activity previously observed in HEK293 and MRC5 cells by Otgaar et al., (2017) is as a result of not only increased hTERT levels but also hTERT import to the nucleus. In addition, Sun et al., (2014) showed that binding of LRP/LR to laminin activates the PI3-kinase/AKT pathway through its interaction with focal adhesion kinases. Also, PI3-kinase /Akt regulation through NF-κB

signalling is reported to increase telomerase activity (Li and Tergaonkar, 2014). Here, we showed an increase in the localisation of active NF-κB to the nucleus (Figure 4.9 B) and together with the increase in hTERT and phospho-TERT (pS824) observed in this study, this may be as a result of LRP::FLAG modulated hTERT phosphorylation which leads to a subsequent increase in telomerase activity and telomere length.

Moreover, confocal microscopy confirmed an increase in hTERT levels on the cell surface, in the cytoplasm and nucleus of cells overexpressing LRP::FLAG (Figure 4.2) as previously observed by Bignoux et al., (revised) and Otgaar et al., (2017). The increase in hTERT levels in the cytoplasm could correspond to other extra-telomeric functions of hTERT such as its anti-apoptotic function in the mitochondria; decreasing ROS production and protection of mitochondrial DNA against ROS induced stress (Ahmed et al., 2008; Ding et al., 2013; Saretzki, 2009). This is important as ROS is a crucial regulator of wound healing however excessive ROS production causes oxidative damage which is the primary cause of chronic wounds (Coluzzi et al., 2014; Rieger et al., 2014). On the other hand, hTERT also plays other roles independent of telomerase activity in the nucleus including the regulation of gene expression. hTERT promotes the expression of genes that promote proliferation, angiogenesis, inflammatory responses and EMT (Ding et al., 2013; Li and Tergaonkar, 2014).

5.2 LRP::FLAG overexpression promotes Epithelial to Mesenchymal Transition (EMT)

Upon confirmation of the relationship between LRP/LR and hTERT, there was further investigation into whether LRP/LR played a role in inducing EMT. In adults, EMT is a crucial step that occurs when epithelial cells dedifferentiate to mesenchymal stem cells in order to promote repair and regeneration of tissue (Ding et al., 2013). hTERT induces EMT via upregulation of Snail1 and Vimentin (Ding et al., 2013). Therefore, the role of LRP/LR in EMT was assessed by analysing the effects that LRP::FLAG overexpression exerted on the epithelial marker, α-E-catenin, and the mesenchymal stem cell markers, N-Cadherin and Vimentin. Western blotting and confocal miscopy showed that HEK293 cells overexpressing LRP::FLAG promoted the mesenchymal phenotype by upregulating N-Cadherin and Vimentin which are mesenchymal markers (Inada et al., 2016), however no α-E-catenin detected before or after transfection (Figure 4.4A and 4.5A). Before transfection HEK293 cells already expressed the neural markers Vimentin and N-Cadherin since they are considered of neuronal lineage because they express signature markers typical of neuronal cells (Inada et al., 2016). Notably, an increase, not loss in E-Cadherin expression was shown (Figure 4.6 A.) however EMT is usually characterised by loss of E-Cadherin, whereas gain in E-

Cadherin is usually observed during mesenchymal to epithelial transition (MET) or cell reprogramming (Gout et al., 2008). Thus, this upregulation of E-Cadherin implies that the increase in Vimentin and N-Cadherin observed after overexpressing LRP::FLAG in HEK293 cells is not as a result of EMT but possibly due to cell reprogramming. The lack of increase in α-E-catenin levels may be a consequence of E-Cadherin preferentially binding to β-catenin in order to promote pluripotency (Soncin and Ward 2011). Moreover, HEK293 cells have previously been shown to produce mesenchymal markers such as N-Cadherin and Vimentin and epithelial markers such as E-Cadherin concurrently, hence our observations confer with current literature (Inada et al., 2016). In MRC5 cells, upregulation of N-Cadherin and Vimentin, as well as downregulation of α-E-catenin (Figure 4.4B and 4.5B) and E-Cadherin (Figure 4.6B), was observed. Since EMT is characterised by loss of proteins that mediate cell-cell adhesion and gain in proteins that promote motility such as Vimentin and N-Cadherin (Gout et al., 2008), this suggests that EMT occurred in MRC5 cells overexpressing LRP::FLAG. Therefore overexpression of LRP::FLAG may act as an inducer of EMT in normal fibroblasts possibly through upregulating TGFβ1, Snail and hTERT which are reported inducers of EMT (Ding et al., 2013; Li and Tergaonkar, 2014). An upregulation of TGFβ1 was observed in MRC5 cells (Figure 4.8B) and this induces Smad 2/3 signalling in order to activate TFs responsible for controlling genes favouring the EMT phenotype, or it induces miRNAs which are known to down-regulate the expression of epithelial markers (De Craene et al., 2013; Thiery and Sleeman, 2006). On the other hand, hTERT upregulation observed in Figure 4.2B may induce the expression of EMT-promoting genes by activating the WNT/β-catenin pathway (Ding et al., 2013; Li and Tergaonkar, 2014). As previously mentioned, LRP/LR directly interacts with β-catenin (Venticinque and Meruelo, 2012), which may be another mechanism by which it induces EMT. Although Snail is a well characterised effector of TGFβ1 induced EMT (De Craene et al., 2013), here we observe its upregulation only in HEK293 cells overexpressing LRP::FLAG. Since both cell lines are stably transfected, we can only postulate that the increase in TGFβ1 resulted in the expected upregulation of Snail, however, this upregulation persisted in order to maintain a stem cell phenotype in HEK293 and possibly decreased after EMT occurred in MRC5 cells in order to promote a mesenchymal stem cell state. Transient transfection will have to be performed in order to confirm this theory.

Moreover, the increased motility associated with EMT is also characterised by morphological modifications as a result of altered cellular adhesion, acquisition of anterior-posterior polarity and cytoskeletal reorganization. LRP/LR is known to play a role in cytoskeletal organisation (Gout et al., 2008). Previous studies have shown that LRP/LR co-localizes and directly interacts with actin, tubulin, focal adhesion complexes, cytoskeletal stress fibres and cell anchorage proteins including

Vimentin (Kim et al., 2005b; Keppel & Schaller, 1991; Venticinque and Meruelo, 2012; Yannariello-Brown et al., 1988). These interactions of LRP/LR with the cytoskeleton are considered to aid cell motility and attachment and may be responsible for the change in morphology observed in mesenchymal stem cells (Venticinque et al., 2011). In addition, LRP/LR promotes the release of matrix metalloproteases (MMPs) such as collagen type IV/ MMP-9. MMPs assist in detachment of cells from the ECM by cleaving cell-ECM interactions protein in order to gain motility (Turpeenniemi-Hujanen et al., 1986). This suggests that overexpression of LRP::FLAG induces EMT by upregulating hTERT, Snail1, Vimentin and MMPs levels.

5.3 LRP::FLAG overexpression promotes the expression of pluripotency markers

As mentioned previously, telomerase is responsible for maintaining the self-renewal capacity of stem cells and the catalytic subunit, hTERT, plays a role in gene expression in order to maintain the stem cell phenotype (Flores et al., 2006). In addition, the overexpression of hTERT is known to lead to the immortalisation of cells and to lead normal somatic cells to develop stem cell like properties (Bodnar et al, 1998). Although LRP/LR plays a role in promoting cell survival and inhibition of apoptosis (Moodley et al., 2013), which are traits of stem cells, not much is known about whether the overexpression of the 37kDaLRP has the ability to promote stem like characteristics without inducing cancer. Therefore, this study was further aimed at investigating whether LRP/LR had an effect, direct or indirect, on the expression of various proteins associated with the stem cell phenotype. An increase in a number of the pluripotency-related markers was observed in both transfected HEK293 and MRC5 cells. Of the 15 pluripotency markers investigated, 14 were upregulated in HEK293 cells and 5 in MRC5 cells after overexpression of LRP::FLAG (Table 4.1). Of note are the increases in the TFs Oct4 and SOX2 in both cell lines which are known to drive cellular reprogramming (Takahashi and Yamanaka, 2006). These TFs bind to a multitude of gene regulatory sites, many of which regulate cell pluripotency and early embryonic development (Avilion et al., 2003; Boyer et al., 2005; Loh et al., 2006). Interestingly, SOX2 expression has been found in adult stem cells that are crucial for normal tissue repair and regeneration and in multipotent progenitors that give rise to some adult epithelial tissues (Arnold et al., 2011). Therefore, the upregulation of SOX2 observed could induce the self-renewal and differentiation capacity characteristic of stem cells. In addition, forced expression of Oct4 and SOX2 with either the combinations of KLF4 and MYC or NANOG and LIN28 has been reported to induce pluripotency in mouse and human somatic cells (Nakagawa et al., 2008). Notably, expression from the Oct4 and SOX2 levels was also increased in comparison to adult mesenchymal stem cells (Figure 4.7). Combined with the increase observed in NANOG expression in HEK293 cells, this suggests that

there is sufficient expression of the reprogramming factors to dedifferentiate the cells. Thus, LRP::FLAG overexpression could induce pluripotency in immortalised cell lines. However, in MRC5 cells the increase in Oct4 and SOX2 expression is accompanied by a strong increase in GATA4 and a decrease in NANOG (Figure 4.6B) which usually denotes incomplete reprogramming. GATA4 regulates genes involved in embryogenesis and in myocardial differentiation and function (Rivera-Feliciano et al., 2006). Therefore, this transcription factor may be promoting the expression of differentiation genes and not de-differentiation genes. Moreover, NANOG expression is known to start decreasing with the onset of differentiation. Therefore, efficient transgene silencing that is essential for the derivation of pluripotent cell lines may not have occurred in MRC5 cells (Takahashi and Yamanaka, 2006). Since MRC5 cells show an increase of some stem cell markers, this implies that overexpression of LRP::FLAG induces transition of fibroblasts into mesenchymal stem cells but is not sufficient to drive complete cell reprogramming into pluripotent cells (Takahashi and Yamanaka, 2006). Other pluripotency markers were increased after LRP::FLAG overexpression especially in HEK293 cells. Increase in foetal proteins AFP and HCG was only observed in HEK293 cells overexpressing LRP::FLAG (Figure 4.6). AFP is a major foetal plasma protein and HCG is a placental hormone that regulates immunity, angiogenesis, implantation and trophoblast stem cell differentiation (Thomason et al., 1998). Thus, the upregulation of these proteins may represent an embryonic phenotype in cells overexpressing LRP::FLAG. In addition, E-Cadherin was also upregulated after overexpression of LRP::FLAG in HEK293 cells (Figure 4.6). Although E-Cadherin is a well-known epithelial marker (Gout et al., 2008), it has been reported to play a crucial role in maintaining pluripotency in embryonic ESC and iPSC through activation of Wnt/β-catenin, PI3K/Akt, and NF-κB signalling (Rdmer et al., 2011). Furthermore, an increase in the expression of TFs: PDX-1, Otx2, GSC, FoxA2, SOX17, TP63, and Snail was observed in HEK293 cells overexpressing LRP::FLAG while transfected MRC5 cells showed an increase only in FoxA2. These proteins are expressed during embryogenesis in order to activate the expression of genes associated with pluripotency (Rigbolt et al., 2011). Of note is the increase in FoxA2 that promotes cellular maintenance, metabolism, differentiation, and organogenesis. FoxA2 is a transcription regulator considered a 'pioneer' factor that opens the compacted chromatin through its interactions with core histones at target enhancer and/or promoter sites thereby allowing access for other proteins (Costa et al., 2003). LRP/LR is also reportedly involved in chromatin remodelling through associations with histones h2a, h2b, and h4 (Kinoshita et al., 1998; Venticinque & Meruelo, 2012). This shows a novel role of LRP/LR in the recruitment of chromatin remodelling proteins such as FoxA2. Another transcription regulator that is upregulated in HEK293 cells after overexpressing LRP::FLAG is SOX17. SOX17 binds the minor groove of DNA and bends the DNA in order to allow for gene expression. It also negatively

regulates Wnt signalling and plays a role in the maintenance of the hematopoietic stem cell pool (Engert et al., 2013). Therefore, it may prevent aberrant Wnt signalling that is observed in cancers. Other pluripotency markers that have been identified as tumour suppressors include PDX-1 and TP63 where loss in function of these proteins has been implicated in carcinogenesis. PDX-1 has been identified as a tumour suppressor in gastric cancer whereby its downregulation has been identified in various gastric cancer types (Ma et al., 2008). TP63, a member of the p53 family is upregulated after overexpressing LRP::FLAG in HEK293 cells. This protein is involved in cellular responses to stress and development through the activation of p53-responsive genes causing cell cycle arrest and apoptosis. Previous studies have reported high TP63 in embryonic tissue where it plays a role in limb development and in adult epithelial tissue where it maintains epithelial stem cell regeneration (Benard et al., 2003; Harms et al., 2004). Hence, since overexpression of LRP::FLAG does not result in the suppression of tumour suppressors, this implies that although LRP/LR upregulation is a tumorigenic marker, it possibly does not induce carcinogenesis.

Another protein that is upregulated in HEK293 cells but downregulated in MRC5 cells overexpressing LRP::FLAG is the TF, Snail1. In embryonic stem cells, Snail1 promotes mesoderm and neural crest formation whereas in adults it regulates the maintenance and size of the stem cell compartments (Mazzolini et al., (2018). Thus, the increase in Snail1 expression observed in HEK293 may promote pluripotency. In addition, Snail expression is positively regulated by NF-κB and TGFβ/SMAD signalling pathways. Studies have shown that NF-κB and TGFβ1 signalling also plays a role in maintaining undifferentiated ESC and IPSC in culture (Amit et al., 2000); Zhang et al., 2011). Conversely, the decrease in Snail expression observed in MRC5 cells may be considered a marker for the mesenchymal stem cell phenotype. Mazzolini et al., (2018) showed that Snail1-depleted mouse MSC exhibit higher levels of both telomerase activity and the long non-coding RNA called telomeric repeat-containing RNA (TERRA), an RNA that controls telomere integrity. However, they also reported that Snail1 repression of TERRA was required not only for telomere maintenance but also for the expression of a subset of mesenchymal genes. Thus the decrease in Snail expression observed in MRC5 cells overexpressing LRP::FLAG may possibly be to promote telomerase activity; however, there is still adequate Snail1 protein to inhibit TERRA and its negative effects on telomere elongation (Mazzolini et al., 2018). Therefore, LRP/LR may also play a role in promoting the stem cell phenotype through its relationship with NF-κB and TGFβ1.

Another indicator of pluripotency is high levels of ALP activity which are exhibited on the cell surface of embryonic stem cells (Stefkova et al., 2015). Hence, an ALP activity assay was performed in order to investigate whether HEK293 cells overexpressing LRP::FLAG could possibly

be pluripotent stem cells. Interestingly, an increase in ALP activity was observed in transfected HEK293 cells (Figure 4.8). Since high levels of ALP activity is usually reported in highly proliferative cells such as germ line cells, embryonic cells, ESCs and IPSCs (Hahnel et al., 1990; Narisawa et al., 1997; Stefkova et al., 2015), this implies that overexpressing LRP::FLAG promotes a pluripotent phenotype in HEK293 cells. Moreover, increased ALP expression is associated with more cell proliferation and migration, where it provides an unlimited supply of phosphates for DNA and RNA synthesis, which are key characteristics of most stem cells (Langer et al., 2007). This suggests that LRP::FLAG may play a role in up-regulating the activity of ALP leading to a stem-cell-like state. Moreover, similar to LRP/LR, ALP is also predominantly located in the lipid raft regions on the cell membrane (Paulick and Bertozzi, 2008). There its activity is responsible for phosphorylating laminin-1, which binds to LRP/LR with high affinity. This leads to increased cell adhesion and proliferation (Trachana et al., 2005) implying that apart from LRP::FLAG inducing the activity of the pluripotency marker, ALP, this phosphatase may in turn play a role in the activities of laminin-1 and LRP/LR interactions.

Moreover, studies have shown that stem cells are also characterised by more a rounded morphology and enlarged nucleus (Becker et al., 1963; Siminovitch et al., 1963). In this study it was also observed that cells overexpressing LRP::FLAG also exhibited similar traits (Figure 4.5), thus further suggesting that these cells may possibly be stem cell like in nature. This change in morphology combined with the upregulation of some pluripotency markers suggests that the increase in hTERT expression in response to LRP::FLAG overexpression results in a stem cell phenotype. The increase in stem cell markers may be due to the involvement of LRP/LR in protein synthesis through its ribosomal function or its ability to remodel chromatin interactions (DiGiacomo and Meruelo, 2015). In addition, since most of the pluripotency markers investigated play crucial roles in embryogenesis, this alludes to LRP/LR having a role in regulating the expression patterns of genes that control embryogenesis apart from acting as a foetal antigen or its laminin binding ability (Biragyn et al., 2007; Rao et al., 1994). Additionally, although VEGF R2 signalling pathway promotes wound healing and cell survival characteristics, its expression was markedly decreased in both cell lines after overexpression of LRP::FLAG. The reason for this is unknown but a decrease in levels may not result in decreased signalling, or this pathway may not be crucial in promoting a stem cell like phenotype in this study.

5.4 LRP overexpression and cancer

Although the upregulation of hTERT and LRP/LR has been implicated in cancer, Otgaar et al., (In preparation) has shown that overexpression of LRP::FLAG upregulates telomerase activity without increasing the occurrence of carcinomas. This could be as a result of the upregulation of the tumour suppressors PDX-1, TP63 (Figure 4.6) and TGFβ1 (Figure 4.8) reported in this study. Overexpression of PDX1 *in vivo* and *in vitro* inhibits cell migration, proliferation, clonal formation and induced apoptosis through the activation of caspases 3, 8, 9 and 10. TGFβ1 expression also halts tumour development in normal cells or acts as a tumour suppressor in early carcinomas (Neel et al., 2012; Yang and Moses 2008). Moreover, deleterious mutations in the TGFβ gene or TGFβ1 protein downregulation are associated with numerous human cancers and aggressive tumours (Neel et al., 2012). TGFβ suppresses tumour progression through increased apoptosis and negative regulation of various growth factors, cytokines, and chemokines expression (Yang and Moses 2008). As previously mentioned, TP63 is a member of the p53 family involved in cellular responses to stress and development through the activation of p53-responsive genes causing cell cycle arrest and apoptosis (Benard et al., 2003; Harms et al., 2004). Thus, TP63 acts as a tumour suppressor by activating p53 which is usually downregulated in most cancers (Nigro et al., 1989). Furthermore, cancer is characterised by the maintenance of critically shortened telomeres in order to drive genome instability whereas a previous study on MRC5 and HEK293 reported an increase in telomere length in response to LRP::FLAG overexpression (Otgaar et al., 2017). Moreover, LRP overexpression appears to decrease the DNA damage that is often seen in tumorigenesis (Otgaar et al., 2017). In this study, they showed a significant decrease in γH2AX, which is considered a marker of DNA damage accumulation (Otgaar et al., 2017). Furthermore, the increase in TERT they illustrated may have also reduced the accumulation of DNA damage as this protein has been implicated in DNA repair (Sharma et al., 2015). Additionally, ectopic expression of TERT in mice without functional loss of tumour suppressor genes has been found to be insufficient to drive tumorigenesis (Mendelsohn and Larrick, 2012). Lastly, although upregulation of the 67kDa LR promotes metastasis of tumorigenic cells, it is also upregulated in normal physiology in response to injury (Baloui et al., 2009: Jovanovic et al., 2015). Therefore we postulate that the overexpression of LRP::FLAG does not induce tumorigenesis, however in the presence of oncogenes it may promote metastasis. Further research will be required to test this theory.

5.5 LRP::FLAG overexpression promotes repair and regeneration through the expression of cytokines

Since the main role of stem cells in adults is to repair and regenerate tissue, the role of LRP in wound healing possibly through cell migration and adhesion was also investigated (Bongso and Lee 2005). It has been previously shown that LRP/LR plays a key role in cell adhesion through its interaction with the ECM protein laminin-1 (Stitt et al., 1998) and cell migration through cytoskeletal rearrangements in order to promote motility (Yannariello-Brown et al., 1988). Therefore, *in vitro* wound healing, cell migration and adhesion potential of cells overexpressing LRP::FLAG was analysed. As preliminary data in HEK293 cells only, it was observed that there was an increase in wound gap closure in transfected HEK293 cells (Figure 4.9B). Thus, overexpression of LRP::FLAG stimulated wound gap closure possibly through increasing cell Migration (Figure 4.9A) and decreasing cell adhesion (Figure 4.9 B). Previous studies have shown that the interaction between the membrane-associated LRP/LR and ECM glycoprotein laminin-1 has been shown to be crucial for, immune responses, angiogenesis, cell adhesion and degradation of the basal lamina in order to allow cell migration (Baloui et al., 2009; DiGiacomo and Meruelo, 2015; Stitt et al., 1998). Thus, the increased cell migration, which is important for response to stimuli of fibroblasts and immune cells during wound healing, may be as a result of the 37kDa and laminin-1 interaction that facilitated proteolytic cleavage of the basement membrane in order to allow cell migration (Fatehullah et al., 2009; Jovanovic et al., 2015; Wenzel et al., 2010). In addition, reorganisation of the cytoskeleton suggested in section 5.2 has previously been associated with increased cell motility. Furthermore, previous studies have shown that blocking the 67kDa LR decreases the adhesive and invasive potential of tumourigenic cells (Munien et al. 2017; Vania et al., 2016). Although this study showed increased cell migration after upregulating LRP::FLAG, the study also reports an unexpected decrease in cell adhesion (Figure 4.9). Since figure 4.1 indicates that upregulation of the 37kDa LRP does not result in increased 67kDa LR, it implies that the 37kDa LRP modulates cell adhesion independent of the 67kDa LR. This could be due to the interaction of the 37kDa LRP to HSPGs in the ECM where Fatehullah et al., (2009) showed that HSPGs which mediate cell binding only interact with the 37kDa LRP and not with the 67KDa LR. Moreover, previous studies have reported increased cell adhesion and decreased cell migration and subsequently delayed wound healing in cells with decreased HSPGs (Fatehullah et al., 2009; Gotte et al., 2008). Thus by upregulating the 37kDa LRP and possibly interaction with HSPGs, this could result in the decreased cell adhesion observed in transfected HEK293 cells. Therefore, LRP/LR may promote wound healing by decreasing the adhesive potential of cells and increasing the migration of cells towards wound healing signals.

However, detachment and migration of cells during wound healing is usually in response to stimuli such as cytokine signalling, (Baloui et al., 2009; Stitt et al., 1998). Therefore, we analysed the expression of two cytokines, TGFβ1 and NFκB, which are important regulators of inflammatory responses using western blotting and immunofluorescence microscopy. Western blotting showed no significant change in the levels of the pro-inflammatory cytokine NFκB1 in both cell lines (Figure 4.10). Inappropriate activation of NF-κB has also been associated with a number of inflammatory disorders and cancer while persistent inhibition of NF-κB leads to inappropriate immune cell development or delayed cell growth (Heng, 2011). Although there was no increase in total levels of NF-κB in both HEK293 and MRC5 cells, we observed increased localisation of the protein in the nucleus of HEK293 cells (Figure 4.11). Activated NF-κB is often translocated to the nucleus where it stimulates the expression of genes involved in inflammation, immune responses differentiation, growth and apoptosis inhibition. Therefore LRP/LR may influence the translocation of NF-κB to the nucleus (Heng, 2011).

In addition, NF-κB plays a role in the translocation of hTERT from the nucleus to the cytoplasm where it can perform telomerase independent functions and in turn also activate hTERT expression thereby forming a feedback loop (Li and Tergaonkar, 2014). Catalytically inactive hTERT can stimulate the proliferative phase of wound healing through activation of the Wnt/β-Catenin signalling pathway. Previous studies have shown that telomerase activity is exhibited in perpetually regenerating human tissue, such as proliferative basal cells and hematopoietic cells where it contributes to the unlimited proliferation capacity of these cell types (Haik et al, 2000). Moreover, TERT plays a role in inflammation by mitigating ROS production during the inflammatory phase in order to avoid the development of chronic wounds (Coluzzi et al., 2014; Haik et al, 2000). Therefore, by overexpressing LRP::FLAG and subsequently hTERT, it is possible to promote the healing of wounds by increasing cell proliferative capacity and protecting against aberrant ROS production

Another pathway involved in modulating inflammation is the TGFβ1 pathway. TGFβ1 is considered to be the most important anti-inflammatory cytokine with regard to wound healing. Following damage to cutaneous tissue, TGFβ1 is promptly up-regulated and secreted by macrophages, platelets, and keratinocytes. It is vital for initiating inflammation, granulation tissue formation and is required to facilitate cell migration (Heng, 2011). Here, overexpression of LRP::FLAG resulted in increased TGFβ1 levels in both HEK293 and MRC5 cells (Figure 4.10). Figure 4.10 A1 showed that there was an increase in the 12.5kDa monomer and 25kDa dimer of mature TGFβ1. Previous studies have reported that upregulation of LRP/LR can reportedly be

triggered by cytokines such as TGFβ (Wenzel et al., 2010). However, it appears that LRP/LR may have the ability to also trigger cytokine release as exhibited by the increase in active TGFβ1 levels observed in this study. Activated TGFβ1 is found usually on the cell surface and in the cytoplasm where it activates various pathways. Therefore, confocal microscopy was employed to determine the expression of this protein in the cells. Translocation of TGFβ1 from the nucleus to the cell surface and cytoplasm of HEK293 cells was observed (Figure 4.11) (this experiment was not performed in MRC5 cells). Activation of TGFβ1 involves proteolytical processing of the TGFβ1 precursor resulting in an N-terminal latency-associated peptide (LAP) non-covalently associated with the TGF-β1 dimer (Shi et al., 2006; Taipale et al., 1994). Release from this complex is mandatory for the release of biologically active TGF-β1. TGF-β1 is activated in various ways including structure modification by ROS or proteolysis by MMPs (Barcellos-Hoff and Dix, 1996; Yu and Stamenkovic, 2000). One such MMP responsible for TGFβ1 activation is MMP-9 (Kobayashi et al., 2014). As previously mentioned LRP/LR promotes the release of MMP-9 (Turpeenniemi-Hujanen et al., 1986) thus, it may indirectly influence TGFβ1 activation. *In vivo* studies have highlighted the potential use of TGFβ1 as a treatment for wound healing. Becks et al., (1991) showed that TGFβ administered to rat incisional wounds accelerated wound healing and increased wound strength. In addition, El Gazaerly et al., (2013) also showed a similar result whereby accelerated wound closure in combination with increased cell proliferation rate and a denser more organized new extracellular matrix was observed in wounds treated with TGFβ1 in comparison to the untreated. Therefore, LRP/LR may promote wound healing through its relationship with TGFβ1. However, persistence of TGFβ1 during wound healing has been identified as one of the culprits for tissue fibrosis (Kim et al., 2017). Therefore, usage of LRP as a therapeutic for wound healing will require precise attenuation of TGFβ1 signalling, or knockdown of LRP may be used to treat disorders characterised by TGFβ1 overexpression.

Moreover, the role of LRP/LR in wound healing can be inferred by its upregulation often observed in relation to hormone or cytokine signalling after injury (Castronovo et al., 1989; Poon et al., 2011; Raghunath et al., 1993; Shi et al., 199; Wenzel et al., 2010). Upregulation of LRP/LR and laminin-1 activates cellular response pathways such, c-Jun N-terminal protein kinase (JNK) and Mitogen-activated Protein Kinases (MAPK) pathways through persistent de-phosphorylation (Givant-Horwitz et al., 2004). Protein phosphatase 1 (PP1) has been shown to be an effector of LRP/LR initiated phosphorylation (Kim et al., 2005a). The activated MAPK pathway promotes cell proliferation, survival and differentiation. In addition, the JNK pathway also promotes cell proliferation and differentiation along with apoptosis and cytokine production (Givant-Horwitz et al., 2004). Furthermore, LRP/LR promotes the release of matrix metalloproteases (MMPs) such as

collagen type IV or MMP-9 (Turpeenniemi-Hujanen et al., 1986). Cells undergoing EMT require MMPs in order to detach from the ECM and gain motility. However, these MMPs are also responsible for N-Cadherin shedding by cleaving N-Cadherin into 90kDa and 40 kDa variants which were observed in Figure 4.4 in this study for both cell lines. The 90kDa N-Cadherin isoform is a soluble N-terminal truncated variant of N-Cadherin. Derycke et al., (2006) illustrated that this isoform induces angiogenesis, cell migration and stimulates phosphorylation of kinases. It is also associated with the activation of microglia that plays a primary role in response to central nervous system injuries. This suggests that LRP/LR plays a role in N-Cadherin shedding through the release of MMPs. In addition, the positive regulation and activity of MMPs are reportedly controlled by TGFβ1 during wound healing (Duflo et al., 2006). Hence, since we have shown that overexpression of LRP::FLAG increases TGFβ1 levels (Figure 4.10), this could be another mechanism by which N-Cadherin shedding is modulated. Furthermore, the LRP/LR and laminin-1 interaction also facilitates angiogenesis in the basal lamina in order to provide oxygen and nutrition to the new granulated tissue (Khusal et al., 2013; Li et al., 2003). The angiogenic response in injured tissue may be stimulated by angiogenesis cytokines such as (TGFβ), which was shown to be upregulated by LRP::FLAG overexpression in this study (Li et al., 2003). Therefore LRP/LR overexpression may play a role in wound healing through the activation of inflammatory response pathways such as MAPK, JNK, NF-κB and TGFβ1.

6 Conclusion

In conclusion, overexpression of LRP::FLAG results in an increase in the protein levels of hTERT and the telomerase active isoform, phopspho-TERT (pS824). Furthermore, LRP also plays a role in the expression of EMT responses that induce cell reprogramming and subsequently promote the expression of pluripotency markers such as Oct4 and Sox2. Due to the expression patterns of the pluripotency and EMT markers investigated plus increased ALP activity, it infers that overexpression of LRP::FLAG reprogrammed HEK293 cells into pluripotent stem cells. However, LRP::FLAG overexpression was found to be insufficient to drive complete cell reprograming of MRC5 cells into pluripotent cells although it did induce transition into mesenchymal stem cells which are the primary stem cells for adult repair and regeneration. Nonetheless, extensive pathway mapping will be required to elucidate the exact nature of the relationship between LRP/LR and these proteins. Moreover, overexpression of LRP::FLAG was shown to promote wound healing *in vitro* by increasing wound gap closure through increased cell migration and decreased cell adhesion in addition to modulating cytokine levels and localisation. In transfected HEK293 cells, NF-κB was translocated to the nucleus where it acts as a transcription factor and the levels of cytosolic and cell surface active TGFβ1 that acts as a ligand for various signalling cascades were increased. Therefore, strategies aimed at elevating LRP levels may provide a potential alternative curative therapy for wound healing by inducing stem-cell-like traits and regulating cytokine expression

Future Work

Since overexpression of LRP::FLAG was able to significantly increase hTERT levels and increase the levels of other pluripotency markers, further experiments will be required to characterise how closely these cells resemble stem cells These may include cell survival assays (clonogenic assay), pathway analysis using proteomics and FACS analysis to determine the cell type specific surface antigens being expressed,. In addition, differential media tests can be performed to determine whether cells overexpressing LRP::FLAG are multipotent/ pluripotent. Moreover, since hTERT upregulation in mesenchymal stem cells has been reported to result in pluripotent stem cells it is imperative to determine whether LRP upregulation also has the potential to change multipotent cells to pluripotent cells. This is because pluripotent cells have an increased ability to regenerate tissue or organs, as well as rejuvenating stem cell niches which confer better whole-body regeneration. This LRP::FLAG treatment can be perfected by tagging the protein with a stem cell antibody that recognises stem cell antigens so that they only promote rejuvenation of stem cells. Lastly, since upregulation of most stem cell markers is related to carcinogenesis further research is required to determine whether overexpression of LRP::FLAG induces cell cycle dysfunction and dysregulation of anti-tumorigenic apoptotic pathways which are fundamental to carcinogenesis.

Moreover, LRP::FLAG has been reported to modulate the expression of key inflammation regulators TGFβ1 and NF-κB, therefore, the role of LRP in wound healing needs to be further elucidated. This can be accomplished by performing an *in vivo* study. To determine its role in wound healing, surgically wounded mice such as C57BL/6J mouse models could be treated either with the pCIneo-moLRP-FLAG plasmid or alternatively utilising a recombinant LRP::FLAG protein. Some of the mice could receive excisional wounds on the skin dorsal surface to mimic acute injury while the other mice may undergo 5/6 nephrectomy also known as the remnant kidney model which is an experimental model for chronic renal failure. Thereafter physiological parameters could be tested and biochemical analysis will be performed. This would give an indication of whether LRP plays a role in the regeneration and functional restoration of an organ, particularly the skin and kidneys which are known to have limited regeneration capabilities. Depending on the results observed, a clinical study could be instigated for the development of LRP::FLAG as a therapeutic for age-related disorders and acute or chronic wounds.

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8 Appendix

8.1 Buffer Compositions

1XPBS (pH 7.2; 1L)

8g NaCl

0.2g KCL

1.44g $NA_2HPO_4.12H_2O$

0.24g KH_2PO_4

1X RIPA lysis buffer (100ml)

150 nM NaCl

1 % Triton X-100

0.5 % (w/v) Sodium Deoxycholate

0.1 % SDS

50 nM Tris-HCl (pH 8)

5m EDTA (pH8)

1X Running Buffer (pH8.3; 1L)

3g Tris

14.4g Glycine

1g SDS

1X Transfer Buffer (pH8.3; 1L)

3g Tris

14.4g Glycine

20 % Methanol

Table 9.1: Composition of polyacrylamide gels (per gel)

	Separating gel		**Stacking**
	12 %	**10 %**	**4 %**
Distilled water	2.15 ml	2.4 ml	1.83 ml
40 % acrylamide	1.5 ml	1.25 ml	313 µl
Separating Tris buffer	1.25 ml	1.25 ml	-
Stacking Tris buffer	-	-	313 µl
10 % SDS	50 µl	50 µl	25 µl
APS	50 µl	50 µl	25 µl
TEMED	2 µl	2 µl	2.5 µl

8.2 8.2 Ethical approval and cell line authentication sheets

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Genetic Resources, Department of Agriculture, Forestry and Fisheries
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Dear Prof Weiss

RE: REGISTRATION OF FACILITY

With reference to the application to register a facility, submitted in terms of the Genetically Modified Organisms Act, 1997 (Act No. 15 of 1997). Registration number **39.2/University of Witwatersrand -19/146.**

The facility is hereby registered; please find attached your certificate which serves as proof of registration. Please familiarize yourself with the Standard Operating Procedure approved for Regulation 2(2) to determine whether your current activities or any future activities would require an additional contained use permit or not.

Please consult the website of the Department at www.daff.gov.za (Branches, Agricultural Production, Health & Food Safety/ Genetic Resources/ Biosafety) for the latest application forms and the SOP document referred to in the above paragraph.

If any of the provisions of the Genetically Modified Organisms Act, 1997 (Act No. 15 of 1997), including any condition of any permit issued in terms of the GMO Act, is not complied with at all times, you will be subject to prosecution in terms of Section 21 of the GMO Act, 1997.

DEPT. AGRICULTURE, FORESTRY & FISHERIES
DIRECTORATE: GENETIC RESOURCES
2019 -05- 15
PRIVATE BAG X973, PRETORIA, 0001
REGISTRAR: GMO ACT

Yours sincerely

Ms N L Mkhonza
Registrar: Genetically Modified Organisms Act, 1997 (Act No. 15 of 1997)

IDEXX BioResearch
FINAL REPORT OF LABORATORY EXAMINATION
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IDEXX BioResearch Case # 5863-2015

Received: 1/16/2015
Completed: 1/22/2015

Submitted By

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Specimen Description

Species: Human
Description: cells
Number of Specimens/Animals: 10

Purchase Order #: 6720010840

ID	Cell Line	Species	ATCC #	Notes
1	A2780 C200	Human		A2780 Drug Resistant Line
2	OVCAR4	Human		
3	OVCAR8	Human		
4	SKOV3	Human	HTB-77	
5	HEK293	Human	CRL-1573	
6	BT-474	Human	HTB-20	
7	ZR-75-1	Human	CRL-1500	
8	LoVo	Human	CCL-229	
9	Capan-2	Human	HTB-80	
10	H6	Human		Subclone of HCT116

Services/Tests Performed: CellCheck 9 (9 Marker STR Profile and Inter-species Contamination Test)

Summary: Cell Check: The samples were confirmed to be of human origin and no mammalian interspecies contamination was detected. The alleles for 9 different markers were determined and the results were compared to the alleles reported for each cell line.

The genetic profiles for samples 2, 4-7, and 9 were identical to the genetic profiles reported for each cell line.

Sample 1: The genetic profile for the sample has several differences compared to the reference profile. The overall identity match of the sample is below the 80% cutoff for authentication of this sample. The A-2780 cell line does not typically demonstrate microsatellite instability and the finding of 3 alleles at multiple markers coupled with the low identity score and the finding of rare allele sizes at marker D16S539 make it unlikely for this sample to have originated from the A-2780 cell line.

Sample 3: The genetic profile is not consistent with the genetic profile reported for the OVCAR-8 cell line. The sample profile is consistent with the genetic profile reported for the A-2780 cell line (ECACC# 93112519). The identity score of the sample is 90% compared to the A-2780 cell line.

Sample 8: The genetic profile is missing an allele at marker D5S818, but the genetic profile is otherwise identical to the profile reported for this cell line.

Sample 10: The genetic profile has an allele size difference at marker vWA and is missing an allele at marker TPOX as well as missing the Y amelogenin allele. Despite this number of changes the identity score is greater than 80% and this cell line is consistent with originating from the HCT-116 parental cell line. These changes are most likely the result of microsatellite instability reported in this cell line.

Please see the report for details.

CELL CHECK

Species-specific PCR Evaluation

Species	1	2	3	4	5	6	7	8	9	10
mouse	-	-	-	-	-	-	-	-	-	-
rat	-	-	-	-	-	-	-	-	-	-
human	+	+	+	+	+	+	+	+	+	+
Chinese hamster	-	-	-	-	-	-	-	-	-	-
African green monkey	-	-	-	-	-	-	-	-	-	-

Marker Analysis

Marker Name	1		2		3		4		5		6	
	Sample Results	A-2780 (ECACC# 93112519)	Sample Results	OVCAR-4 (Mol Cancer Ther 2009;8:713-724)	Sample Results	OVCAR-8 (Mol Cancer Ther 2009;8:713-724)	Sample Results	SK-OV-3 (ATCC# HTB-77)	Sample Results	HEK293 (ATCC# CRL-1573)	Sample Results	BT-474 (ATCC# HTB-20)
AMEL	X	X	X	X	X	X	X	X	X	X	X	X
CSF1PO	10, 11, 12	10, 11	10	10	10, 11	11	11	11	11, 12	11, 12	10, 11	10, 11
D13S317	13	12, 13	9	9	12, 14	12	8, 11	8, 11	12, 14	12, 14	11	11
D16S539	15, 16	11, 13	11	11	11, 13	13	12	12	9, 13	9, 13	9, 11	9, 11
D5S818	12, 13	11, 12	13	13	11, 12	12	11	11	8, 9	8, 9	11, 13	11, 13
D7S820	10	10	10, 11	10, 11	10	12	13, 14	13, 14	11, 12	11, 12	9, 12	9, 12
TH01	6	6	9	9	6	7	9, 9.3	9, 9.3	7, 9.3	7, 9.3	7	7
TPOX	8, 10	8, 10	8	8	8, 10	8	8, 11	8, 11	11	11	8	8
vWA	14, 15, 16	15, 16	14, 18	14, 18	15, 16, 17	16, 17	17, 18	17, 18	16, 19	16, 19	15, 16	15, 16

Marker Name	7		8		9		10	
	Sample Results	ZR-75-1 (ATCC# CRL-1500)	Sample Results	LoVo (ATCC# CCL-229)	Sample Results	CAPAN-2 (ATCC# HTB-80)	Sample Results	HCT-116 (ATCC# CCL-247)
AMEL	X	X	X, Y	X, Y	X	X	X	X, Y
CSF1PO	10, 11	10, 11	10, 11, 13, 14	10, 11, 13, 14	11, 12	11, 12	7, 10	7, 10
D13S317	9	9	8, 11	8, 11	11, 12	11, 12	10, 12	10, 12
D16S539	11	11	9, 12	9, 12	9, 13	9, 13	11, 13	11, 13
D5S818	13	13	11, 13	11, 12, 13	11, 12	11, 12	10, 11	10, 11
D7S820	10, 11	10, 11	9.3, 10, 11	9.3, 10, 11	9, 11	9, 11	11, 12	11, 12
TH01	7, 9.3	7, 9.3	9.3	9.3	9.3	9.3	8, 9	8, 9
TPOX	8	8	8, 9	8, 9	8	8	8	8, 9
vWA	16, 18	16, 18	17, 18	17, 18	17	17	16, 22	17, 22

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FINAL REPORT OF LABORATORY EXAMINATION
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IDEXX BioResearch Case # 10622-2015

Received: 3/18/2015
Completed: 3/25/2015

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Specimen Description

Species: Human
Description: cells
Number of Specimens/Animals: 10

Purchase Order #: 6720012920

ID	Client ID	Cell Line	Species	ATCC #	other
1	A2780 C30	A2780 C30	Human		Cisplatin Resistant A2780
2	FC-IBC02	FC-IBC02	Human		FCCC Establsihed Line - Profile Sent
3	ASPC-1	ASPC-1	Human	CRL-1682	
4	BxPC-3	BxPC-3	Human	CRL-1687	
5	CFPAC-1	CFPAC-1	Human	CRL-1918	
6	HPAF-II	HPAF-II	Human	CRL-1197	
7	DLD-1	DLD-1	Human	CCL-221	
8	MRC-5	MRC-5	Human	CCL-171	
9	SW48	SW48	Human	CCL-231	
10	SW480	SW480	Human	CCL-228	

Services/Tests Performed: CellCheck 9 (9 Marker STR Profile and Inter-species Contamination Test)

Summary: Cell Check: The samples were confirmed to be of human origin and no mammalian interspecies contamination was detected. The alleles for 9 different markers were determined and the results were compared to the alleles reported for each cell line.

The genetic profiles for samples 2, 4-8, and 10 were identical to the genetic profiles reported for each cell line.

Samples 1 and 9: The genetic profiles of the samples have slight differences compared to the established profiles. However, the identity match is above 80% confirming the samples are consistent with the cell lines of origin.

Sample 3: The genetic profile is not consistent with the genetic profile reported for the AsPC-1 cell line (ATCC# CRL-

1682). The sample profile is identical to the genetic profile reported for the MIA PaCa-2 cell line (ATCC# CRL-1420). This sample appears to be misidentified.

Please see the report for details.

CELL CHECK

Species-specific PCR Evaluation

Species	1	2	3	4	5	6	7	8	9	10
mouse	-	-	-	-	-	-	-	-	-	-
rat	-	-	-	-	-	-	-	-	-	-
human	+	+	+	+	+	+	+	+	+	+
Chinese hamster	-	-	-	-	-	-	-	-	-	-
African green monkey	-	-	-	-	-	-	-	-	-	-

Marker Analysis

Marker Name	1		2		3		4		5		6	
	Sample Results	A-2780 (ECACC# 93112519)	Sample Results	FC-IBC02 (ATCC 003079)	Sample Results	AsPC-1 (ATCC# CRL-1682)	Sample Results	BxPC-3 (ATCC# CRL-1687)	Sample Results	CFPAC-1 (ATCC# CRL-1918)	Sample Results	HPAF-II (ATCC# CRL-1997)
AMEL	X	X	X	X	X	X	X	X	X, Y	X, Y	X	X
CSF1PO	10, 11	10, 11	10, 12	10, 12	10	10, 13	13	13	10	10	10, 11	10, 11
D13S317	12, 13	12, 13	9, 13	9, 13	12, 13	9, 12	11	11	12	12	12	12
D16S539	11, 15, 16	11, 13	11, 14	11, 14	10, 13	11	9, 11	9, 11	9, 11	9, 11	11, 13	11, 13
D5S818	11, 12	11, 12	12, 13	12, 13	12, 13	12	11	11	10, 11	10, 11	11, 13	11, 13
D7S820	10	10	8, 11	8, 11	12, 13	12, 13	10, 13	10, 13	8, 10	8, 10	10, 13	10, 13
TH01	6	6	7, 9.3	7, 9.3	9, 10	7, 9.3	9	9	8	8	9	9
TPOX	8, 10	8, 10	8, 11	8, 11	9	8, 10	8	8	8	8	8	8
vWA	14, 16	15, 16	17	17	15	17	14, 18	14, 18	17	17	17	17

Marker Name	7		8		9		10	
	Sample Results	DLD-1 (ATCC# CCL-221)	Sample Results	MRC-5 (ATCC# CCL-171)	Sample Results	SW-48 (ATCC# CCL-231)	Sample Results	SW-480 (ATCC# CCL-228)
AMEL	X, Y	X, Y	X, Y	X, Y	X	X	X	X
CSF1PO	11, 12	11, 12	11, 12	11, 12	9, 10	9, 10	13, 14	13, 14
D13S317	8, 11	8, 11	11, 14	11, 14	11, 12	11, 12	12	12
D16S539	12, 13	12, 13	9, 11	9, 11	11, 13	11, 13	13	13
D5S818	13	13	11, 12	11, 12	10, 14	10, 14	13	13
D7S820	10, 12	10, 12	10, 11	10, 11	9, 10	9, 10	8	8
TH01	7, 9.3	7, 9.3	8	8	6, 9.3	6, 9.3	8	8
TPOX	8, 11	8, 11	8	8	8	8	11	11
vWA	18, 19	18, 19	15	15	18, 19, 20	18, 20, 21	16	16