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Culture Expansion of Undifferentiated Human Mesenchymal Stem/Stromal Cells for Management of Gynaecological Diseases

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Table of Contents

Abstract

Mesenchymal stem cells (MSCs) are a type of adult stem cell of mesodermal origin. In addition to their adult stem cell properties of self-renewal, proliferation, and multi-lineage differentiation, MSCs display a range of other properties including, homing to sites of injury, and secretion of bioactive factors with angiogenic, anti-inflammatory, immunomodulatory and regenerative properties. MSCs are considered immuno-privileged cells as they do not express co-stimulatory molecules. First identified in bone marrow as colony-forming unit fibroblasts (CFU-F), MSCs have now been identified in almost all postnatal tissues. There are no ethical issues associated with obtaining MSCs providing an advantage in their use for cell-based therapies as autologous cells. The beneficial properties have ensured that MSCs have been used in clinical trials as cell-based and regenerative medicine therapies to treat many diseases related to neurological, optical, cardiovascular, musculoskeletal, vascular and immune-related diseases. Surprisingly, few trials have been conducted for gynaecological diseases such as thin endometrium, Asherman's syndrome, infertility and none for pelvic organ prolapse.

There are still many hurdles associated with the clinical application of MSCs. Although present in almost all adult tissues, they are rare cells and need extensive culture expansion to generate clinically relevant numbers. Despite their highly proliferative ability *in vitro*, the unavoidable drawback is their spontaneous differentiation, replicative senescence and cell death in culture resulting in a generation of a heterogeneous population of cells with variable and decreased potency.

There are multiple signalling pathways responsible for maintaining the MSC phenotype, including transforming growth-beta factor (TGF-β), platelet-derived growth factor (PDGF), Wnt and Notch. Therefore, there is the possibility of generating an undifferentiated homogeneous population of MSCs with high potency by targeting the signalling pathways to mitigate the unwanted effects with small molecules during culture expansion.

At the beginning of my study, there were a number of publications describing the use of small molecule/s to maintain embryonic and induced pluripotent stem cells in an undifferentiated state during culture expansion, but not for MSCs. However, macromolecules such as heparan sulphate enhance self-renewal of bmMSCs in vitro and in vivo. Thus, in this thesis, a TGF-βR inhibitor A83-01 was evaluated for its ability to maintain MSC properties and prevent spontaneous differentiation, apoptosis and senescence of pre-menopausal $SUSD2^+$ endometrial MSCs (eMSCs) to generate homogeneous undifferentiated MSCs. A83-01 dose dependently promoted $SUSD2^+$ eMSC proliferation and blocked senescence and apoptosis via the SMAD 2/3 pathway. A83-01 also enhanced the colony forming ability of eMSCs. Further, I identified that the SUSD2 adhesion molecule used to purify eMSCs were regulated by TGF-βR signalling pathway for the first time. I also demonstrated for the first time that eMSCs survive for 30 days in immunocompromised mice using cells transduced with a lentiviral vector containing the mCherry reporter gene.

To further understand the mechanism of action of A83-01, a transcriptional study was undertaken on long term passaged control and A83-01 treated eMSCs which identified genes regulating stemness, proliferation, inflammation, angiogenesis and apoptosis.

The pleiotropic nature of the TGF-βR signalling pathway acting via canonical as well as non-canonical signalling pathways was also confirmed. This indicated that treating eMSCs with A83-01 changed them into an anti-inflammatory and wound healing phenotype, further supporting their utility as cell therapy for the treatment of a wide range of diseases.

MSCs from different tissues are currently being assessed in clinical trials. Therefore, we evaluated the effect of A83-01 on MSCs sourced from post-menopausal endometrium, placenta, menstrual blood, adipose tissue and bone marrow as autologous cells for potential clinical use. A83-01 was more effective in maintaining MSCs from the reproductive tissues than those from adipose tissue and bone marrow.

This thesis also investigated the development of a high content screening (HCS) assay of A83-01 analogues to identify improved molecular structures for maintaining eMSCs stemness during culture expansion. The cell density, duration of culture and SUSD2 immunofluorescence staining assay were optimised. However, the assay was not robust enough to proceed with the screening of the chemical library. Future studies should consider the findings from the transcription study to look for molecules influencing differences in cytoplasmic and nuclear β-catenin for optimising HCS assay.

In conclusion, this study confirms the loss in functional ability of culture expanded MSCs. It identified a small molecule, A83-01 that enables the expansion of undifferentiated MSCs from reproductive tissues with increased potency for use in cell-based and tissue engineering therapies..

General Declaration

Monash University

In accordance with Monash University Doctorate Regulation 17 Doctor of Philosophy (PhD) regulations, the following declarations are made:

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original paper published in peer reviewed journal (Chapter 2) and two unpublished manuscripts in preparation for eventual publication (Chapter 3 & 4) and two review manuscripts published in peer reviewed journals (included in the appendices). The core theme of the thesis is human mesenchymal stem cells. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Ritchie Centre, Hudson Institute of Medical Research under the supervision of Associate Professor Caroline Gargett and Professor Jerome Werkmeister.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

Thesis Chapter	Publication Title	Publication status	Nature and extent of candidate's contribution
1	Stem Cells in Endometrial Physiology	Published	70%
	Endometrial mesenchymal stromal cell and tissue engineering for pelvic organ prolapse repair		60%
2	Inhibition of Transforming Growth Factor-beta Receptor signaling promotes culture expansion of undifferentiated human Endometrial Mesenchymal Stem/stromal Cells	Published	90%
3	In vivo survival of eMSC cultured in A83-01	In preparation	80%
4	Transcriptome sequencing of human endometrial mesenchymal stem cells cultured under TGF-βR inhibition	In preparation	80%

Student signature: … [redacted] ……Date: …28/06/2017…

Main Supervisor signature:… [redacted] ……..Date: 28/06/2017……

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Structure of Thesis

In compliance with Monash University Doctorate regulations, this thesis consists of unpublished work relating to “Culture Expansion of Undifferentiated Human Mesenchymal Stem/Stromal Cells for Management of Gynaecological Diseases”.

Chapter 1: General Introduction. *Written as Chapter.*

Chapter 2: Inhibition of Transforming Growth Factor-beta Receptor signaling promotes culture expansion of undifferentiated human Endometrial Mesenchymal Stem/stromal Cells. *Written as a manuscript, published in Scientific Reports.*

Chapter 3: *In vivo* survival of eMSC cultured in A83-01 Written as a manuscript in preparation for publication.

Chapter 4: Transcriptome sequencing of human endometrial mesenchymal stem cells cultured under TGF-βR inhibition. *Written as a manuscript in preparation for publication.*

Chapter 5: Effect of A83-01 on mesenchymal stem cells from different tissues. *Written as Chapter.*

Chapter 6: Development of high content screening assay of A83-01 analogues for eMSCs culture expansion. *Written as Chapter.*

Chapter 7: General discussion. *Written as a chapter*

List of Publications

Gurung, S., Werkmeister, J.A., and Gargett, C.E. 2015. Inhibition of Transforming Growth Factor-beta Receptor signaling promotes culture expansion of undifferentiated human Endometrial Mesenchymal Stem/stromal Cells. Scientific reports 5:15042. doi:10.1038/srep15042.

Gurung, S., Deane, J.A., Masuda, H., Maruyama, T., and Gargett, C.E. 2015. Stem Cells in Endometrial Physiology. Seminars in reproductive medicine 33:326-332. doi:10.1055/s-0035-1558405.

Gurung, S., Werkmeister, J. A. & Gargett, C. E. 2016. Book chapter “Endometrial MSC and Tissue Engineering for Pelvic Organ Prolapse Repair” Editor Kerry Atkinson in “The Biology and Therapeutic Application of Mesenchymal Cells” John Wiley & Sons.

Gargett, C.E., and **Gurung, S.** 2016. Endometrial Mesenchymal Stem/Stromal Cells, Their Fibroblast Progeny in Endometriosis, and More. Biol. Reprod. doi:10.1095/biolreprod.116.141325.

Letouzey, V., Tan, K.S., Deane, J.A., Ulrich, D., **Gurung, S.**, Ong, Y.R., and Gargett, C.E. 2015. Isolation and characterisation of mesenchymal stem/stromal cells in the ovine endometrium. PloS one 10:e0127531. doi:10.1371/journal.pone.0127531.

Patent

Provisional application filed at the Australian Patent Office titled “A METHOD FOR CULTURING MESENCHYMAL STEM CELLS” on behalf of Shanti Gurung, Jerome A. Werkmeister and Caroline E. Gargett on 27/08/2014. “lasped”.

Patent Cooperation Treaty application filed titled “A METHOD FOR CULTURING MESENCHYMAL STEM CELLS” (AU2015/050498) on behalf of Shanti Gurung, Jerome A. Werkmeister and Caroline E. Gargett on 27/08/2015. “lasped”.

International Conferences

S. Gurung, J. Deane, J. A. Werkmeister and C. E. Gargett. “A Small Molecule Inhibitor for Culture Expansion of Undifferentiated Human Mesenchymal Stem/Stromal Cells”. International Society for Stem Cell Research, San Francisco, California, USA, 2016.

S. Gurung, J. A. Werkmeister and C. E. Gargett. “Culture Expansion of Undifferentiated Human Endometrial MSC using a Small Molecule Inhibitor”. 4th World triennial Congress (Tissue Engineering and Regenerative Medicine International Society) Boston, USA, 2015.

National Conferences

S. Gurung, J. Deane, J. A. Werkmeister and C. E. Gargett. “Culture Expansion of Undifferentiated Human Mesenchymal Stem/Stromal Cells for Management of Pelvic Organ prolapse”. Australasian Gynaecological Endoscopy and Surgery Society, Melbourne, Victoria, Australia, 2016. Oral presentation

S. Gurung, J. A. Werkmeister and C. E. Gargett. “Use of small molecules to improve culture expansion of eMSC for clinical use”. Australasian Society for Biomaterials and Tissue Engineering, Sydney, Australia. April/2015. Oral presentation

S. Gurung, J. A. Werkmeister and C. E. Gargett. “Inhibition of Transforming Growth Factor ß Receptor Signaling Promotes Culture Expansion of Undifferentiated Human Endometrial MSCs”. Australasian Society for Stem Cell Research, Lorne, Victoria, 2014.

Local Conferences

S. Gurung, J. Deane, J. A. Werkmeister and C. E. Gargett. A Small Molecule Inhibitor for Culture Expansion of undifferentiated Human Mesenchymal Stem Cells. Monash Health Research Week, Clayton, Australia, 2016. Oral presentation

S. Gurung, J. A. Werkmeister and C. E. Gargett. "Culture Expansion of Undifferentiated Human Endometrial MSC Using a Small Molecule Inhibitor". Monash Health Research Week, Clayton, Australia, 2015.

S. Gurung, J. A. Werkmeister and C. E. Gargett. "Inhibition of Transforming Growth Factor β Receptor Signaling Promotes Culture Expansion of Undifferentiated Human Endometrial MSCs". The Ritchie Centre Colloquium, Monash Medical Centre, Melbourne, 2014.

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Presentation by my supervisor from my work

S. Gurung, J. A. Werkmeister and C. E. Gargett. “Endometrial Mesenchymal Stem Cells as Cell-Based therapy for POP”. 62nd SRI Annual Scientific Meeting, San Francisco, CA, March 25-28/2015

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S. Gurung, J. A. Werkmeister and C. E. Gargett. “A83-01, a Small Molecule TGF-beta Receptor Inhibitor Maintains Mesenchymal Stem Cell Phenotype and Function during Serum-Free Culture Expansion” International Society for Cellular Therapy ANZ Regional Meeting: Translating Cell Therapy, Adelaide, Australia. October/2015.

Abbreviations

^{o}C	Degree Celsius
dw	dry weight
μm	micron
μg	microgram
mg	Milligram
ml	Millilitre
mM	Millimolar
ad	Adipose
ALK	Activin receptor-like kinases
ANOVA	Analysis of variance
AS	Asherman's syndrome
bm	Bone marrow
BM	Bench medium
BrdU	Bromodeoxyuridine
CO_2	Carbon dioxide

CD	Cluster of differentiation
CFU-F	Colony-forming unit fibroblast
Ct	Cycle threshold
DMEM	Dulbecco’s modified eagle’s medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EGF	Epidermal growth factor
ESCs	Embryonic stem cells
FACS	Fluorescence activating cell sorting
FCS	Fetal calf serum
FDA	Food and Drug Administration
FGF2	Basic fibroblast growth factor
FSC	Forward scatter
g	Gravitational force
gDNA	Genomic deoxyribonucleic acid

GVHD	Graft-versus-host disease
HCS	High Content Screening
IDO	Indoleamine 2,3-dioxygenase
IFATS	International Federation for Adipose Therapeutics and Science
ISCT	International Society for Cellular Therapy
iPSCs	Induced pluripotent stem cells
IVF	In vitro fertilisation
Lgr5	Leucine-rich repeat containing G protein-coupled receptor 5
mb	Menstrual blood
MHC	Major histocompatibility complex
MFI	Mean fluorescent intensity
MSC	Mesenchymal stem/stromal cell
NCS	Newborn calf serum
NSG	NOD scid gamma
N_2	Nitrogen
O_2	Oxygen

p	Placenta
P	Passage
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGFRβ	Platelet derived growth factor beta
PE	Phycoerythrin
PFA	Paraformaldehyde
PGE2	Prostaglandin E2
PI	Propidium Iodide
pm	Postmenopausal
POP	Pelvic organ prolapse
qRT-PCR	Quantitative reverse transcription-polymerase chain reaction
RBCs	Red Blood Cells
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute

RT	Room temperature
SFM	Serum free medium
SM	Stromal medium
SVF	Stromal vascular fraction
TDE	Thin dysfunctional endometrium
TGF-β	Transforming growth factor beta
TGF-βR	Transforming growth factor beta receptor

Chapter 1

General Introduction

Table of Contents

1.1. Stem Cells

Stem cells are an undifferentiated population of quiescent cells with the potential to self-renew, differentiate and give rise to more specialised cells (Weissman, 2000). Although rare in adult tissues, they are responsible for maintaining homeostasis and regeneration of damaged tissues. There are mainly three groups of stem cells, according to the cell of origin and their differentiation potency: embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and adult stem cells. ESCs are pluripotent stem cells derived from the inner mass of the pre-implantation embryo. They are able to generate cell types derived from all three germ layers; ectoderm (skin), mesoderm (bone and muscle) and endoderm (lungs and gastrointestinal system). ESCs are highly proliferative and expandable in culture and capable of undergoing continuous proliferation without senescence (Trounson and DeWitt, 2016). iPSCs are pluripotent cells generated through forced expression of specific transcription factors (*OCT4*, *SOX2*, *KLF*4 and *c-MYC*) that reprogram differentiated cells (Takahashi et al., 2007). iPSCs portray similar properties to ESCs in terms of morphology, proliferative ability, telomerase activity, immortality and surface antigen expression (Takahashi et al., 2007). Pluripotent stem cells can undergo genetic mutations depending upon the *in vitro* culture conditions and extensive expansion (Simonson et al., 2015, Trounson and DeWitt, 2016). Adult stem cells are rare but found in almost all adult tissues and include haematopoietic stem cells of the bone marrow, intestinal stem cells, epidermal stem cells and mesenchymal stem cells in bone marrow, and most tissues and organs (Armstrong et al., 2012, Lv et al., 2014). Even though ESCs provide an unlimited source of cells with broad potency, obtaining them is ethically controversial. The efficacy of differentiation protocols for ESCs and iPSCs is not yet absolute and they can form teratomas in patients (Prokhorova et al.,

2009, Gutierrez-Aranda et al., 2010, Simonson et al., 2015). Unlike ESCs and iPSCs, adult stem cells are limited by their proliferative capacity and differentiation potential, but they do not have the negative concerns of ESCs and iPSCs, thus making them more attractive for cell-based and tissue engineering therapies.

1.1.1. Mesenchymal stem/stromal cells (MSCs)

1.1.1.1. Properties of MSCs

Mesenchymal stem/stromal cells (MSCs) are a type of adult stem cells. They were first identified in bone marrow by Friedenstein et al as adherent fibroblast-like colony-forming cells (Friedenstein et al., 1974). MSCs are defined as a plastic adherent clonogenic, non-haematopoietic cell population, which undergo extensive proliferation in culture and differentiate into multiple mesodermal lineages: fat, bone, cartilage, stroma, tendon and muscle *in vitro* under specific culture media **(Figure 1.1)**, and have specific phenotypic surface markers during culture (Dominici et al., 2006, Bourin et al., 2013). MSCs have been identified in tissues other than bone marrow, such as adipose tissue (Zuk et al., 2002), dermal tissue (Young et al., 2001), dental pulp (Perry et al., 2008), umbilical cord tissue (Romanov et al., 2003), placenta and endometrium (da Silva Meirelles et al., 2006, Schwab and Gargett, 2007, Gargett et al., 2016). They have a perivascular location in tissues and specific markers suggest that they are pericytes (Caplan and Bruder, 2001, Gargett, 2007, Sacchetti et al., 2007, Crisan et al., 2008). The role of pericyte/MSCs is to maintain tissue homeostasis and repair by dividing and replenishing themselves as well as mature cells (Mills et al., 2013).

MSCs also show plasticity, with the ability to differentiate into cells from ectodermal and endodermal lineages such as neuronal-like cells (Zhang and Alexanian, 2014), hepatocytes and renal tubular epithelial cells (Grove et al., 2004). The differentiation and transdifferentiation ability of MSCs has been exploited for cell-based therapies and there has been an increasing interest in their use in tissue engineering (TE). For TE applications MSCs are delivered locally, bypassing the cells in lungs and loss that occur when injected systemically (de Girolamo et al., 2013).

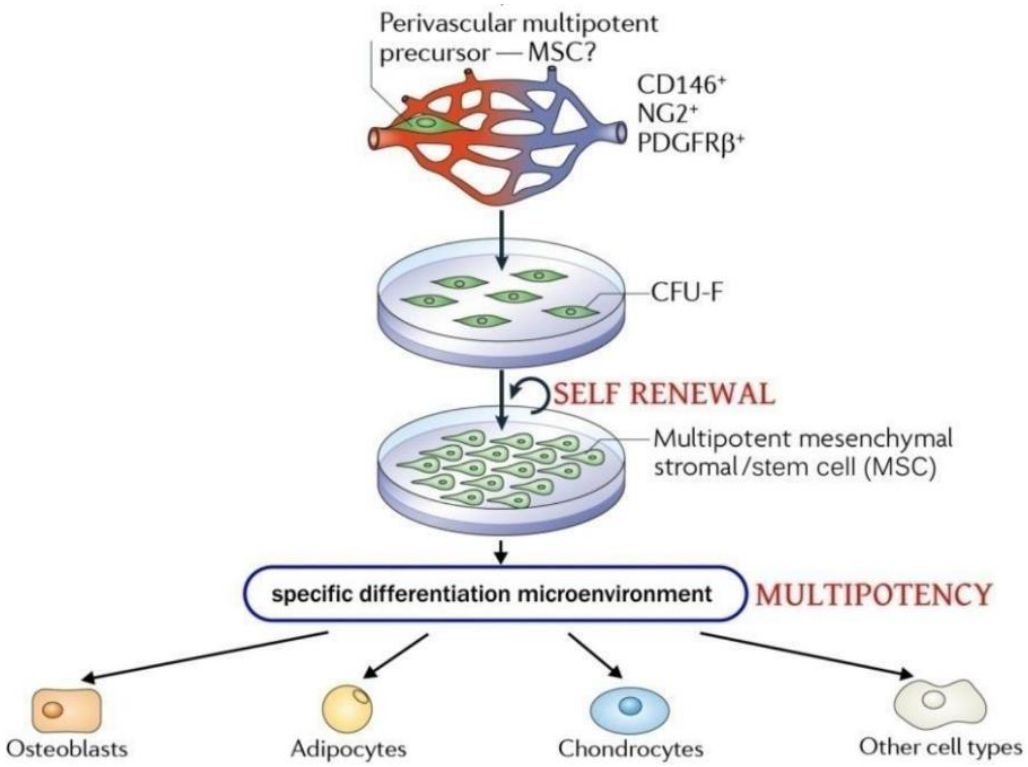

Figure 1.1: Properties of Mesenchymal stem cells (MSCs). Mesenchymal stem cells are perivascular cells expressing pericyte markers such as CD146, NG2 and PDGFRβ. They are plastic adherent and can give rise to fibroblastic colony-forming units. They can self-renew, proliferate and commit to mesodermal lineages in specific differentiation microenvironment. Reproduced from Nombela-Arrieta et.al., 2011 with permission from Nature Reviews Molecular Cell Biology.

1.1.1.2. MSC Surface Markers

As defined by the International Society for Cellular Therapy (ISCT), ≥95% of the MSCs must express surface markers CD105, CD90 and CD73 in culture, and not express the haematopoietic and their lineage markers CD34, CD45, CD14, CD11b, CD19 and HLA class II **(Table 1.1)** (Dominici et al., 2006). This definition was initially designated for cultured bone marrow MSCs and the negative markers were used to exclude the haematopoietic stem cells and their differentiated progeny (leucocytes). Although this definition was adopted to maintain uniformity and facilitate the exchange of results among MSC investigators, with the increasing knowledge of their secretome, transcriptomes, anti-inflammatory profiles and role in immunomodulation through paracrine effect, this definition needs reappraisal to better define the direction of research (Keating, 2012). Defining MSCs with these markers do not separate MSCs from differentiated fibroblasts which spontaneously form in cultures (Spitzer et al., 2012, Barragan et al., 2016, Gargett and Gurung, 2016). In addition, there is neither a single marker or a set of markers that fully defines MSCs from multiple tissues from which they are now obtained nor are these markers associated with MSC stemness properties especially in culture. Surface markers such as SSEA-4 (stage specific embryonic antigen 4), Stro-1, CD146 (melanoma cell adhesion molecule) and CD271 (low-affinity nerve growth factor receptor) (Lv et al., 2014) have been proposed as stemness markers, however, while they are invariably expressed by MSCs from various tissues, their expression varies or disappears completely during culture expansion depending on the conditions. This has led to difficulty in defining efficacious MSCs in cultures in terms of the traditional surface markers. Newer surface markers have been identified, such as SUSD2 or combinations of markers such as $CD271^{+}CD90^{+}VCAM^{+}$ and $CD146^{+}CD140b^{+}$, to identify MSCs. These markers

together with cell transcriptome profile such as Wnt, Notch and other potency related genes and proteome profile such as platelet derived growth factor, epidermal growth factor and fibroblast growth factor (Anderson et al., 2016) can be used to monitor them *in vitro* for clinical use (Keating, 2012).

Table 1.1: Minimal criteria for MSC identification (Dominici et al., 2006)

Plastic adherence in standard culture condition	
Phenotypic expression	
Positivity (>95%)	Negative (<2%)
CD105, CD73, CD90	CD34, CD45, HLA-DR, CD14 or CD11b CD79a or CD19
In vitro multipotent differentiation to osteoblasts, adipocytes and chondroblasts demonstrated by specific staining	

1.1.1.3. Homing ability to injured sites

Homing is the ability of MSCs to migrate to sites of tissue damage where they exert their reparative effect, when administered systemically (Chamberlain et al., 2007). Migration of MSCs is vital for effective systemically administered therapy. An *in vitro* migration study showed that passage 2 (P2) bone marrow MSCs (bmMSCs) migrated towards various growth factors (insulin-like growth factor-1 and platelet-derived growth factor-AB) and chemokines (stromal-derived factor-1, SDF-1), with migration towards the latter further enhanced by priming MSCs with the inflammatory cytokine TNF-

α (Ponte et al., 2007). TNF-α-primed MSCs cultured in 30% fetal calf serum (FCS) increased expression of macrophage-derived chemokine (MDC) receptors - C-C chemokine receptor type 2/3/4 (CCR2/3/4) which increased MSCs migration to chemokines RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted), MDC and SDF-1 (Ponte et al., 2007). Adhesive molecules such as P-selectin and the vascular cell adhesion molecule 1 (VCAM-1) are also increased when MSCs are placed in an inflammatory environment thus promoting their migration. SDF-1, a key MSC homing factor increased during myocardial infarction and promoted migration of mouse bmMSCs to the damaged heart and improved cardiac function *in vivo* (Askari et al., 2003). However, SDF-1 levelled off 7 days after the infarct, indicating the importance of timing of MSC transplantation in disease conditions (Askari et al., 2003). These studies indicate that mobilisation and homing of MSCs to the injured site depend upon the local and systemic inflammatory state and can be improved by timing their administration with the inflammatory milieu of patients and therefore making the best use of MSC-cell-therapy.

1.1.1.4. Tissue repair mediated by MSCs-derived bio-active soluble factors

Recently, MSCs have been recognized for their biological effects in repairing damaged tissues through secretion of soluble bioactive molecules such as growth factors, anti-fibrotic factors, angiogenic factors, chemokines and cytokines that inhibit apoptosis and activate tissue specific endogenous stem cells (Caplan, 2009). MSC-conditioned medium recapitulates the activity of MSCs *in vitro*, indicating a paracrine effect through soluble mediators and initiates cellular signalling that ultimately enhances tissue repair. Cells delivered through the systemic route are trapped in the capillary system of the lungs and the majority of studies have reported very low detection of cells in

target tissues as early as 24 hours after infusion (Parekkadan and Milwid, 2010, Eggenhofer et al., 2014). However, these studies have still detected significant tissue repair and recovery long after the cells were undetectable (Kurtz, 2008, Eggenhofer et al., 2014). Therefore, the paracrine effect of MSCs *in vivo* has been hypothesised to mediate their reparative effect.

1.1.1.5. Immunomodulatory and anti-inflammatory properties

MSCs are considered immuno privileged due to their non-expression of human leucocyte antigen-class antigen 1 (HLA class I). However, they express normal levels of MHC class I, thus are protected against natural killer cells, a component of the innate immune system. They also lack expression of co-stimulatory surface molecules CD80, CD86 and CD40, which are necessary for full activation of T-cells (Menard et al., 2013). Therefore, they are hypoimmunogenic and not detected by the host immune cells.

In vitro and *in vivo* studies have shown that MSCs have immunomodulatory properties, suppressing the activity of T cells, B cells, dendritic cells, macrophages and natural killer cells. Baboon MSCs inhibited allogeneic non-stimulated as well as IL-2 stimulated lymphocytes proliferation *in vitro* (Bartholomew et al., 2002). When MSCs were administered intravenously to baboons receiving skin grafts, the grafts survived for modest but significantly longer period of time indicating the immuno suppressive property of MSCs (Bartholomew et al., 2002). When co-cultured with peripheral blood mononuclear cells (PBMCs), MSCs are known to secrete immunosuppressive mediators such as nitric oxide (NO), indoleamine 2,3, dioxygenase (IDO) and

prostaglandin E2 (PGE2) *in vitro* which are responsible for inhibiting the innate immune cells as well as modulate their functions (Le Blanc and Mougiakakos, 2012).

MSCs also mediate anti-inflammatory effect through mediating phenotypical changes in macrophages from M1 (pro-inflammatory) to M2 (anti-inflammatory) type via action of PGE2 (Nemeth et al., 2009). Therefore, in an inflammatory environment, M2 macrophages produce IL-10, an anti-inflammatory cytokine thus promoting healing. *In vitro* co-culture studies have also indicated that IDO and COX2 expression in MSCs are increased which further polarises macrophages to M2 phenotype (Nemeth et al., 2009). Thus, administration of MSCs can modulate the host immune response in a favourable manner to promote healing.

1.2. Use of MSCs for Gynaecological Disorders

MSCs are an attractive cell type for cell-based therapies based on their differentiation potential, modulation of the immune system and the ability to secrete a wide variety of cytokines, growth factors and chemokines to promote healing. They promote tissue regeneration, home to injury sites and modulate immune cells suppressing the inflammatory environment to promote healing and recovery. MSCs are very attractive cell type for various diseases, immunologic as well as non-immunologic disorders **(Figure 1.2)**7(Trounson et al., 2011, Squillaro et al., 2016). Indeed, MSCs have been used in clinical trials for diseases related to heart, bone and cartilage, gastro-intestinal tract, neurodegenerative and immune related and inflammatory disorders (Trounson et al., 2011). Of the ~660 ongoing MSCs clinical trials listed on the NIH Clinical Trials website (https://clinicaltrials.gov, 2017, search criteria “mesenchymal stem cells”), 272 use autologous MSCs whereas 182 used allogeneic MSCs, 22 used both and the rest

are of unknown status. Despite MSCs generally being considered immuno privileged, they nonetheless have recently been found to express detectable levels of HLA Class I antigens (Murphy et al., 2013) and activated natural killer cells are able to recognise different ligands (UL 16-binding proteins and Nectin-2) expressed by MSCs (Spaggiari et al., 2006). Repeated use of allogeneic MSCs for *in vivo* differentiation and contribution to new tissues raises a possibility of activation of the host immune system to elicit a graft-versus-host response, albeit engraftment is generally very low (Eliopoulos et al., 2005, Murphy et al., 2013). Autologous MSCs, on the other hand, avoid possible immune-mediated MSC depletion of administered dose upon differentiation. Animal studies show allogeneic MSCs trigger a significant increase in $CD8^+$ killer T cells and natural killer cells compared to syngeneic MSCs (Eliopoulos et al., 2005). Similar reports have also been observed in human clinical trials where 13% of patients injected with allogeneic MSCs developed an anti-HLA antibody against the donor cells, which persisted for more than a month (Murphy et al., 2013, Perin et al., 2015). While business models favour allogeneic MSC therapies, current literature and clinical-trials outcomes so far have not yet been sufficient to make definitive conclusions about the utility of allogeneic in preference to autologous MSCs. Therefore, academic experts are in favour of autologous cell therapies but companies such as Mesoblast would hold that allogeneic are the favoured approach (Murphy et al., 2013).

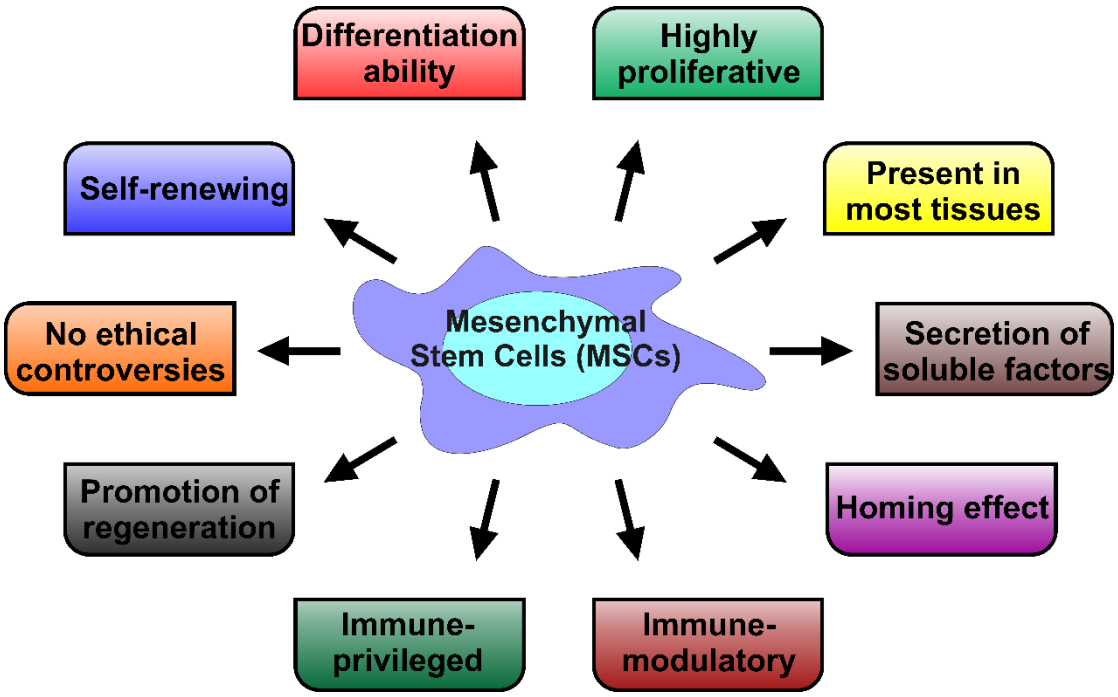

Figure 1.2: Characteristics of MSCs. Mesenchymal stem cells display properties such as self-renewal, proliferation and differentiation ability and secrete immunoregulatory cytokines which augment their potential for use in cell-based and tissue-engineering therapies.

1.2.1. Pelvic Organ prolapse

In women, pelvic organs (bladder, uterus, vagina, small bowel, and rectum) are well supported by the cardinal ligaments, levator ani skeletal muscles and the endopelvic fascia comprising the Pelvic Floor (DeLancey, 1992). Pelvic Organ Prolapse (POP) is a very common condition affecting millions of women worldwide. It is the downward descent of the pelvic organs and/or vault into the vagina due to the weakened or damaged pelvic floor support structures **(Figure 1.3)** (Jelovsek et al., 2007). There are different types of POP depending upon the organs that herniate through the vagina. A

cystocele occurs when the bladder herniates through the weakened anterior vaginal wall. Enterocele is the descent of small bowel into vagina and rectocele occurs when the posterior vaginal wall weakens, allowing the rectum to protrude **(Figure 1.3A)**. The weakening of the ligaments, muscles and vaginal walls also lead to prolapse of the uterus or the vault. In 2010, 3.3 million American women had POP and by 2050 it is estimated that the number will increase by 46% to 4.9 million (highest projection being as many as 9.2 million with POP) (Wu et al., 2009).

POP is a multifactorial condition. Pregnancy, prolonged labour and vaginal delivery are the main factors that injure the support structures due to immense stretching or tearing, causing damage or denervation especially to the levator ani **(Figure 1.3B)**. Subsequent pregnancies, childbirth, being overweight and menopausal further weaken the tissues (Jelovsek et al., 2007, Delancey et al., 2008, Ashton-Miller and Delancey, 2009, Miller et al., 2015).

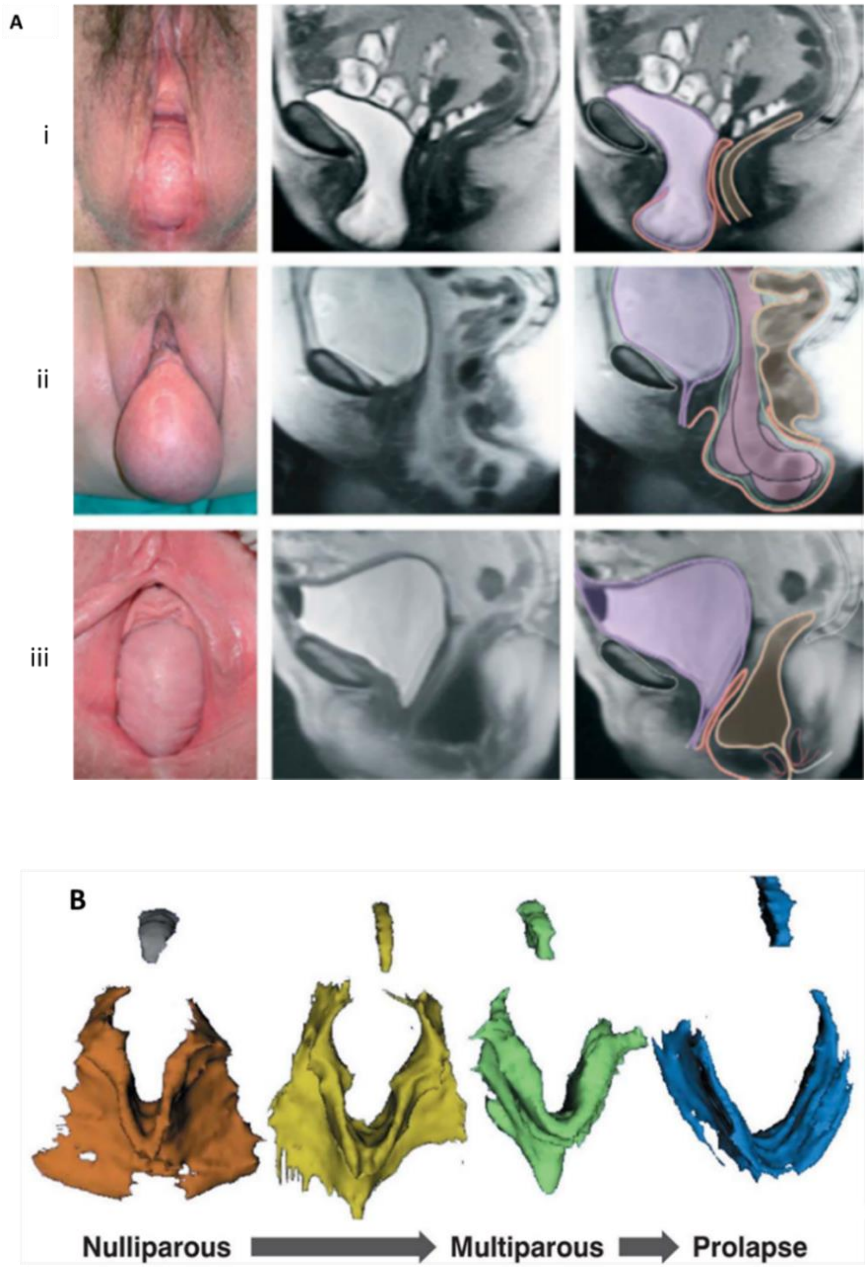

Figure 1.3: Photographs and MRI pictures showing different forms of pelvic organ prolapse. A) Cystocele (i, bladder, purple), enterocele (ii, small intestine, green) and rectocele (iii, rectum, orange) through vaginal wall (orange) are shown. The left panel is the photographs and the middle and right panels are MRI images. Reproduced from Jelovsek et al., 2007 with permission from The Lancet. **B)** Increase laxity and distension of the levator ani muscle complex resulting in a change in the shape of the muscle with increasing parity. Reproduced from Hoyte et al., 2015 with permission from Contemporary OB/GYN.

Women with POP suffer urinary and bowel and/or sexual dysfunction (Lowder et al., 2010). These symptoms affect day-to-day activities and can be quite embarrassing. Frequently women do not talk about them or wait for a long time to see a doctor. These symptoms can lead to social exclusion, loss of productivity and lower quality of life.

Surgical intervention is required in a majority of women with POP (Olsen et al., 1997, Wu et al., 2014a). The lifetime risk of women needing to undergo surgery for POP is as high as 19% in the general Australian female population (Smith et al., 2010) and 20% in the US by the age of 80 years (Wu et al., 2014a). Due to the relatively high failure rate of native tissue surgery, synthetic meshes were introduced in 2004 to treat POP. The introduction of synthetic mesh augmentation surgery for management of the POP has delivered seemingly higher 'cure' rates, especially for anterior vaginal wall damage compared to anterior colporrhaphy (vaginal wall repair) using native tissue repair. In the US, meshes were used in as many as third of POP surgeries and inserted transvaginally in ~75% of cases (Chughtai et al., 2015). More recently, serious complications such as mesh erosion into the vagina, bladder or bowel, pain, dyspareunia, mesh shrinkage and infections have led to multiple surgical interventions in as many as 18% of cases (Ellington and Richter, 2013, Maher et al., 2016). Therefore, within four years of approval for the use of vaginal mesh, the FDA posted several public notifications warning of the complications associated with transvaginal placement of surgical mesh in 2008 and 2011. In 2016 it reclassified vaginal mesh from class II moderate risk devices to high-risk class III devices. This has led to the withdrawal of vaginal meshes from the market by several leading companies (Barski and Deng, 2015) There is currently no ideal surgical management approach for treating POP, and current mesh operations require improvements to achieve better

long-term outcomes with a concomitant decrease in complications. Until this is achieved, there remains a real risk that successful treatment options will be reduced and relatively ineffective native tissue surgery will be the only option available for a large number of women with POP. There is a great need for a new approach by either targeting women early with prophylactic treatment and/or to manage women with POP using new therapeutic approaches. Recently, as a proof of principle of POP repair, our group showed a tissue engineered construct of SUSD2 enriched endometrial MSCs and gelatin-coated polyamide scaffold was able to induce tissue regeneration with enhanced healing through an anti-inflammatory effect in a wound repair model in rats (Ulrich et al., 2014a). In a similar rat model, poly-L-lactic acid scaffolds seeded with adipose MSCs also showed neovascularisation and new collagen deposition (Roman Regueros et al., 2014). Therefore, tissue engineering approaches using mesenchymal stem cells provide potential new avenues for treating POP (Ulrich et al., 2013, Roman et al., 2014, Darzi et al., 2016).

1.2.2. Asherman's Syndrome and Thin endometrium

Asherman's syndrome (AS) or intrauterine adhesion (IUD) is a condition characterised by the formation of fibrotic tissue, leading to the complete obliteration of the uterine cavity and/or cervix (Gargett and Healy, 2011). Depending upon the degree of adhesion, obliteration and site, women with AS/IUD can present with a range of menstrual problems from amenorrhea and infrequent bleeding to recurrent pregnancy loss and infertility (Gargett and Healy, 2011, Alawadhi et al., 2014).

Asherman's syndrome is an acquired disease and depending upon the severity affects 2-22% of infertile women. It commonly results due to infection or severe damage to

the basal layer of the endometrium sustained during surgery, such as dilatation and curettage following post-partum or termination of pregnancy when the circulating estrogen level is low (Gargett and Healy, 2011, Alawadhi et al., 2014). This leads to the severe damage to the endometrial stem/progenitor cells residing in the basalis layer of the endometrium, which are essential for the normal endometrial regeneration and healing without scarring (Gargett and Healy, 2011, Gargett and Ye, 2012, Alawadhi et al., 2014, Gargett et al., 2016). The resultant scant/thin functional endometrium is incapable of supporting embryo implantation (Gargett and Ye, 2012). There is no clear consensus about the treatment for Asherman's syndrome. The limited therapy available is to do surgical removal of the fibrotic tissues to restore the uterine cavity. However, the endometrial regeneration is limited by the decreased number of stem/progenitor cells in the basal endometrium.

Endometrial thickness is vital for uterine receptivity. In thin dysfunctional endometrium (TDE), the endometrium is unresponsive to exogenous estrogen stimulation and unable to generate thick enough endometrium to support embryo implantation (Hunter et al., 2015). Therapies to improve the endometrial thickness using drugs such as estradiol, low-dose aspirin, and pelvic floor neuromuscular stimulation have proven to be unreliable. Similar to AS, it is postulated that in TDE there are insufficient endometrial stem/progenitor cells.

Intravenous transplantation of bone marrow-derived stem cells in a mechanically induced murine model of Asherman's syndrome resulted in the recruitment of the cells in the endometrium with a significant decrease in fibrosis (Alawadhi et al., 2014). It also significantly improved pregnancy outcome (90%) compared to the untreated

group (30%) and the results were comparable to the normal mice. However, in this study there was the unknown identity of the incorporated cells. Similarly, in a chemically-induced rat model of Asherman's syndrome, cultured rat adipose tissue MSCs labelled with bromodeoxyuridine (BrdU) were injected locally and intraperitoneally (Kilic et al., 2014). After 15 days, around 6% of BrdU positive cells were detected in the uterine tissue sections. There was a decrease in fibrosis with increased proliferating cells and furthermore increase in vasculogenesis when oral estrogen was given concurrently (Kilic et al., 2014).

There are clinical cases reporting the delivery of stem cells into the uterine cavity with success in treating Asherman's syndrome (Nagori et al., 2011, Singh et al., 2014, Santamaria et al., 2016). A 33-year-old woman with severe Asherman's syndrome treated with $CD90^{+}CD133^{+}CD9^{+}$ autologous bone-marrow mononuclear cells and hormonal therapy was able to regenerate her endometrium thick enough to implant a donor embryo and support the pregnancy for at least 8 weeks (Nagori et al., 2011). Despite the excitement of success, this study neither expanded nor followed up on the outcome of the pregnancy and did not elaborate on how or which cell-type was involved in regenerating the thin endometrium. In a pilot study of six women with refractory AS, autologous $CD34^{+}$ mononuclear cells were implanted in the sub-endometrial zone followed by administration of oral estrogen (Singh et al., 2014). This resulted in a significant increase in the endometrial thickness and the commencement of the menstrual cycle in five of the women for at least 9 months. Despite the improvement, the endometrium was not thick enough to commence IVF treatment (Singh et al., 2014). Animal experiments of whole uterine transplantation using re-

cellularised uterus with MSCs have shown hope to women with endometrial-related infertility (Miyazaki and Maruyama, 2014, Hellstrom et al., 2016).

Recently a pilot study was reported using MSCs in women with refractory AS and thin endometrium (Santamaria et al., 2016). Autologous $CD133^+$ bone marrow-derived endothelial progenitor cells were introduced directly into the spiral arterioles through catheterization of each uterine artery. This procedure resulted in a significant increase in mature blood vessels formation expressing CD31 and alpha-smooth muscle actin (α-SMA) in the endometrium six months following cell therapy. There was a regeneration of functional endometrium assessed by a resumption of menstruation in all women. In addition, eight out of sixteen women achieved pregnancy spontaneously or through artificial reproductive therapy after cell therapy with outcomes ranging from premature rupture of membranes to successful delivery of healthy term babies in two cases (Santamaria et al., 2016). These pilot studies demonstrate the promising use of adult stem cells to regenerate endometrium (Gargett et al., 2012, Gargett and Ye, 2012). Further mechanistic studies ought to be conducted to determine the role of different tissue-sourced stem cells and how they are able to regenerate thick endometrium capable of carrying conceptus to term.

1.3. Sources of MSCs for management of Gynaecological disorders

Cell-based therapies and tissue engineering are new therapies proposed for various disease conditions and may provide a new approach in the future for the treatment of gynaecological diseases (Gargett and Chan, 2006, Ulrich et al., 2013, Roman et al., 2014). To date, there are three clinical trial studies on the use of adult stem cells for Asherman's syndrome and thin dysfunctional endometrium (https://clinicaltrials.gov,

2017, search criteria “Asherman’s syndrome and mesenchymal stem cells”). Despite POP being a very common and debilitating disorder, no clinical trials using cell-based therapy for POP have been registered in the NIH clinical trials website. Various cell sources have been proposed for treating POP using animal models such as murine skeletal-muscle derived stem cells (Roman et al., 2014), buccal fibroblasts (Roman et al., 2014) and endometrial mesenchymal stem cells (Ulrich et al., 2012, Edwards et al., 2013, Ulrich et al., 2013). As an autologous source of cells for treatment of gynaecological disorders, women can access MSCs from their endometrium (pre-menopausal as well as post-menopausal), placenta, menstrual blood, bone marrow and adipose tissue **(Figure 1.4)**.

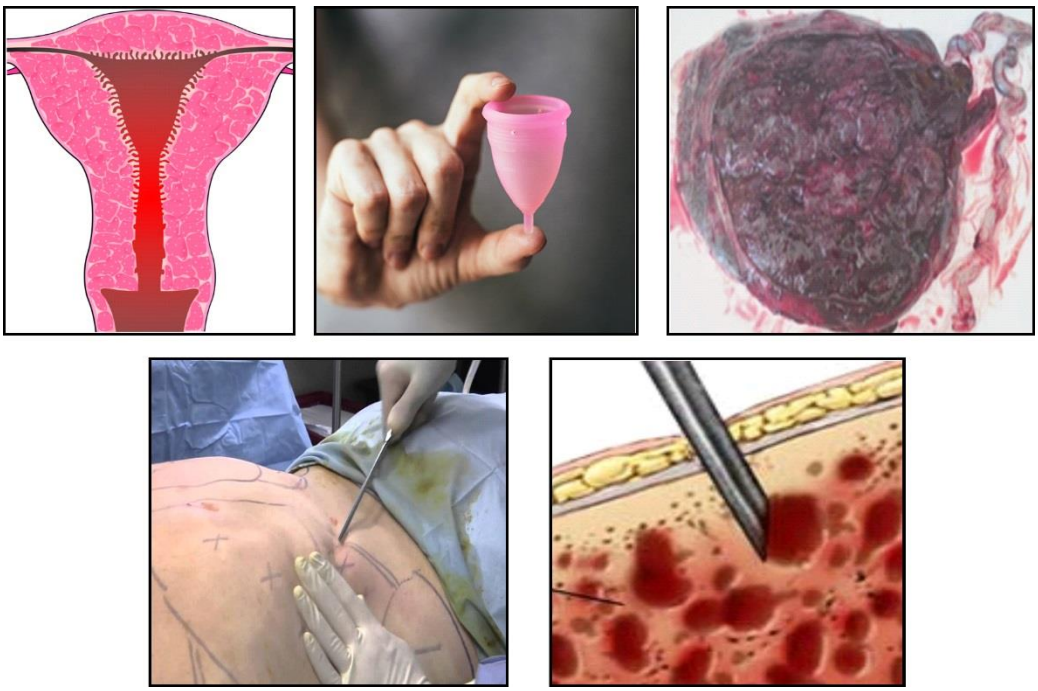

Figure 1.4: Autologous sources of MSCs for women. MSCs can be sourced from the endometrium, maternal placenta, menstrual blood, bone marrow and adipose tissue as autologous sources of cells for cell-therapy and regenerative therapy for women

1.3.1. Endometrial MSCs (eMSCs)

The human endometrium is a dynamically remodelling mucosa, undergoing 400 to 500 monthly cycles of morphological and functional changes during a woman's reproductive life (Gargett et al., 2007). Every month cyclical changes in sex steroid hormones regulates these processes to prepare a receptive endometrium for embryo implantation. In the absence of an embryo implantation, the functional layer is shed during menstruation, whereas the basalis layer is retained. A new functional layer regrows from the basalis, generating 4 to 10 mm of mucosal tissue in the first half of the subsequent cycle in response to rising circulating estrogen level (Gargett et al., 2016). Endometrial regeneration is also observed postpartum and in postmenopausal women treated with estrogen therapy for a short duration of 6-8 weeks (Ulrich et al., 2014b). Pregnancies have been reported following endometrial ablation or resection (Wood and Rogers, 1993, Gargett, 2007). These observations suggest that stem/progenitor cell populations reside deep in the basalis layer of the endometrium (Gargett, 2007).

Endometrial MSCs were first identified as rare clonogenic stromal cells, comprising 1.25% colony forming stromal cells (Chan et al., 2004). Those initiating the large colony forming unit (CFU) exhibited typical MSC properties. Transplantation of unfractionated human endometrial epithelial and stromal single-cell suspensions (5×10^5 cells) beneath the kidney capsule of ovariectomized and estrogen-supplemented immunocompromised NOG (NOD/Shi-scid/IL-2Rγnull) mice reconstituted functional endometrium comprising cytokeratin and $CD9^+$ glandular structures, $CD10^+CD13^+$ stroma, and α-SMA^+ myometrial layers (Masuda et al., 2007). The endometrial

xenografts responded to cyclical exogenous sex hormones producing tortuous glands and differentiated decidual cells followed by large blood-filled cysts suggestive of menstruation (Masuda et al., 2007).

Human eMSCs can be purified using co-expression of CD140b (PDGFRβ) and CD146 (Schwab and Gargett, 2007), and a single perivascular marker, SUSD2 (Masuda et al., 2012). These markers identified a perivascular location closely associated with endothelial cells in endometrium, pointing to a likely pericyte identity of human eMSCs **(Figure 1.5)** (Schwab et al., 2005, Masuda et al., 2012, Spitzer et al., 2012). Indeed, transcription profiling of $CD140b^{+}CD146^{+}$ and $SUSD2^{+}$ eMSCs compared to their negative counterparts shows the expression of pericyte markers and identify them as pericytes (Spitzer et al., 2012, Murakami et al., 2013).

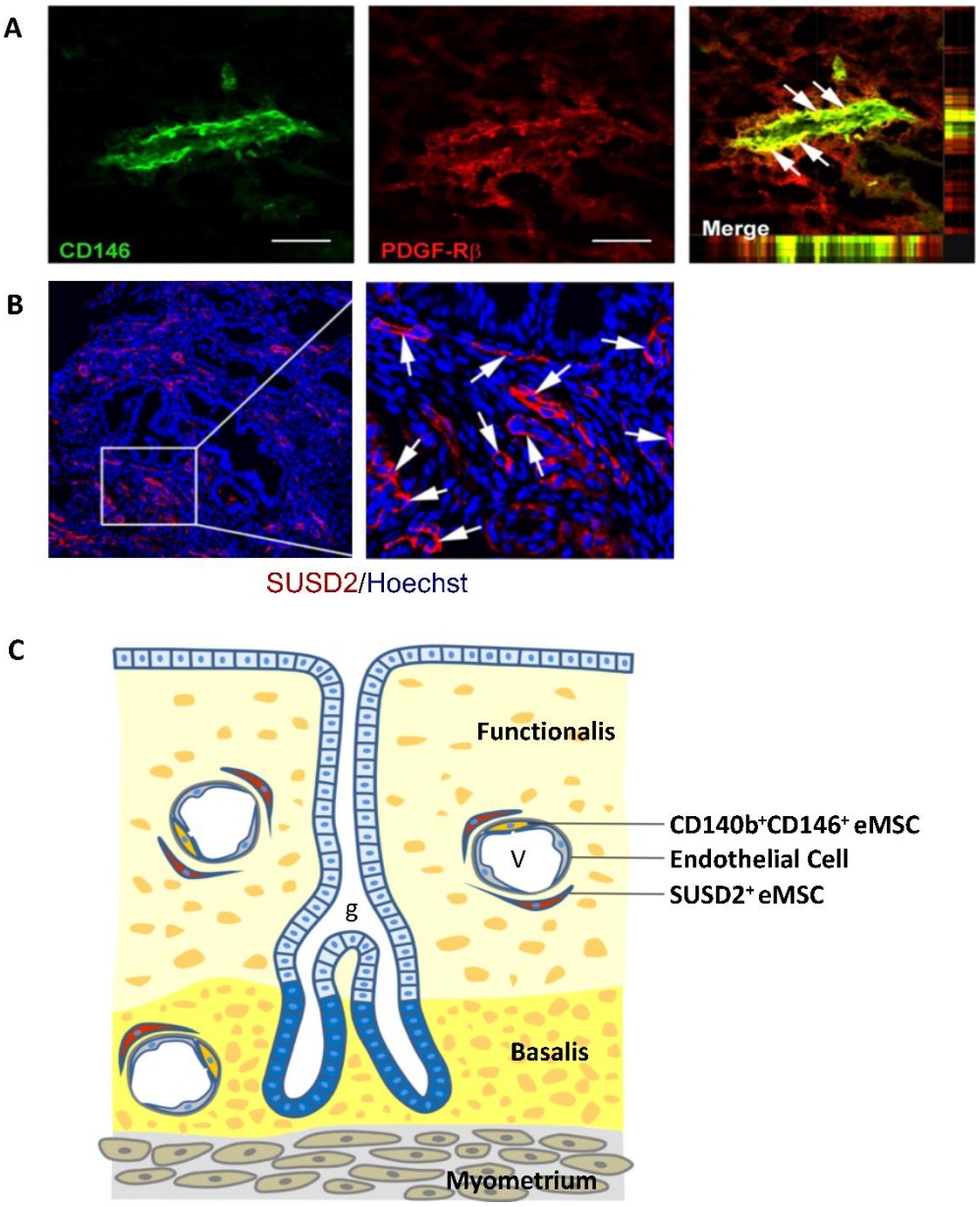

Figure 1.5: Location of human endometrial mesenchymal stem cells. Human mesenchymal stem cells are found in perivascular locations in the endometrium. **A)** CD146 (first panel), CD140b (second panel) and their co-localisation expression (arrows, last panel) around blood vessels of the functional layer of the endometrium. Reproduced from Schwab and Gargett, 2007 with permission from Human Reproduction. and **B)** SUSD2 localisation throughout the vessels in human endometrium. **C)** Schematic showing the location of the MSCs in the functionalis and basalis layer in the human endometrium (g, gland). Reproduced from Gurung et al., 2015 with permission from Seminars in Reproductive Medicine.

SUSD2 is a novel marker, recently identified as an alternative to CD271 for purifying human bone marrow MSCs (Hans-Jorg Buhring, 2007, Sivasubramaniyan et al., 2013) and endometrium using W5C5 antibody (Masuda et al., 2012). SUSD2 is a type I transmembrane protein that has a large extracellular region with domains containing a Somatomedin B, an adhesion-associated domain in MUC4 and other proteins, a von Willebrand factor and a Sushi domain (Sivasubramaniyan et al., 2013). These domains have roles in cell adhesion, homodimerisation, homing, signal transduction and migration through interaction with LGALS1 (Lectin, galactosidase binding soluble, 1) and UGGT1 (UDP-glucose ceramide glucosyltransferase-like 1) proteins (Nadjar et al., 2015). Adult human spermatogonial progenitors are also identified as $CD49f^{+}/CD49a^{-}/SSEA\text{-}4^{+}/SUSD2^{+}$ cells in the testis (Harichandan et al., 2013). SUSD2 protein is highly expressed in brain, especially in the hippocampus, where it plays a role in the neuritic growth and extracellular synapses (Nadjar et al., 2015). However, the pathways through which SUSD2 signals are not yet known.

$SUSD2^{+}$ eMSCs are clonogenic and differentiate into adipocytes, osteocytes, chondrocytes, myocytes and endothelial cells (Masuda et al., 2012). They also differentiate into smooth muscle cells (SMCs) and fibroblasts no longer expressing SUSD2 when cultured on gelatine-coated polyamide meshes (Su et al., 2014). SMCs and fibroblasts generate collagenous extracellular matrix that provide tissue elasticity and create a physiologic environment, demonstrating the therapeutic potential of eMSCs (Su et al., 2014). Human $SUSD2^{+}$ eMSCs transplanted under the kidney capsule of NSG mice also generated human $vimentin^{+}$ endometrial stroma-like tissue and migratory endothelial cells in the kidney parenchyma similar to xenografted endometrial Side Population (SP) cells (Masuda et al., 2012). These studies

demonstrate that SUSD2^{+} eMSC contribute to the regeneration of functionalis stromal fibroblasts (SFs) and blood vessels each menstrual cycle. Gene expression profiling of freshly isolated CD140b^{+}CD146^{+} eMSCs showed their similarity to endometrial fibroblasts (CD140b^{+}CD146^{-}), although there were also 762 differentially expressed genes (Spitzer et al., 2012). Principal components analysis confirmed the relatively close relationship between eMSCs and endometrial fibroblasts compared with endothelial cells (CD140b^{-}CD146^{+}) (Spitzer et al., 2012), indicating that fibroblasts are lineage cells of eMSCs. The gene expression of human eMSCs highlights their pericyte identity as genes involved in angiogenesis, inflammation, and immunomodulation were established as signature eMSC genes, together with steroid hormone/hypoxia response, cell communication, proteolysis, self-renewal, and multipotency genes. A secretome and RNA-seq analysis of passage 2 decidualized SUSD2^{+} eMSCs revealed increased immunomodulatory and trophic factor genes chemokine ligand-7, leukemia inhibitory factor (LIF), and CXCR4 receptor, but lower MHC class I and no class II genes compared with SUSD2^{-} endometrial fibroblasts (Murakami et al., 2014). Collectively these data suggest that eMSC are clonogenic, multipotent pericytes, capable of responding to hormonal changes during the menstrual cycle and having roles in regenerating the endometrial stroma and vasculature monthly. eMSCs are also responsible for optimal endometrial stromal decidualization to support embryo implantation and establishing placenta. The immunomodulatory factors secreted by eMSCs confirm their likely immunosuppressive properties similar to the bone marrow and adipose tissue MSCs and indicate a role for eMSCs in supporting the survival of the implanted allogeneic embryo.

1.3.2. Menstrual blood MSC (mbMSCs)

Every month during menstruation the functionalis is shed indicating that eMSCs can be harvested and isolated from menstrual blood. The specific markers used to isolate eMSCs (co-expression of $CD140b^{+}CD146^{+}$ or the single marker SUSD2) show their perivascular location both in the functionalis and basalis layers **(Figure 1.5)** (Schwab and Gargett, 2007, Masuda et al., 2012). eMSCs can be isolated from the endometrial biopsy or curette which sample the functionalis layer using the specific eMSC markers, CD140b/CD146 and SUSD2 (Schwab and Gargett, 2007, Masuda et al., 2012). Menstrual blood is body waste that is available every month and requires no invasive procedures (Gargett et al., 2012). Menstrual blood is best collected on the second menstrual day in a silicone menstrual cup over few hours (Koks et al., 1997). With appropriate hygienic education to donor women, there is minimal risk of infection through vaginal contact.

Menstrual blood stromal cells have been described as endometrial regenerative cells or multipotent menstrual blood stromal cells (Meng et al., 2007, Patel et al., 2008). Viable menstrual blood stromal cells (mbSCs) have been isolated from menstrual blood by culturing plastic adherent cells. They produce heterogeneous cultures of MSCs as well as stromal fibroblasts (Gargett and Masuda, 2010). Cultured mbSCs express the ubiquitous MSC markers (CD90, CD73 and CD105) and are negative for CD34, CD45 and CD14. They are also highly proliferative and karyotypically stable in long term culture (Meng et al., 2007). mbSCs differentiated into lineages from all three germ layers including neuronal, hepatic pancreatic and cardiomyocytes (Meng et al., 2007, Patel et al., 2008). Some have also shown that cultured mbSCs express pluripotency markers OCT-4, SSEA-4 as well as c-kit, and have no MHC class II but

surprisingly 100% of them were highly positive for MHC class I (Patel et al., 2008, Patel and Silva, 2008). Comparing micro RNA, gene expression profiles and growth factor levels between passage 3 mbSCs and bone marrow stromal cells showed some similarities as well as differences (Wang et al., 2012a). The gene expression analysis showed that mbSCs expressed genes more involved in immunity and inflammation-related pathways than bone marrow stromal cells. mbSCs also had very high expression of *PDGF*, angiopoietin-1 and *MMP3* genes that are vital for angiogenesis and tissue remodelling (Wang et al., 2012a).

In an animal model of myocardial infarction, mbSCs transdifferentiated into cardiomyocytes and induced neo-vascularisation, secreting paracrine factors which promoted cardiac remodelling and function (Hida et al., 2008). mbSCs fused with host muscle cells and differentiated into dystrophin-expressing skeletal muscle in a mouse model of Duchene muscular dystrophy (Cui et al., 2007). Therefore, mbSCs have potential utility in cell-based therapies for muscle injury or chronic muscular diseases (Cui et al., 2007). As proof of principle, patients suffering from multiple sclerosis were treated with mbSCs (Zhong et al., 2009). This treatment was well tolerated and safe, demonstrating the feasibility of mbSCs as a cell source for cellular therapy. Similarly, they also differentiated into dopaminergic neurons *in vitro* and in an animal model of Parkinson disease *in vivo* (Wolff et al., 2011). They migrated, engrafted in the lesion site and increased production of striatal dopamine and its metabolites. Overall, these studies show menstrual blood derived MSC/SFs as a renewable source of cells for child-bearing women.

1.3.3. Placental MSCs (pMSCs)

The placenta is a readily available biological waste tissue. The human placenta contains both maternal (decidua basalis) and fetal component (amnion and chorion). Placental MSCs can be isolated from amnion, chorion (of fetal source) and decidua basalis (of maternal source) portions of the placenta. MSCs in the feto-maternal interface highlights their role in immune tolerance and in prevention of fetus rejection (In 't Anker et al., 2004, Huang et al., 2009). pMSCs isolated from third trimester placental cotyledons were all of the maternal origin without fetal cell contamination (In 't Anker et al., 2004). They were highly proliferative than adult bmMSCs (In 't Anker et al., 2004, Barlow et al., 2008). They were also less immunogenic and more immunosuppressive than bmMSCs. Similarly, they showed typical MSCs properties, differentiating into mesodermal lineages as well as into neuronal cells (Portmann-Lanz et al., 2006). They also facilitated $CD34^+$ cord blood haematopoietic stem cell engraftment in NOD SCID mice (Hiwase et al., 2009). However, the majority of these studies used the terminology placental MSCs which included fetal as well as maternal MSCs as they were isolated from the whole placenta. Since these cultures contained cells of fetal and maternal origin, they comprised a heterogeneous population of stromal cells. Decidua basalis is derived from the basalis endometrium where $SUSD2^+$ and $CD146^+$/$CD140b^+$ eMSCs reside. Therefore, pMSCs can be easily isolated from maternal placental tissue in a similar manner as eMSCs using the same markers.

1.3.4. Adipose tissue MSCs (adMSCs)

Adipose tissue contains vascular stroma which is of mesodermal origin. The adipose-derived stromal vascular fraction (SVF) has been characterised similarly to bmMSCs (Zuk et al., 2002). They are plastic adherent cells and express typical MSC markers CD90, CD105 and CD73 with tri-lineage differentiation properties (Zuk et al., 2002). SVF also expresses CD34 and ATP-binding cassette sub-family G member 2 (ABCG2) (haematopoietic and endothelial stem/progenitor cell markers) which decreased significantly over four passages in cultures despite high expression of typical MSC markers indicating spontaneous differentiation (Mitchell et al., 2006). Adipose tissue stromal cells also differentiated into a neurogenic phenotype *in vitro*, suggesting their potential for cell therapies targeting the neuronal system. Growth factors such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF) as well as dexamethasone enhanced adherent SVF cell growth in culture (Lee et al., 2009, Chieregato et al., 2011). They are also more proliferative in 5% O_2 than in the normal 20% O_2 environment (Yang et al., 2012). The adipose-derived SVF population secret higher levels of anti-inflammatory cytokines such as IL-6 and have more potent immunomodulatory effects than bmMSCs (Melief et al., 2013). However, the SVF is a heterogeneous population of cells, including hematopoietic lineage cells (stem and progenitors, granulocytes, monocytes and lymphocytes), endothelial cells, erythrocytes, pericytes and stromal cells (Bourin et al., 2013). This is because most investigators use a plastic adherent protocol to collect the adipose tissue derived stromal cells (Bourin et al., 2013). To develop international standards for generating reproducible parameters of adipose tissue cell therapies, ISCT in conjunction with the International Federation for Adipose Therapeutics (IFATS) published a guide for isolation for MSCs from adipose tissue

and assays to assess their properties (Bourin et al., 2013). This recent guideline suggests the use of combination of positive markers CD34 to distinguish adMSCs from the rest of the cells in SVF and negative markers CD235a/CD45/CD31 to remove red blood, cells leukocytes and endothelial cells respectively (Bourin et al., 2013).

CD34 is a family of transmembrane protein with three members; CD34, podocalyxin and endoglycan (Nielsen and McNagny, 2008, Maumus et al., 2011). The CD34 family of proteins is proposed to be involved in proliferation, blocking differentiation, enhancing migration, chemotaxis and asymmetric cell division (self-renewal) (Nielsen and McNagny, 2008). They are expressed by various cell types, specifically by multipotent precursors, mast cells, eosinophils, hair follicle stem cells, vascular endothelial cells and some neuronal cells (Nielsen and McNagny, 2008).

CD34 is used to isolate haematopoietic stem cells and has been defined as a negative marker to distinguish culture-expanded bmMSCs from haematopoietic stem cells and endothelial cells (Dominici et al., 2006). Recently, the consensus in the absence of CD34 expression by MSCs has been challenged (Lin et al., 2012). CD34 has also been used to identify stem cells populations, including muscle satellite cells, adipose tissue and bone marrow. Multipotent progenitors have been reported in human $CD34^+$ bmMSCs (Lin et al., 2012). The Stro-1 antibody, which has been used exclusively to isolate bmMSCs, was generated against a population of CD34 positive bone marrow cells (Simmons and Torok-Storb, 1991, Lin et al., 2012). It was therefore hypothesised that MSCs in situ express the CD34 antigen and the lack of CD34 expression in MSCs is a cell culture phenomenon. The new adMSCs guidelines also highlight that class and clone of CD34 antibody determines the intensity of detection and recommended

the use of class III CD34 antibody to increase efficiency (clone 581 or 4H11) (Bourin et al., 2013). Indeed, all the native adMSCs were found in the $CD34^+$ fraction of the SVF (Maumus et al., 2011). The $CD34^+$ adMSCs, and not $CD34^-$ cells, have colony forming ability and are multipotent (Maumus et al., 2011). AdMSCs proliferate in serum-free, xeno-free culture media *in vitro* while maintaining multipotency (Lindroos et al., 2009, Efimenko et al., 2011). In adipose tissue, CD34 expressing MSCs are present in the stroma and around the capillaries of subcutaneous adipose tissue (Maumus et al., 2011). There is still controversy that adMSCs have perivascular origin and pericyte features. Some studies show that adMSCs co-express CD140b and NG2 pericyte markers (Traktuev et al., 2008, da Silva Meirelles et al., 2015) while others have demonstrated otherwise, despite their presence near capillaries, indicating the expression of these markers increases during cell culture (Maumus et al., 2011). As for eMSCs (Chan et al., 2004, Schwab et al., 2005), FGF2, EGF, PDGF-BB supported adMSCs-growth confirming the functionality of corresponding receptors FGFR, EGFR and PDGFRβ in adMSCs (Traktuev et al., 2008).

1.3.5. Bone marrow MSCs (bmMSCs)

Since the identification of bone marrow CFU-fibroblasts, there has been a large number of studies on bmMSCs. Multipotent MSCs in bone marrow are very rare, accounting for <1% of nucleated cells and this decreases significantly with age (Stolzing et al., 2008). Advancing age directly associate with not only the loss of bmMSC number, but also a decrease in their lifespan and proliferative ability due to replicative senescence (Stolzing et al., 2008, Caplan, 2009). bmMSCs have been isolated using various markers including STRO-1 (Simmons and Torok-Storb, 1991), CD271 (Quirici et al., 2002, Hans-Jorg Buhring, 2007) and SUSD2 (Sivasubramaniyan

et al., 2013). They self-renew and are able to differentiate into mesodermal lineages (Pittenger et al., 1999) as well as ectodermal (Schwartz et al., 2002) and endodermal lineages (Schwartz et al., 2002). They can also be expanded in serum-free and hypoxic (physiologic normoxic) environment for clinical applications (Grayson et al., 2006, Chase et al., 2010, Tsai et al., 2011). As early as 1950, bone marrow transplants were used for leukemia patients who had been treated with chemotherapy (Blau, 2013). bmMSCs secrete extracellular matrix proteins, growth factors and anti-apoptotic factors (Baraniak and McDevitt, 2010). They have been used in numerous clinical trials including intervertebral disc repair and Chronic Ischemic Stroke (Orozco et al., 2011). Presently, there are 323 registered clinical trials using bmMSCs (https://clinicaltrials.gov, 2017, search criteria “bone marrow mesenchymal stem cells). bmMSCs were the first MSC approved by Health Canada for the treatment of acute graft-versus-host disease (GVHD) in children under the age of 18 years (Le Blanc et al., 2008, Cyranoski, 2012).

1.4. Issues with MSC therapies.

The majority of studies and clinical trials with MSCs have used plastic adherent stromal cell populations expanded in culture therefore the starting cell population is heterogeneous comprising progenitors and committed fibroblasts that are less efficacious. By immunoselecting clonogenic MSCs with surface markers specific to adult stem cells, a homogeneous population of potent starting cells can be obtained in order to generate progeny with similar potency (Lv et al., 2014). As mentioned in section 1.1.1. MSCs are a rare population of cells in tissues, accounting for 1-4% of cells, which decrease with increasing age (Stolzing et al., 2008). Due to their rarity, MSCs require extensive culturing *in vitro* to generate sufficient numbers for clinical

transplantation, especially for use in adult. As an example multiple doses of ~ 9 million cells/kg body weight were given to patients with steroid-resistant, severe, acute GVHD (Le Blanc et al., 2008). Similarly, 79-300 million MSCs were used to re-cellularised mouse and rat decellularised uteri respectively (Miyazaki and Maruyama, 2014, Hellstrom et al., 2016). However, MSCs undergo spontaneous differentiation to fibroblasts and committed cells during the culture expansion process, decreasing their purity, clonogenicity and efficacy (Baxter et al., 2004, Sethe et al., 2006). MSCs become larger and also undergo replicative senescence (Zaim et al., 2012, Bellayr et al., 2014), lose differentiation potential and undergo shortening of telomeres (Parsch et al., 2004, Wagner et al., 2009). Therefore, extensive expansion increases number of cells but results in heterogeneity and experimental inconsistency.

Comparing gene expression profiling of late and early passaged bmMSCs has confirmed the impact of cell culture on the stem cell properties of expanded MSCs and identified various age-induced genes that distinguish them (Wagner et al., 2009). There were 721 genes upregulated and 481 genes downregulated in late passage compared to the early passaged MSCs (Wagner et al., 2009). These differentially expressed genes correlated with the differential gene expression observed in MSCs sourced from older individuals. Studies have shown that the survival rate was significantly less in GVHD patients treated with late passage MSCs compared to early passage MSCs (von Bahr et al., 2012). Heterogeneity and senescence decrease efficiency of culture-expanded MSCs with reduced and/or variable clinical outcome as has been the experience to date with bmMSCs (von Bahr et al., 2012, Prockop et al., 2014).

Freshly isolated transgenic MSCs injected into irradiated mice showed 55-65% recovery from bone marrow, which decreased to 10% and 0% after only 24 hours and 48 hours of culture respectively, showing both the homing capacity of bmMSCs and the shortness of their survival *in vivo* on intravenous infusion (Rombouts and Ploemacher, 2003). The authors also suggested that cell cycle stage of bmMSCs at the time of transplantation may be a factor influencing survival, whereby the primary quiescent MSCs in G0 phase of cell cycle survive longer, while the cultured cells in G1 or later stages of the cell cycle have limited survival (Rombouts and Ploemacher, 2003). The homing ability of MSCs decreases significantly if they have been pre-cultured (Sohni and Verfaillie, 2013). This shows that culture conditions likely modify the homing receptors for chemokines.

The MSC markers CD73, CD90 and CD105 appear to be expressed consistently regardless of the culture environment, passage stage or the functional ability of MSCs (Maumus et al., 2011, Lo Surdo et al., 2013). Furthermore, gene expression analysis of different passaged MSCs show no differences in genes corresponding to the markers established by the ISCT for MSC identification (Bellayr et al., 2014). Therefore, the ISCT MSC makers cannot be used to monitor multipotent MSCs during culture expansion. STRO-1, SSEA-4, CD271 and CD146 are some of the markers reported to isolate MSCs with high stemness (Lv et al., 2014). However, there is a large difference in their expression in MSCs from different tissues (Lv et al., 2014) and their expression is lost early during the culture expansion process (Jin et al., 2016). This downregulation of surface markers *in vitro* suggests early differentiation, generation of more committed cells, such as fibroblasts with concomitant loss of clonogenicity (Bellayr et al., 2014) or the influence of culture condition and the

environment on marker expression, thus they cannot be used for assessing MSCs function and phenotype *in vitro*. Similarly, CD34 expression in adMSCs, at cellular and protein level, negatively correlates as a consequence of cell culture (Maumus et al., 2011).

A comparison of transcriptional profiles of primary $CD271^{high}$ bmMSCs with culture-expanded $CD271^{+}$ MSCs using 96 gene expression arrays showed a decrease in transcriptional activity in the latter, especially Wnt-related genes (>1000 fold) and Wnt inhibitory factor 1 (Churchman et al., 2012). Similarly, of the 51 genes overexpressed in $CD271^{+}$ bmMSCs compared to the haematopoietic lineage cells, 24 genes (CD271 specific marker) were downregulated in the cultured bmMSCs indicating loss of stemness properties. A further 10 more genes, additional CD271 specific markers, were downregulated in culture-expanded MSCs. Stroma, adipogenesis and osteogenesis-related genes were also lowered in culture-expanded MSCs compared to CD271 cells, indicating loss of potency (Churchman et al., 2012). A gene expression profiling study combining hierarchical clustering and principal component analysis identified a unique set of lineage genes for eMSCs and endometrial stromal fibroblasts respectively (eSFs) (Spitzer et al., 2012). Comparison of freshly isolated eMSCs phenotypes with their short- and long-term clonal-culture counterparts confirmed the lineage commitment of eMSC to eSF, demonstrating that eMSCs rapidly and spontaneously differentiated into eSF, adopting the eSF lineage genes in culture. This suggests that MSCs lose their multipotency and undergo differentiation *in vitro* by acquiring cumulative changes towards senescence during the expansion process (Gargett and Gurung, 2016). Therefore, there is a great need to update the standard markers used to identify MSCs from all sources in culture and include a set of known

transcriptional factors directly related to MSC-potency such as TWIST-1, Id1, BMi-1, and assess functionally by colony forming ability to directly reflect stemness and *in vivo* efficacy.

1.5. Signalling pathways in MSCs

The integrity and function of adult stem/progenitor cells in tissues are regulated by intrinsic and extrinsic factors (Jones and Wagers, 2008). The extrinsic component of stem cell environment, also known as “stem cell niche” includes cellular as well as acellular components, including the extracellular matrix. The stem cell niche concept was first introduced by Schofield as a milieu where progeny maturation is prevented and stemness is maintained (Schofield, 1978). The stem cell niche is a specialised tissue structure that not only gives a physical support but also provides molecular signals for proper functioning of stem cells and determines their fate. The intrinsic component is a very complex network of signalling pathways, regulated by various transmembrane and intracellular receptors and transcription factors augmenting and/or curtailing one another (Kahn, 2014). Analysis of undifferentiated and differentiated progeny of stem cells has demonstrated that involvement of transforming growth factor (TGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), Wnt, Notch and a large number of transcriptional factors are responsible for stem cell self-renewal, proliferation, differentiation, cell death and growth arrest **(Figure 1.6)** (Conboy et al., 2005, Liu et al., 2010, Coutu and Galipeau, 2011, Bjornson et al., 2012). An imbalance between these pathways can lead to deregulated biological outcomes such as cell death, tumours or loss of organ function. Therefore, understanding the signaling pathways involved in MSC function, their niche and identifying important factors helps the understanding of process leading to stem cell

differentiation and maintenance of the stem cell population for cell-based therapy, tissue engineering and regenerative medicine applications.

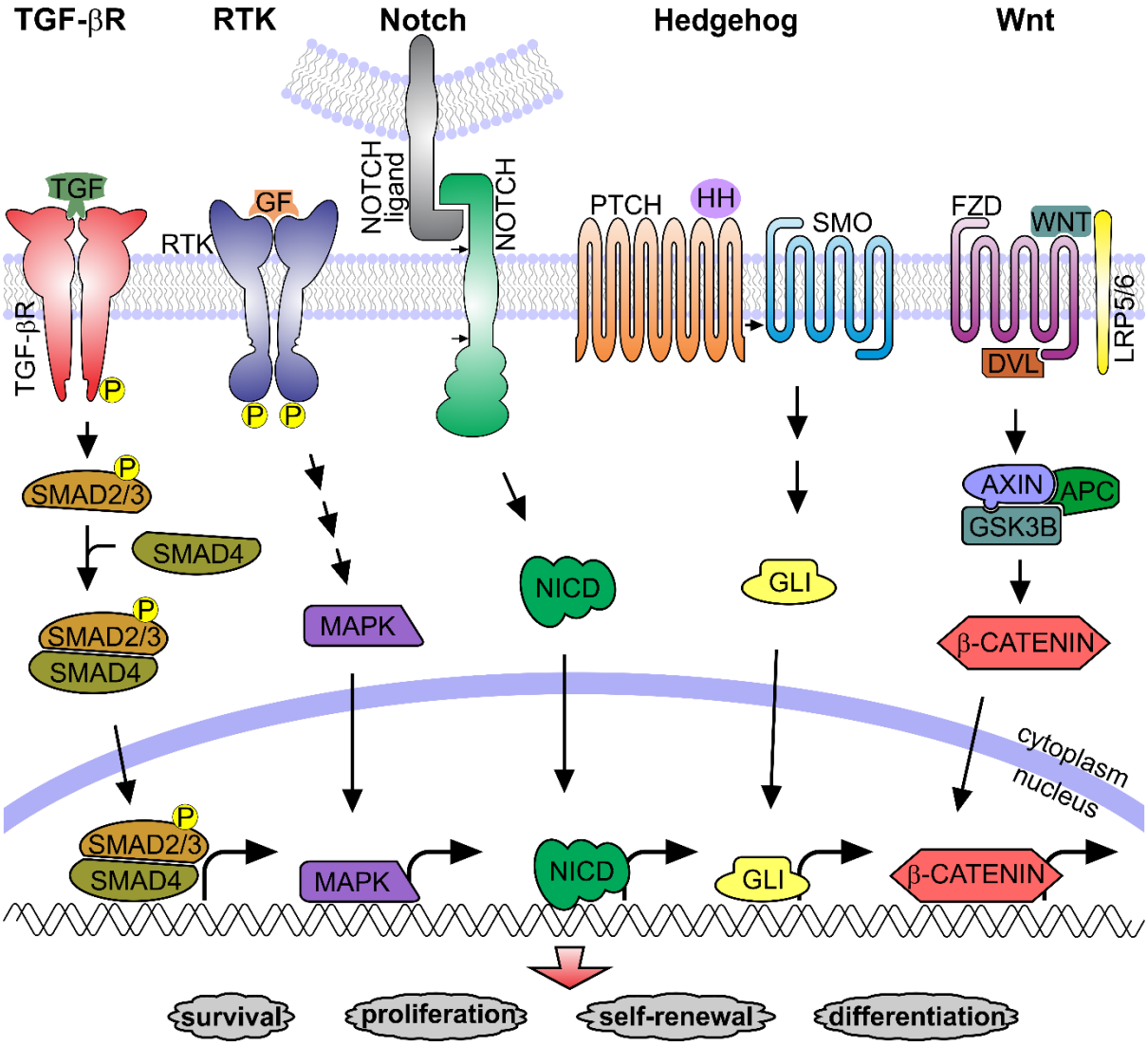

Figure 1.6 Signalling pathways involved in maintenance of homeostasis in MSCs. Various signalling pathways are involved in regulation of MSCs including Transforming growth factor beta, Receptor tyrosine kinase, Notch, Hedgehog and Wnt signalling pathways. Fine balance between these pathways and their key transcription factors is vital in sustaining homeostasis in MSCs. In addition, these pathways can also crosstalk with each other to maintain a fine regulatory network.GF, growth factor. HH, hedgehog. NICD, Notch Intracellular Domain.

Tissue oxygen concentration is comparatively low, ranging from 1% in the cellular micro environment to 14% in the arterial circulation (physiological oxygen concentration) compared to the atmospheric concentration of 21% **(Figure 1.7)** (Stamati et al., 2011). These levels allow optimal mitochondrial respiration for generating sufficient energy for cellular functions. It is apparent that physiological oxygen concentration promotes cell homeostasis (Ivanovic, 2009). Oxygen utilisation in cells produces reactive oxygen species as by-product which can cause cellular damage and induce signals for apoptosis and differentiation (Ivanovic, 2009). Human bmMSCs cultured in 5% O_2 enhanced bFGF, VFGF-A, IL-6 and IL-8 production *in vitro*. Condition medium (CM) from these cultures increased proliferation of keratinocytes, fibroblasts and endothelial cells (Chen et al., 2014). Endothelial cell migration and tubular formation were also enhanced compared to the CM from normoxic MSC cultures. These results were also supported by *in vivo* studies showing accelerated wound healing in a mouse model (Chen et al., 2014).

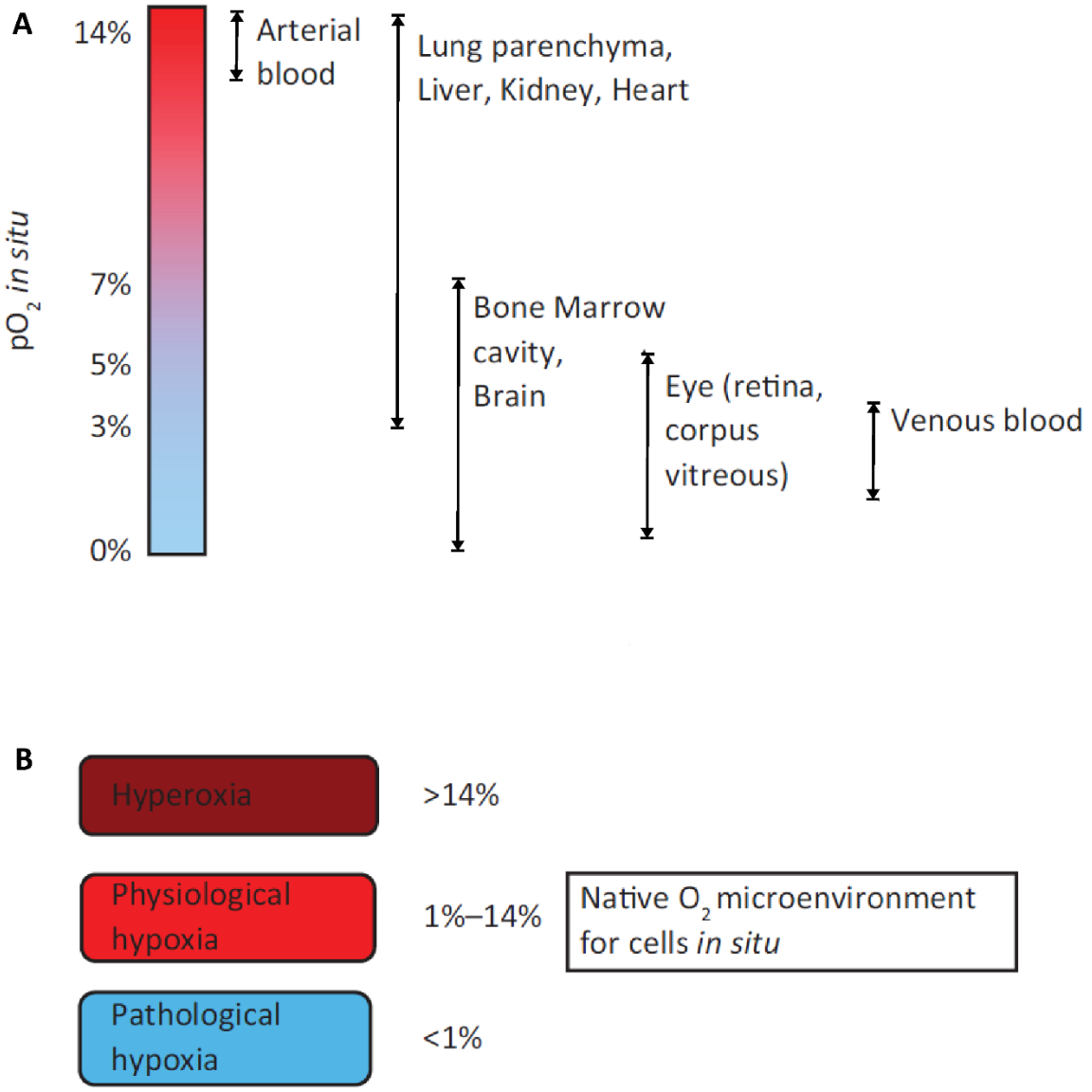

Figure 1.7: In situ tissue oxygen concentration. a) In situ oxygen concentration in different tissues b) Cellular native oxygen microenvironment in different tissues in the mammalian body. Modified from Stamati et al, 2011 with permission from Journal of Tissue Engineering.

1.5.1. Transforming growth factor receptor beta pathway

Transforming growth factor-beta (TGF-β) is a pleiotropic cytokine involved in regulation of various cellular responses (Ng et al., 2008, Wang et al., 2012b, Zhang, 2017). TGF-β is secreted by many cell types including MSCs in a latent precursor form, which is processed extracellularly to generate mature active form. In the canonical pathway, the ligands bind to the membrane-bound TGF-β type II receptor which allows dimerisation with TGF-β type I and this receptor complex initiates signal transduction through phosphorylation and activation of Smad2/3 (Derynck and Zhang, 2003). Smad2/3 activation can also result via the binding to p38MAPK and JNK (Derynck and Zhang, 2003, Neuzillet et al., 2014). Together with the common partner Smad4, the Smad-protein complex translocates and accumulates in the nucleus where it regulates transcription of target gene, Smad binding element (SBE) **(Figure 1.8)**. In the non-canonical pathway, TGF-β receptor signalling can activate Smad-independent cascades such as Erk, JNK and p38 MAPK kinase pathways (Derynck and Zhang, 2003). Activation of tyrosine kinase receptor can also activate the above pathways and regulate the Smad proteins as well as transcription of target gene, transcription factor binding element (TBE) **(Figure 1.8)**.

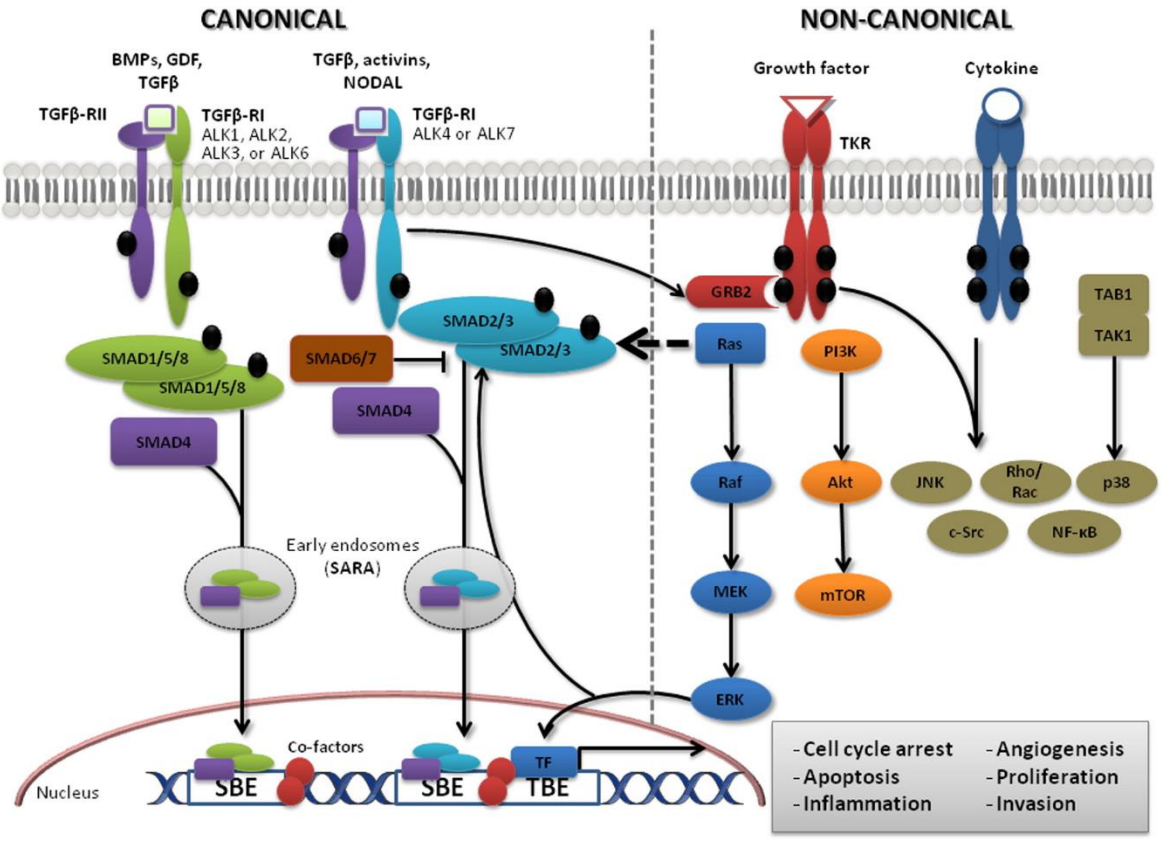

Figure 1.8: Transforming growth factor beta signalling. TGF-β family signals via SMADS dependent canonical pathway, regulating transcription of target gene, Smad binding element (SBE). It also signals in a SMADS independent non-canonical pathway to regulate transcription of target gene, transcription factor binding element (TBE). Reproduced from Neuzillet et.al., 2015 with permission from Pharmacology and Therapeutics.

TGF-β is secreted by MSCs (Popova et al., 2010, Kyurkchiev et al., 2014) and leads to their differentiation via an autocrine loop (Mishra et al., 2005, Kurpinski et al., 2010, Popova et al., 2010, Zhao and Hantash, 2011). It is one of the main cytokines

promoting differentiation of MSCs into osteoblasts, chondrocytes and adipocytes (Pittenger et al., 1999, Ng et al., 2008). Human neonatal lung MSCs produce TGF-β1 which promotes myofibroblast differentiation (Popova et al., 2010). This finding was confirmed in lysophosphatidic acid stimulated adMSCs which secreted TGF-β1 that led to myo-fibroblast differentiation (Jeon et al., 2008). TGF-β1 concentration is substantially increased in subchondral bone in humans with osteoarthritis, attracting nestin positive MSCs to the damaged site. This was associated with increased osteoid formation and high vascularity an effect, reversed with an inhibitor as well as an antibody against TGF-β1 (Zhen et al., 2013). Collectively, these studies indicated that the TGF-β signalling pathway is important in MSC differentiation.

TGF-β also participates in apoptosis via Smad activation, in association with Death associated protein 6 and TGF-βR inducible transcription factor (Ribeiro et al., 1999, Black et al., 2007). TGF-β apoptosis effects are cell context dependent, and in hepatocytes and epithelial cells, it acts through the activation of TGF-β-inducible gene-1 and production of reactive oxygen species, activating caspase and increasing the mitochondrial permeability (Ribeiro et al., 1999, Black et al., 2007). In MSCs, it acts via increasing ROS production. TGF-β signalling also induces senescence in bmMSCs via the production of mitochondrial ROS (Wu et al., 2014b).

In the human endometrium, TGF-β and their high affinity receptors are expressed by endometrial cells and macrophages under the influence of physiological female hormones, fluctuating during different phases of menstrual cycle. TGF-βs are expressed at the highest level in late secretory and menstruation and decrease in the

proliferative phase (Omwandho et al., 2010). TGF-β transducers, pSmad2/3/4 are expressed by endometrial stromal cells. TGF-β1 is also expressed by post-menopausal endometrium, especially in the stromal compartment (Loverro et al., 1999). These observations suggest that TGF-β signalling is important in the cyclical differentiation and scarless healing of the endometrium (Trounson and McDonald, 2015). TGF-β also promoted differentiation of human $SUSD2^{+}$ eMSCs into smooth muscle actin-2a (SMA2a) and Smooth Muscle-Myosin Heavy Chain (SM-MHC) expressing smooth muscle cells when cultured on polyamide/gelatine meshes (Su et al., 2014). This differentiation process also demonstrated the loss of SUSD2 expression indicating that SUSD2 is downregulated upon cellular differentiation.

Depending upon the cell-type, TGF-β are important regulators of growth stimulation or inhibition, apoptosis, angiogenesis, immune responses, cell differentiation and tumorigenesis.

1.6. Improving potency of MSCs with small molecules

Accumulating evidence has highlighted the potential of MSC-based therapy with more than 600 clinical trials either completed or ongoing currently using MSCs for many disease conditions worldwide (https://clinicaltrials.gov/). So far, no serious adverse effects have been documented using MSC therapy in these clinical trials (Trounson and McDonald, 2015). Despite these positive results, the majority of MSC-clinical trials have not progressed beyond phase I/II due to the underwhelming effects. Culture-expanded MSCs are senescence, committed cells in a heterogeneous population with a varying hierarchy of stemness and multipotency. There is a significant scope for

improvement in generation of more effective MSCs for better outcomes in cell therapies.

An alternative approach is to manipulate MSCs with drugs-like small molecules. Small molecules are readily available to the cells and their effects can easily be manipulated because of their selective and reversible nature. They can replace recombinant proteins and growth factors that promote long-term self-renewal and thus are cost effective. Small molecules can activate/stimulate proliferation, block differentiation and stimulate cells to produce desired soluble factors, prevent apoptosis during culture expansion or in an *in vivo* inflammatory environment (Lairson et al., 2013). Small molecules have been screened to identify chemicals to replace the four Yamanaka factors (Oct3/4, Sox2, Klf4, c-Myc) to generate induced pluripotent stem cells chemically. They have been used to promote differentiation of MSCs into cartilage, bone, cardiac cells, neurones (Cao et al., 2013, Ma et al., 2013). Furthermore, there are numerous reports of employing small molecules in pluripotent stem cells for their reprogramming, culture maintenance and differentiation into various cell types (Amit et al., 2000, Takahashi et al., 2007, Cao et al., 2013). At the commencement of my PhD candidature, little work had focused on screening small molecules to prevent spontaneous MSC differentiation during culture-expansion to maintain their stemness. Small molecules can be used to target known signalling pathways using cell-based high-throughput or high content screening. Approaches used depend upon the cell-related process to generate a desired outcome such as cell surface/intracellular marker expression or functional outcome (Lairson et al., 2013).

Small molecules/chemical cocktails have been used in MSCs to differentiate them to desired lineages. For instance Purmorphamine promotes osteogenesis (Wu et al., 2002), Rosiglitazone to adipogenesis (Styner et al., 2010) and Kartogenin to chondrogenesis (Johnson et al., 2012). NR-101, a small molecule c-MPL agonist, was identified through screening a chemical library assessing $CD34^+$ haematopoietic stem cell proliferation (Nishino et al., 2009). It promoted net expansion of $CD34^+$ haematopoietic stem cells and improved the yield of colony forming units. Similarly, Bmi-1 was identified as an essential component for maintenance of self-renewing haematopoietic stem cells (Park et al., 2003). Short term and long term treatment of $CD34^+$ cord blood cells with the copper chelator, TEPA, increased the proliferation of early progenitors and prolonged the *in vitro* expansion. Treatment of $CD34^+$ cells with TEPA also significantly enhanced their engraftment potential in sublethally irradiated NOD/SCID mice (Peled et al., 2004). This expansion protocol has been used to expand $CD34^+$ cord blood cells *ex vivo* for phase II/III clinical trials for treating haematopoietic malignancies (Patrick, 2014, Mehta et al., 2015)

Recently there has been an increased interest in the use of small molecules to regulate MSCs by targeting pathways, such as TGF-β and PDGF-β (Ball et al., 2012), to improve functional ability of MSCs in culture. This will ensure homogeneity and primitive phenotype of culture expanded MSCs which will retain similar potency as primary MSCs and will have more efficacious therapeutic capability.

1.7. TGF-βR inhibitor, A83-01

A83-01 is a selective inhibitor of TGF-β signalling pathway with high potency. It blocks signaling of type I serine/threonine kinase receptors for cytokines TGF-β superfamily

and phosphorylation of Smad2/3. A83-01 has a selective blocking activity of the kinase activity in TGF-beta type I receptor ALK5, ALK 4 and ALK7 (Tojo et al., 2005). It is more potent than a previously described ALK5 inhibitor, SB-431542, and prevented the growth inhibitor activity by TGF-β (Tojo et al., 2005, Mahmood et al., 2010).

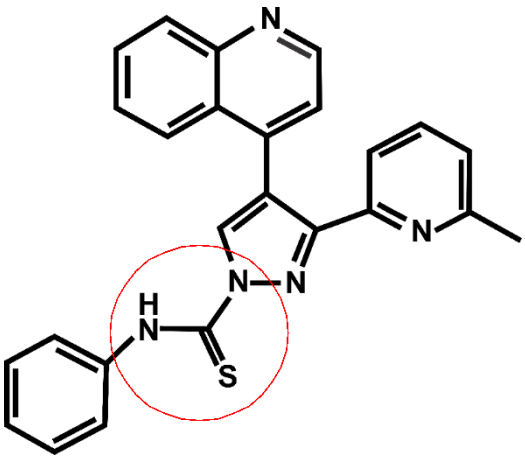

Figure 1.9: Structure of A83-01. Structure of small molecule A83-01 TGF-βR inhibitor is shown with thiourea moiety circled.

A83-01 has a thiourea moiety which has copper chelating properties **(Figure 1.9)**. Thiourea derivatives have been used in medicine and agriculture. It prevents copper mediated oxidative cellular damage by chelating cuprous ion in the media and the formation of a redox-inactive thiourea-copper complex (Zhu et al., 2002). In addition, thioureas are known to have antimicrobial, antimalarial and anti-HIV properties (Ghorab et al., 2016). They are incorporated in many tyrosine kinase inhibitors because of their strong hydrogen bonds in the ATP binding pocket of the enzymes (Ghorab et al., 2016).

Copper is an essential element. Serum contains approximately 50-100ng/ml elemental copper. Albumin, which is used as serum replacement in serum free medium is a physiological copper transporter. Copper can contribute to oxidative stress which is toxic to the cells unless it is chelated with ceruloplasmin and transported to the cells (Linder, 2012). Copper deficiency leads to failure of cells to differentiate, for example patients with copper deficiency have anaemia, neutropenia and thrombocytopenia, with intact progenitors. Supplementing with copper therapy fully resolves the condition (Harless et al., 2006). Copper plays a vital role in cellular differentiation, especially towards osteogenic lineage (Rodriguez et al., 2002). Treating $CD34^+$ cord blood cells with TEPA, a copper chelator, delayed differentiation (assessed by markers), and enhanced cell expansion and CFU (Peled et al., 2004). TEPA pre-treatment also increased $CD34^+$ cells engraftment in NSG mice.

A83-01 is quite unique in that it is the most potent TGF-β inhibitor as well as containing a thiourea moiety with a copper chelating properties that may be beneficial for cell expansion. Human $Lgr5^+$ liver stem cells cultured in mouse liver stem cells medium could not be cultured beyond three weeks and revealed highly active TGF-βR signalling (Huch et al., 2015). This was mitigated by blocking TGF-β signalling with A83- 01, which improved cloning efficiency and extended the lifespan of Lgr5 cells in culture (Huch et al., 2015). $Lgr5^+$ human colonic epithelial cells were maintained in medium containing A83-01 for extensive periods of time, retaining their stem cell markers (Sato et al., 2011). In a mouse model of myocardial infarction, A83-01 facilitated cardiac repair by inducing expansion and enhancing cultured $NKx2.5^+$ cardiomyoblast population viability through increasing Birc5 expression via MEK/ERK-dependent pathway (Chen and Wu, 2012, Chen et al., 2015). In an isoprenaline-

injected mouse model of myocardial infarction, intra-peritoneal injection of A83-01 inhibited Fzd6 (Wnt receptor) and Wnt1 signaling and reversed Wnt3a induced decrease in cardiac progenitor cells through increasing autocrine survivin release which enhanced myocyte survival. There was also decreased cardiac fibrosis and improved cardiac function, indicating A83-01 mediates cardioprotection (Ho et al., 2016). A cocktail of A83-01, GSK3 inhibitor (CHIR) and BMP inhibitor (Dorsomorphin) in culture medium enabled robust self-renewal of cardiovascular progenitor cells (CVPCs) derived from human embryonic stem cells and human induced pluripotent stem cells and retained a high level expression of MESP1/2 (mesodermal posterior BHLH transcription factor ½), a key CVPC marker (Cao et al., 2013). The absence of these inhibitors lead to the loss of colony morphology, decreased CVPC proliferation and MESP1/2 expression, highlighting the importance of these co-inhibitors in maintaining undifferentiated CVPCs (Cao et al., 2013).

1.8. Rationale

Gynaecological disorders such as POP, TDE and AS affect millions of women worldwide and are a burden to the health care system in addition to the mental health of women. Current treatments available for these conditions have very low success rates or are not effective. Cell therapy using MSCs and a tissue engineering approach provides an attractive alternative. There is an urgent need for an alternative approach to manage them. An alternative approach using cell sources will promote repair through tissue regeneration. MSCs are present in almost all post-natal tissue and women can source them from their own endometrium, menstrual blood, placenta, adipose tissue or bone marrow for use as an autologous cell-based therapy.

Due to the inherent rarity of MSCs in tissues, *in vitro* expansion is a requisite. Generating millions of cells for clinical applications has been hindered by their spontaneous differentiation, senescence and loss of functional ability during culture expansion. Understanding the pathways regulating these *in vitro* changes is vital if we wish to manipulate them. Targeting these pathways with small molecules during the culture expansion process is effective, specific and more economically feasible than using growth factors or cytokines which are expensive and may be non-specific. Culture expansion of MSCs with small molecules can generate large homogeneous populations of MSCs with equivalent functional ability *in vitro* as the freshly isolated primary MSCs.

1.9. Aims and Hypotheses

This thesis aimed to investigate the potential role of a small molecule A83-01 in generating a homogeneous population of undifferentiated mesenchymal stem cells from different tissues to be used as a potential source of autologous mesenchymal stem cells for the management of various gynaecological disorders.

Hypothesis I

Spontaneous differentiation of eMSCs in culture can be prevented by inhibition of the transforming growth factor beta receptor pathway with inhibitor A83-01.

Aim I

In **chapter 2**, the aim was to determine whether A83-01 maintained growth and prevented spontaneous differentiation of eMSC during culture expansion.

Hypothesis II

eMSC treated with A83-01 will have similar stem cell properties to the parent cells *in vivo* in a xenogeneic small animal model.

Aim II

In **chapter 3**, the aim was to determine whether cultured expanded eMSCs treated with A83-01 survived for longer duration *in vivo* in an immunocompromised mouse.

Hypothesis III

RNA sequencing will reveal differentially expressed genes in the A83-01 treated late passage eMSCs compared to control cells.

Aim III

Chapter 4 aimed to map the signalling pathway networks to identify mechanism of action of A83-01 treated and control eMSCs using transcriptome analysis.

Hypothesis IV

A83-01 will have similar effects on MSCs from other tissue sources.

Aim IV

In **chapter 5**, the aim was to determine if A83-01 promoted proliferation and blocked apoptosis and senescence of post-menopausal endometrial, menstrual blood, placental, bone marrow and adipose MSCs.

Hypothesis V

The structure function relationship between A83-01 and its analogues will identify the active moiety and determine mechanism of action.

Aim V

In **chapter 6**, the aim was to optimise an assay for a small molecule library of A83-01 analogues and identify the optimal small molecules for eMSC growth.

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Chapter 2

Inhibition of Transforming Growth Factor-β Receptor signaling promotes culture expansion of undifferentiated human Endometrial Mesenchymal Stem/stromal Cells

Shanti Gurung[1, 2], Jerome A. Werkmeister[1,3] and Caroline E. Gargett[1, 2]

I have renumbered sections of this published paper in order to generate a consistent presentation within the thesis. Please see Appendix 1 for published PDF.

Table of Contents

Declaration

Monash University

Declaration for Thesis Chapter 2

In the case of Chapter 2, my contribution to the work involved the following:

Name	Co-author name(s), Nature and % of Co-author's contribution*	Co-author(s), Monash
Shanti Gurung	Performed the experiments, collected and assembly data, analysed the data, and wrote manuscript	90%
Jerome Werkmeister	Collaborated by designing, data analysis and interpretation and final approval of manuscript	N
Caroline Gargett	Conceived and designed the experiments, provided the financial support, helped with data analysis and	N

Student signature: [redacted] Date: 28/06/2017

Abstract

Human endometrial MSC (eMSC) are a novel source of MSC easily harvested from the highly regenerative uterine lining. We have developed protocols for eMSC isolation from single cell suspensions using magnetic bead-sorting using a perivascular marker antibody to SUSD2 and culture expansion in serum free medium (SFM). Similar to other MSC, eMSC spontaneously differentiate into fibroblasts during culture expansion decreasing their purity and efficacy. The aim of this study was to determine if A83-01, a TGF-β receptor inhibitor prevents eMSC differentiation in culture. $SUSD2^+$ eMSC were cultured in SFM with bFGF/EGF in $5\%O_2/5\%CO_2$. At passage 6, eMSC were incubated with or without A83-01 for 7 days, then analysed for MSC properties. A83-01 dose dependently promoted $SUSD2^+$ eMSC proliferation and blocked apoptosis via the SMAD 2/3 pathway. Fewer A83-01 treated cells were autofluorescent or stained with β-galactosidase, indicating reduced senescence. A83-01-treated cells had higher cloning efficiency, differentiated into mesodermal lineages and expressed MSC phenotypic markers. These data suggest that A83-01 maintains $SUSD2^+$ eMSC stemness, promoting proliferation by blocking senescence and apoptosis in late passage cultures through binding to TGF-β receptors. Small molecules such as A83-01 may enable the expansion of undifferentiated MSC for use in tissue engineering and cell-based therapies.

2.1 Introduction

Mesenchymal stem/stromal cells (MSC) have been identified in almost all adult human tissues (Crisan et al., 2009) since Friedenstein and colleagues discovered colony-forming fibroblasts in bone marrow in the 1970s (Friedenstein et al., 1974). MSC are typically characterised by their clonogenicity, multipotency (Pittenger et al., 1999) and surface phenotype (Dominici et al., 2006). In addition, MSC home to damaged tissues (Caplan and Bruder, 2001), and have anti-inflammatory and immunomodulatory properties (Bianco, 2014). Increasingly, MSC are recognized for their biological effects in repairing damaged tissues through secretion of soluble bioactive molecules, including growth factors such as vascular endothelial growth factor (Burlacu et al., 2013), anti-fibrotic factors such as hepatocyte growth factor and prostaglandin E2 (Dong et al., 2015), angiogenic factors (Kuchroo et al., 2015) and molecules that inhibit apoptosis and activate tissue specific progenitor cells. MSC-conditioned medium recapitulates the activity of MSC in vitro indicating a paracrine effect that initiates cellular signalling that ultimately enhance tissue repair (Takahashi et al., 2006, Katagiri et al., 2013). These MSC properties have led to their use in numerous clinical trials for a variety of diseases, including graft versus host disease (Le Blanc et al., 2008), cardio-vascular disease as a cell-based therapy (Psaltis et al., 2008) or in tissue-engineered constructs for bone (www.clinicaltrials.gov).

MSC have recently been identified in the highly regenerative uterine lining (endometrium). Human endometrial mesenchymal stem/stromal cells (eMSC), like other mesenchymal stem/stromal cells are a rare group of quiescence cells (~1–4%) found in a perivascular location (Schwab and Gargett, 2007, Masuda et al., 2012). In the endometrium, eMSC are found in the functionalis layer that is shed during

menstruation and in the remaining basalis layer from which the new functionalis grows each month (Gargett, 2007, Gargett et al., 2008). eMSC can be prospectively isolated from endometrial biopsy tissues using co-expression of the MSC markers, CD140b and CD146 by flow cytometry sorting or with a single marker SUSD2 using magnetic beads (Schwab and Gargett, 2007, Masuda et al., 2012). eMSC isolated using the W5C5 antibody that recognises the SUSD2 antigen have typical in vitro MSC properties, in addition to reconstituting stromal tissue in vivo and significantly reducing inflammation and promoting neovascularisation when delivered as a tissue-engineering construct in an animal model of wound repair (Masuda et al., 2012, Ulrich et al., 2014). SUSD2 is a novel marker, recently identified, as an alternate to CD271 for purifying human bone marrow MSC (bmMSC) (Buhring et al., 2007). SUSD2 is a type I transmembrane protein that has a large extracellular region with domains known to have roles in cell adhesion, homodimerisation, signal transduction and migration (Sivasubramaniyan et al., 2013) through interaction with LGALS1 (galactosidase-binding, soluble, 1) and UGGT1 (UDP-glucose ceramide glucosyltransferase-like 1) proteins (Nadjar et al., 2015). SUSD2 is also highly expressed in brain especially in the hippocampus where it plays a role in neuritic growth and excitatory synapses which involve its cell adhesive properties (Nadjar et al., 2015).

eMSC require expansion for use in clinical applications similar to bmMSC (Masuda et al., 2012, Rajaraman et al., 2013, Ulrich et al., 2013). However, like other MSC, eMSC undergo spontaneous differentiation to fibroblasts during the culture expansion process, decreasing their purity (Baxter et al., 2004). Heterogeneity and decreased efficacy of culture-expanded MSC result in reduced clinical effect. In addition, the regenerative potential of MSC declines with age (Stolzing et al., 2008). Freshly

isolated, culture expanded SUSD2$^+$ eMSC underwent spontaneous differentiation indicated by decreasing proportions of SUSD2$^+$ cells and increasing SUSD2$^-$ cells with increasing passage (Ulrich et al., 2014). The MSC markers designated by the International Society of Cellular Therapy (ISCT) do not indicate the “stemness” of culture expanded MSC. During culture expansion, MSC age losing CFU activity, tri-lineage multipotency, telomere length and ability to generate neotissue *in vivo*, despite expressing the standard ISCT MSC markers (Digirolamo et al., 1999, Banfi et al., 2002, Baxter et al., 2004). For example, bmMSC lose differentiation and proliferative capacity even though expressing high levels of CD44, CD271, CD90 and CD105 during extended culture (Gharibi and Hughes, 2012). Thus, these typical ISCT MSC markers cannot be used to monitor the differentiation state of MSC during culture. The novel marker SUSD2 and CD146 may be superior markers to monitor the status of MSC during the culture expansion process (Rajaraman et al., 2013).

The loss of clonogenicity, multipotency and onset of senescence upon extensive culture of bmMSC results in increased senescence-associated beta-galactosidase and p16 gene expression, as well as changes in DNA methylation, limiting the utility of MSC as a cell-based therapy (Bork et al., 2010). The maintenance of a stem/progenitor cell population during culture expansion requires activity of signalling pathways involved in self-renewal and proliferation while preventing differentiation (Xu et al., 2008). Several small molecules targeting signaling pathways involved in maintaining pluripotency or blocking differentiation have been used for pluripotent cell cultures. Inhibition of the GSK3β, MEK and TGF-β signalling pathways have been used in rat and human induced pluripotent stem cells (iPSCs) to prevent spontaneous differentiation and maintain their stemness during prolonged culture (Li and Ding,

2010). The ROCK inhibitor, Y27632, has been used to prevent dissociation-induced cell death of human embryonic stem cells (Watanabe et al., 2007). The PDGFR-IV tyrosine kinase inhibitor (#521233, Calbiochem) increased expression of pluripotency genes *OCT4* and *NANOG*, and increased MSC potency from multipotent to a pluripotent state (Ball et al., 2012). Transforming-growth factor beta receptor (TGF-βR), platelet-derived growth factor receptor (PDGF-R) and basic-fibroblast growth factor receptor (bFGFR) pathways have crucial roles in specifying MSC differentiation into osteogenic, myogenic, adipogenic and chondrogenic lineages (Ng et al., 2008). Controlling MSC self-renewal and differentiation with small molecule inhibitors or activators of one or more of these key signalling pathways, should generate a homogeneous MSC population during culture expansion.

The use of eMSC for cell-based therapy requires their expansion in culture conditions that supports homogenous growth and maintains self-renewal and multipotency. A83-01 is a potent selective inhibitor of the TGF-βRs ALK4, 5, and 7. A83-01 inhibits SMAD2 phosphorylation (Tojo et al., 2005, Li et al., 2009), maintains self-renewal and proliferation of rat and human induced pluripotent stem cells in cultures without feeder layers (Li et al., 2009). The aim of this study was to determine whether A83-01 maintained growth and prevented spontaneous differentiation of eMSC during culture expansion. In this study, we showed that A83-01 promotes proliferation of late passage SUSD2^{+} cells in serum free medium (SFM), an effect mediated by SMAD2/3 signaling. A83-01 also prevented senescence and apoptosis of cultured eMSC, suggesting that TGF-β has a role in regulating SUSD2 expression and eMSC growth, apoptosis and senescence and therefore may have a role in spontaneous MSC differentiation during culture expansion. Small molecules such as A83-01 may provide

an approach for the expansion of undifferentiated MSC for use in tissue engineering and cell-based therapy.

2.2 Materials and Methods

2.2.1 Human endometrial tissue samples

The experimental protocols were conducted under the ethical guidelines according to the National Health and Medical Research Council (NHMRC) of Australia's National Statement on Ethical Conduct in Human Research. Human ethics approval was obtained from the Monash Health and Monash University Human Research Ethics committees (09270B). Human endometrial tissues samples were collected from pre-menopausal women who were undergoing endometrial curette or hysterectomy for non-endometrial pathologies and who were not taking any exogenous hormones for three months prior to the surgery, following informed patient consent.

2.2.2 Isolation and magnetic bead sorting of SUSD2^{+} eMSC

eMSC were isolated according to our previously published protocol (Masuda et al., 2012). Briefly, endometrial tissues from hysterectomy sample were carefully scraped off the underlying myometrium. Both hysterectomy and curette tissues were mechanically minced and digested with 0.5% collagenase type I and 40 μg/ml deoxyribonuclease type I (both Worthington Biochemical Corporation) in Dulbecco's modified Eagle's medium (DMEM/F12) for 90 and 60 minutes, respectively in a humidified incubator at 37^{o}C on a rotating MACSmix (Miltenyi Biotech). The tissue digest was filtered through 40 μm cell strainer (BD Biosciences) to separate the epithelial gland fragments and undigested tissues. The red blood cells in the filtrate were separated from the single stromal cells by density gradient centrifugation using Ficoll-Paque (GE healthcare Bio-science). eMSC were obtained by incubating the stromal cells in Phycoerythrin (PE)-conjugated anti-human SUSD2 (10 μg/ml,

BioLegend)) in 0.5% FCS/PBS (bead medium) and anti-PE magnetic-activated cell sorting microbeads (Miltenyi Biotec) for 30 minutes each in the dark on ice. The conjugated pellet was resuspended in bead medium and applied to a Miltenyi column (Miltenyi Biotec, #130-042-201) in a magnetic field. The separated cells, containing the $SUSD2^+$ eMSCs in the column were eluted in bead medium and the cells counted.

2.2.3 Cell culture and assessment of cell proliferation

The $SUSD2^+$ eMSC were initially maintained in DMEM/F12 medium containing 10% Fetal calf serum (FCS) (Invitrogen, Lot #769369), 1% antibiotic-antimycotic (Life Technologies) and 2mM glutamine and slowly changed to an in-house DMEM/F12 serum free medium supplemented with basic fibroblast growth factor (FGF2, 10ng/ml) and epidermal growth factor (EGF, 10ng/ml) (SFM) at 37ºC in 5%O_2/5%CO_2/90%N, as described previously (Rajaraman et al., 2013). The cells were seeded at 5000 cells/cm^2 density at subsequent passages in fibronectin (10ug/ml) pre-coated culture flasks. Cell proliferation assays were performed at passage 3 by seeding 1000 cells in 100 μl SFM per well in fibronectin pre-coated 96-well plates with or without A83-01, concentrations varying from 0-10 μM. Media was changed every second day and contained A83-01 at the same concentration. Following 7 days of culture, 20μl of MTS tetrazolium reagent (Promega) was added to each well and incubated for 2 hours and the soluble formazan product was quantified using a micro plate reader (SpectraMax Plus384; Molecular Devices) at 490nm. Further experiments were done at passage 6 where the cells were separated into two groups, one group was treated with 1μM A83-01 and the control with (0.01% DMSO) vehicle. The data was normalised to the control and reported as a percentage.

2.2.4 Immunophenotyping

eMSC were trypsinised with TrypLE™ (Life technologies, #12604-021)) and resuspended at 10^5 cells/tube. Cells were washed with 5% heat-inactived newborn calf serum in DMEM (bench medium) and incubated with PE-, APC- or FITC-conjugated primary antibodies or matched-isotype controls in bench medium for 30 minutes in the dark on ice. Primary antibody used was CD146 (1:1 supernatant, clone CC9, kind gift from Prof David Haylock CSIRO). PE-conjugated antibodies were SUSD2 (1:20, Biolegend, #327406), CD140b (1:20, R&D systems FAB1263P) and CD271 (1:20, Miltenyi Biotec). APC-conjugated antibody was CD90 (1:20, BD Pharmingen). Isotype control antibodies at the same concentration as the primary antibody were included for each run and were used to set the electronic negative control gate on the flow cytometer. Finally, cells were washed with bench medium and fixed with 4% paraformaldehyde (PFA) in 2%FBS/PBS. Samples were analysed using a MoFlo Flow Cytometry (Beckman Coulter) and Summit software (version 5.2., Beckman Coulter).

2.2.5 Immunofluorescence microscopy

Passage 6 (P6) eMSC were cultured on coverslips with or without 1 μM A83-01 for 7days and then fixed in 4% PFA followed by protein block (Dako, X0909) for 10 minutes each at room temperature with washing in between with PBS. PE-conjugated SUSD2 antibody (1:200, BioLegend, #327406) in 2% FCS/PBS was incubated for 2 hours at room temperature in dark. Isotype control IgG1 antibody was used as a negative control. Hoechst 33258 (1:2000, Molecular Probes) was used to visualise nuclei. Images were visualised and photographed using a Delta Vision microscope, and analysed using ImageJ software (ImageJ-win32.Ink).

2.2.6 Immunoblotting

Cell lysates were prepared using lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% triton X-100) with mini protease inhibitor cocktail tablet (Roche) and phosphatase inhibitor sodium vanadate (2 mM). The following antibodies were used: anti-SMAD 2/3 antibody (#3102S), antiphospho-SMAD 2/3 (#8828S), horseradish peroxidise conjugated secondary antibody (#7074S) from Cell Signalling Technology. The specific protein was detected by treating the membrane for two minutes with enhanced chemiluminescence (# 133406, Abcam) which provides the HRP substrate, and capturing the signal in X-ray films.

2.2.7 Quantitative RT-PCR

RNA was isolated using PureLink® RNA mini Kit (Life technologies, #12183018A) and further treated with DNase (PureLink™ DNase, Invitrogen) to obtain DNA-free total RNA. First-strand cDNA was synthesized using SuperScript III first-strand synthesis system (Invitrogen). 50 ng of cDNA was amplified and detected using TaqMan probes for *OCT4, NANOG* and *SOX2* (pluripotent associated genes), and SYBR Green Super Mix for *SUSD2, CD146, AOC3 MMP3* (MSC associated genes), *FRZB, DKK1* (Wnt related genes), *NOTCH3, NOTCH2* and *NESTIN* (NOTCH related genes). The PCR conditions consisted of initial denaturation at 95^{o}C for 10 minutes, followed by 40 cycles of denaturation at 95^{o}C for 15 seconds and annealing/polymerisation at 60^{o}C for 60 seconds. Primer sets are detained in **Table 2.1**. GAPDH or β-Actin was used as an endogenous control to normalise the target gene expression and fold change was calculated using the $2^{-\Delta\Delta CT}$ method.

Table 2.1 . RT-PCR Primers Used

GENE	PRIMER SEQUENCE
OCT4	F:CAGTGCCCGAAACCCACAC
	R: GGAGACCCAGCAGCCTCAAA
NANOG	F: TAATAACCTTGGCTGCCGTCTCTG
	R: GCCTCCCAATCCCAAACAATACGA
SOX2	F: ACACCAATCCCATCCACACT
	R: GCAAACTTCCTGCAAAGCTC
SUSD2	F: AGAGCTGGATGGACCTGAAA
	R: ATGCCAGCATGATGGAGAC
CD146	F:GAAGCATGGGGCTTCCCAG
	R:CCTCCGGAGCTTTGTAGACG
AOC3	F:TCAGCTGGGAGAGGATTTGG
	R:CGGAAGTAGATGGAGTCGGC
MMP3	F:AGCAAGGACCTCGTT TTCATT
	R:GTCAATCCCTGGAAAGTCTTCA
FRZB	F:CCTGCCCTGGAACATGACTAA
	R:CAGACCTTCGAACTGCTCGAT
NESTIN	F:GAAACAGCCATAGAGGGCAAA
	R:TGGTTTTCCAGAGTCTTCAGTGA
DKK1	F:GATCATAGCACCTTGGATGGG
	R:GGCACAGTCTGATGACCGG
NOTCH2	F: GTTTGTGTGGATGGGGTCAA
	R: TCCACATCCTCTGTGCAGAA
NOTCH3	F:GGACCTGCCGTGGCTATA
	R:ACGTCGTCCTCACAGTTATCA
GAPDH	F:TGTGGGCATCAATGGATTTGG
	R:ACACCATGTATTCCGGGTCAAT
β-ACTIN	F:GGGCATGGGTCAGAAGGATT
	R:AGTTGGTGACGATGCCGTG

2.2.8 Mesenchymal stem/stromal cell properties

To assess colony-forming ability, P6eMSC pre-treated and untreated with 1μM A83-01 for 7 days were seeded at 50 and 100 cells/cm^2 on fibronectin-coated 100 mm culture dishes (BD Falcon) in SFM in a tri-gas incubator 5%O_2/5%CO_2/90%N_2 for four weeks. The cells were then formalin-fixed for 10 minutes and stained with haematoxylin (Amber Scientific). The colonies were washed twice with distilled water and counterstained with Scott's tap water to develop the blue colour. Colony efficiency was calculated by counting the number of colonies divided by the number of cells seeded and the percentage determined.

To assess multipotency, the remaining cells were cultured in adiopogenic and osteogenic, and control medium (1% fetal calf serum) on 13-mm coverslips, and for chondrogenic differentiation the cells were cultured as 3D pellets in chondrogenic induction media for 4 weeks at 37^{o}C in 5%CO_2/5%O_2 as described previously (Rajaraman et al., 2013). To detect the differentiation, the cells were fixed with 4% PFA and incubated with 1% Oil Red O for adipogenesis, 4% Alizarin Red (pH 4.1) for osteogenesis and 1% Alcian blue (pH 2.5) on paraffin embedded sections (5 μm) of the micromass pellet for chondrogenesis. Stained cells were examined under an Olympus BX41 microscope (Olympus) and images were taken with 10X objective lens using the DP25 digital camera (Olympus).

2.2.9 Cell cycle analysis and apoptosis by flow cytometry

To assess the cell cycle status, P6 A83-01 treated and untreated cells were detached, pelleted and fixed in ice-cold 70% ethanol at 4^{o}C overnight. They were washed with 2%FBS/PBS and incubated with 50 μl RNAse (100 μg/ml, Sigma) at room temperature

for 15 minutes. 200 μl of propidium iodide (PI) (50 μg/ml, Sigma P4170) was added and the cells were analysed immediately by flow cytometry using BD FACS Canto™ II on PI-linear scales. The data were analysed using FlowJo 7.6.3.

To assess apoptosis, P6 A83-01 treated and untreated cell-pellets were stained with Annexin V-APC/PI kit following the manufacturer's protocol (# 88-8007, eBioscience). Briefly, cells were trypsinised and resuspended in 100 μl binding buffer, 5 μl of Annexin V-APC solution was added to the cell suspension and incubated for 15 minutes at room temperature protected from light. Following washing with the binding buffer, 5 μl of PI was added to the cells suspended in 200 μl binding buffer and events immediately acquired by flow cytometry using BD FACS Canto™ II and analysed with FlowJo 7.6.3.

2.2.10 Cell senescence by β-galactosidase and auto-fluorescence

Senescent cells were assessed by staining for beta-galactosidase activity. P6 A83-01 treated and untreated eMSC were cultured on coverslips for 7 days as described above, then fixed in 4% PFA for 10 minutes and stained in freshly prepared X-Gal (1mg/ml in DMSO) staining reagent (5mM $K_3Fe(CN)$, 5mM $K_4Fe(CN)$, 2 mM $MgCl_2$, 150mM NaCl) in citrate buffer at pH6 for 24 hours at 37°C. The cells were washed twice with PBS and counter stained with nuclear fast red (Sigma-Aldrich, 0.1% w/v) for 10 minutes, then examined under an Olympus BX41 microscope (Olympus). Images were taken with 10X objective lens using the DP25 digital camera (Olympus).

2.2.11 Statistical Analysis

Non-parametric Friedman's test with Dunn's multiple comparison post hoc tests were used to test for multiple groups and Wilcoxon matched-pairs signed rank tests were used to test for statistical significance between treated and control groups. Data are presented as mean ± standard error of mean. Differences were considered statistically significant at $p<0.05$.

2.3 Results

2.3.1 A83-01 dose dependently promotes eMSC proliferation

In our earlier studies, we observed that $SUSD2^+$ cells diminished in number with increasing passage (Ulrich et al., 2014), despite their high purity on initial seeding following SUSD2 magnetic bead sorting (Masuda et al., 2012). To examine the effect of the TGF-βR inhibitor, A83-01 on eMSC proliferation, passage 3 eMSC were cultured in SFM in 5% O_2 with A83-01 concentrations ranging from 0-10 μM for 7 days. Control medium was supplemented with vehicle. The MTS cell viability end-point assay was used to assess the effect of A83-01 on eMSC growth. As shown in **Figure 2.1A**, A83-01 dose dependently increased the number of viable cells with maximal effect at 1 μM concentration ($p<0.05$) by day 7. This result suggests that TGF-βR signaling regulates cell growth in negative manner. All further experiments were carried out with P6 eMSC using A83-01 at 1 μM concentration. A83-01 blocks the phosphorylation of SMAD2/3 **(Figure 2.1B)** thus indicating its activity via SMAD pathway.

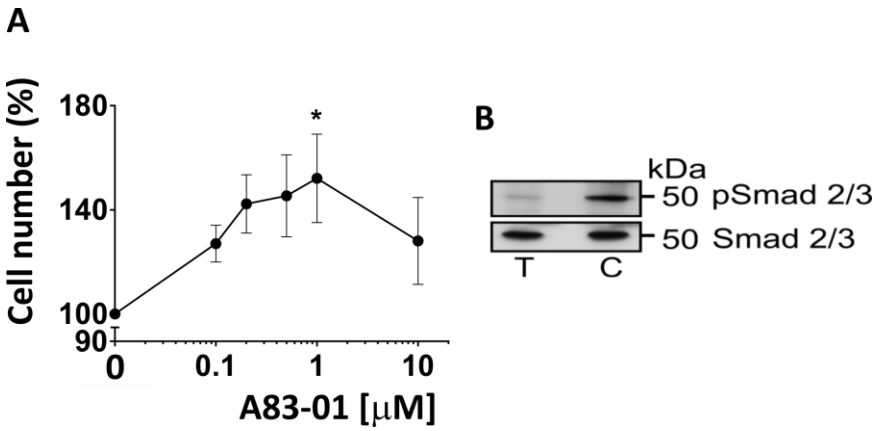

Figure 2.1 Dose Response curve of A83-01 promotion of eMSC proliferation. (A) Passage 3 eMSC incubated A83-01 in SFM in 5%O_2/5%CO_2 at 37ºC for 7 days was assessed by MTS cell viability assay. Means for triplicates were obtained for each sample at each concentration, then normalised to vehicle control DMSO (100%) and plotted as mean ± SEM of n=6 patient samples. **(B)** Passage 6 eMSC lysates with or without 1 μM A83-01 were immunoblotted with anti-SMAD 2/3 or anti-pSMAD 2/3 antibodies. A83-01 inhibited TGF-βR-induced phosphorylation of SMAD 2/3. (C=control, T= treated).

2.3.2 Surface Phenotype Expression of A83-01 treated eMSC

We next examined the phenotype of A83-01 treated eMSC. Single-color flow cytometry analysis of 5 MSC markers showed that untreated P6 eMSC cultures comprised 69%-SUSD2$^+$, 53%-CD140b$^+$, 1%-CD146$^+$, 95%-CD90$^+$ and 0%-CD271$^+$ positive cells **(Figure 2.2A)**, suggesting loss of the MSC phenotype and spontaneous differentiation. It is noteworthy that CD90, the representative ISCT MSC marker (Dominici et al., 2006) did not change over the period of culture, and that P6 eMSC did not express CD271 (bmMSC marker) whether incubated with or without A83-01. There was a significant increase in the percentage of SUSD2$^+$ (94%, $p<0.05$) and

CD140b$^+$ (83%, $p<0.05$) cells when the P6 cells were treated with 1μM A83-01 for at least 7 days. There was also an increase in the mean fluorescence intensity for the SUSD2 marker on the SUSD2$^+$ cells **(Figure 2.2B, C)**, indicating an increase in the number of SUSD2 molecules per cell ($p<0.05$). This was also evident by immunofluorescence **(Figure 2.2D)**. However, A83-01 had no effect on the CD146 expression, which was downregulated during culture expansion **(Figure 2.2A)**.

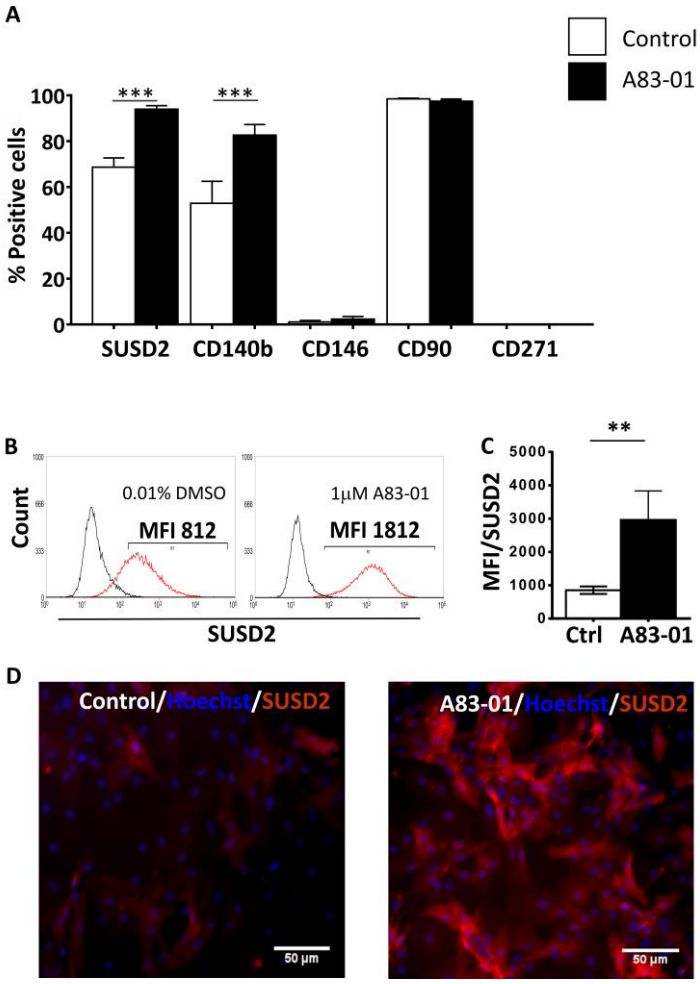

Figure 2.2 Phenotype of P6 eMSC cultured with or without A83-01 in serum free medium in 5%O2. (A) % Positive cells for MSC surface markers on passage 6 eMSC (n=8) cultured in 1μM A83-01(black bars) or in 0.01% DMSO (white bars) for 7 days and assessed by single-colour flow cytometry. **(B)** Representative flow cytometric histograms of SUSD2^{+} eMSC treated with (black bar) and without (white bar) 1 μM A83-01 and MFI summarised in **(C)**. **(D)** SUSD2 expression on control (left panel) and A83-01 treated (right panel) eMSC by immunofluorescence (red). Data are mean ± SEM of n=8 different patient samples. **p<0.01, ***p<0.001.

2.3.3 A83-01 maintained functional properties of late passage eMSC

We next investigated whether inhibition of the TGF-βR signalling pathway in late passage eMSC cultures altered their MSC functional properties. The cloning efficiency of P6 A83-01 pre-treated eMSC was significantly greater ($p<0.05$) than control cells **(Figure 2.3A, B)**. The A83-01 pre-treated eMSC also generated larger colonies than untreated eMSC.

Next, we tested whether A83-01 pre-treated eMSC retained MSC multilineage differentiation capacity. P6 eMSC pre-treated with or without 1 μM A83-01 were cultured in differentiation induction media or 1% FCS growth medium (control) to assess differentiation into adipocytes, osteocytes and chondrocytes **(Figure 2.3C)**. A83-01 pre-treated and untreated cells showed similar phenotype changes in adiopogenic medium with similar numbers of cells containing Oil Red O stained lipid droplets. Similarly for osteogenic differentiation, the amount of Alizarin Red stained calcium deposits was comparable. In contrast, chondrogenic differentiation of the cell pellets was greater for the A83-01 pre-treated cells, as a strong Alcian Blue stained matrix in a cartilage-like organoid was observed, while the untreated eMSC pellet disintegrated easily with little evidence of chondroitin sulphate matrix deposition **(Figure 2.3C)**. There was no differentiation in non-induction medium.

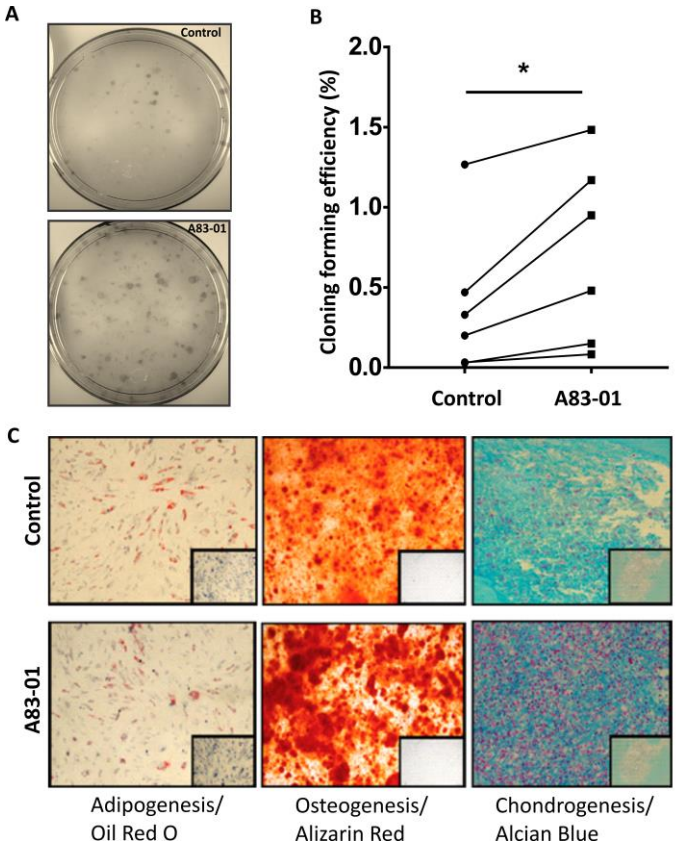

Figure 2.3 Functional MSC properties of P6 eMSC cultured with or without A83-01 in serum free medium. (A) Representative culture plates seeded at clonal density (50 cell/cm^2) for 3 weeks. **(B)** Graph shows Colony Forming Efficiency of P6 eMSC pre-treated with 1μM A83-01 or 0.01% DMSO vehicle for 7 days in 5% O_2 in SFM followed by clonal culture at 50 cells/cm^2 in SFM for 4 weeks. **(C)** Multilineage mesodermal differentiation of 0.01% DMSO treated control and 1 μM A83-01 treated P6 eMSC showing adipogenic, osteogenic, and chondrogenic differentiation (controls were cultured in 1% serum media) for four weeks in 5%O_2. Oil Red O was used to visualise cellular lipid vesicles for adipogenic differentiation, Alizarin Red to detect calcium mineralisation for osteogenic differentiation and Alcian Blue to detect acidic polysaccharides in the extracellular matrix for cartilage differentiation. Representative images from n=3 samples. Data are mean ± SEM of n=6 different samples *p<0.05.

2.3.4 A83-01 effect on pluripotency and stem cell gene expression

We next examined the expression of pluripotency and MSC genes suggested to have a role in maintaining MSC self-renewal. Quantitative RT-PCR of A83-01 treated and untreated cells failed to detect pluripotency genes *OCT4*, *SOX2* and *NANOG* in either group (results not shown) although they were demonstrated in the human iPS cells positive control. Consistent with the flow cytometry data, *SUSD2* was downregulated in the control group and highly expressed in the A83-01 treated cells (p=0.0078) **(Figure 2.4)**. The expression of *CD146* and *MMP3* genes was reduced in A83-01 treated cells (p=0.04 and p=0.0078, respectively) **(Figure 2.4)**. There was also an increase in the expression of *AOC3* (p=0.031), a marker of $SUSD2^{+}$ cells (Murakami et al., 2014) and *FRZB* (p=0.015) **(Figure 2.4)**, a gene encoding for a Wnt ligand binding protein, in A83-01 treated group while no difference was observed in the expression of *NOTCH2, NOTCH3* and *DKK* genes **(Figure 2.4)**.

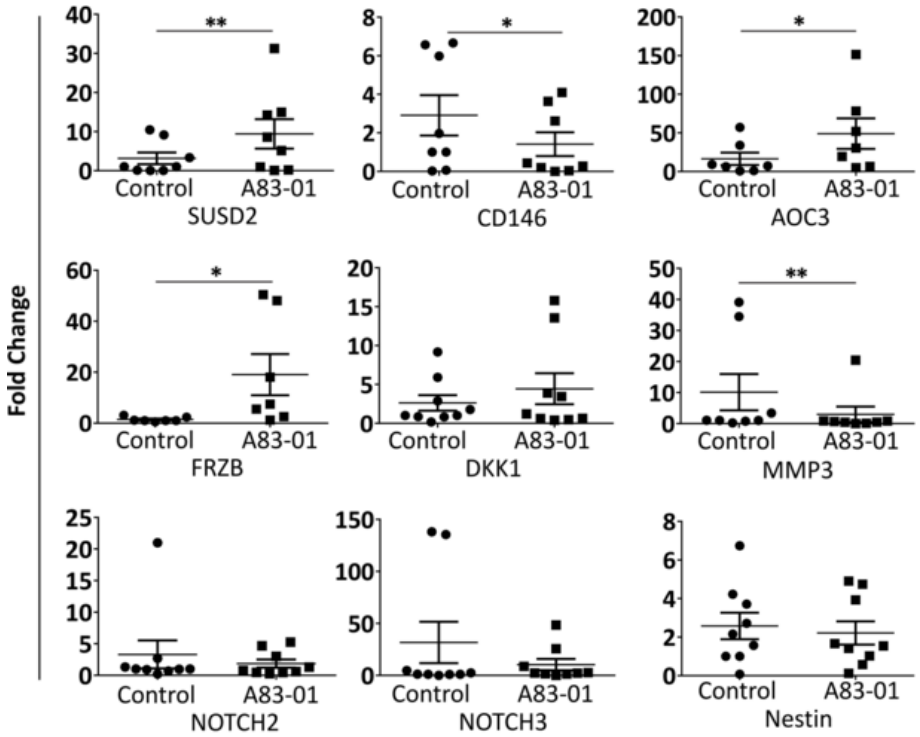

Figure 2.4 Quantitative RT-PCR analysis of MSC genes. P6 eMSC cultures treated (black squares) or untreated control (black circles) with 1 μM A83-01 in 5%O_2/5%CO_2/90%N at 37ºC for 7 days. qRT-PCR analysis of *SUSD2*, *CD146, AOC3, FRZB*, *MMP3, DKK1, NOTCH3, NOTCH2* and *NESTIN*. β-Actin or *GAPDH* were used to normalise the mRNA level, and fold change was calculated using $2^{-\Delta\Delta CT}$. Data are mean ± SEM on n=7 different tissue samples**.** *p<0.05; **p<0.01.

2.3.5 A83-01 blocks apoptosis and senescence in P6 eMSC

To identify the mechanism of action of A83-01 in increasing eMSC proliferation **(Figure 2.1)**, we undertook cell cycle analysis **(Figure 2.5A)** with propidium iodide to label DNA. Figure 5A and B shows that A83-01 treatment increased the proportion of cells in G2/M phase ($p<0.05$) indicative of an increased rate of cell division. There were also significantly fewer A83-01 treated cells in the sub G1/G0 phase of the cycle compared with control cells indicating fewer apoptotic cells with fragmented DNA content in the A83-01 treated cells **(Figure 2.5A, B)**. We then quantified the apoptotic cells using Annexin V flow cytometry to assess early phase apoptosis. The inclusion of PI was to detect late apoptotic and necrotic cells. Culture expanded P6 eMSC significantly reduced the percentage of live cells and increased the proportion of apoptotic cells as shown by the increased binding of Annexin V to exposed phosphatidylserine (PS) on the outer leaflet of the plasma membrane **(Figure 2.5C, D)**. The increased PI staining in the untreated eMSC also indicated increased necrotic cells. These changes were mitigated by pre-treatment with 1μM A83-01 ($p<0.05$) **(Figure 2.5C, D)**.

To further understand the action of A83-01, we examined unstained P6 eMSC from treated and control groups by UV light to quantify autofluorescence, as a measure of senescence. As shown in **Figure 2.5E**, control P6 eMSC were significantly more autofluorescent than the A83-01 treated P6 eMSC ($p=0.001$). Therefore, we measured senile associated β-Gal (SAβ-Gal) activity by incubating cells with X-Gal. As shown in **Figure 2.5E**, A83-01 treated P6 eMSC showed little β-Gal staining whereas the untreated control eMSC displayed blue staining indicative of senescent cells.

Furthermore, the A83-01-treated cells were smaller and more numerous, in agreement with our findings above.

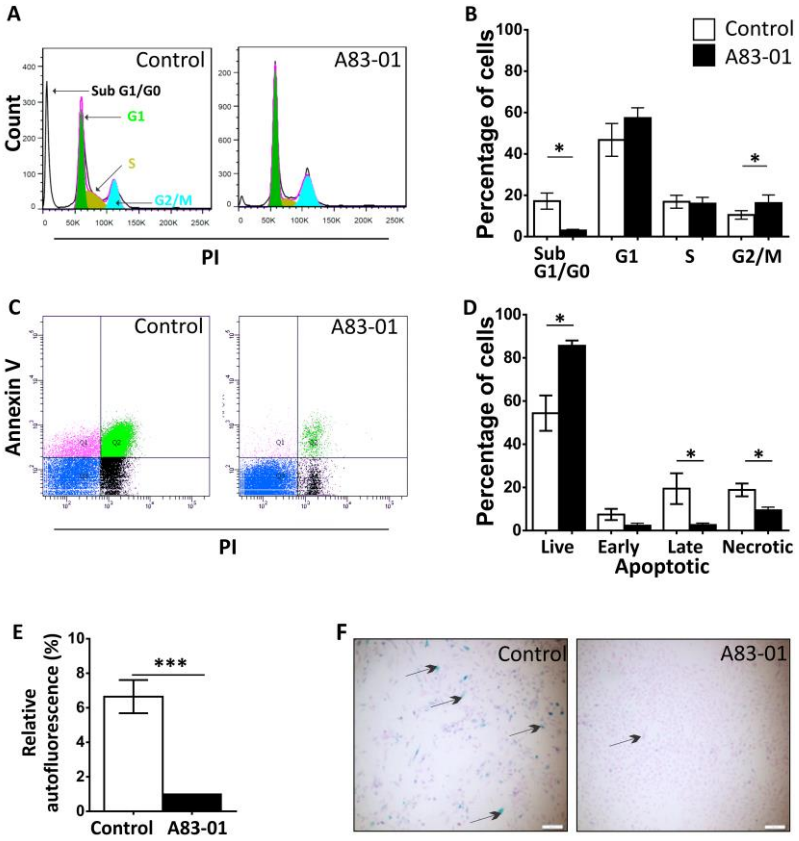

Figure 2.5 A83-01 blocks apoptosis and promotes eMSC proliferation. P6 eMSC treated with 1 μM A83-01 or 0.01% DMSO cultured for 7 days in SFM in 5%O_2/5%CO_2/90%N were assessed by **(A)** Cell cycle analysis of PI stained cells. Shows representative PI staining on a linear axis of a flow cytometry plot **(B)** the percentage of cells in SubG1/G0, G1, S and G2/M stages of the cell cycle (black bar A83-01 treated, White bar control). Data are mean ± SEM, n=7 patient samples; *p<0.05. **(C)** Annexin-V and PI staining. Representative flow cytometric plots. The lower left quadrant of each panel shows the viable cells, upper left early apoptotic; upper right late apoptotic and lower right necrotic cells and **(D)** graph showing the percentage of live, apoptotic and necrotic cells. Data are mean ± SEM, n=6 patient samples, *p<0.05. **(E)** Relative autofluorescence by flow cytometry of unstained P6 cells treated (black bar) and untreated (white bar) with 1 μM A83-01. Data are mean ± SEM, n=10, ***p<0.005. **(F)** Representative images showing the staining of senescence-associated β-galactosidase (SA-β-gal) in cultured P6 eMSC treated with or without 1 μM A83-01.

2.4 Discussion

The main findings from this study are that A83-01, a small molecule TGF-βR inhibitor, prevented the typical loss of undifferentiated MSC during culture expansion. Specifically, we showed that A83-01 treatment prevented loss of SUSD2$^+$ eMSC in late passage cultures by promoting the mitosis and proliferation of P6 SUSD2$^+$ eMSC and by preventing their apoptosis and senescence. A83-01 treated SUSD2$^+$ cells in late passage culture retained their MSC properties, showing greater clonogenicity then untreated cells. In particular, there were greater numbers of large colonies which undergo serial cloning and are more proliferative than those initiating small colonies (Gargett et al., 2009). Their multilineage differentiation capacity was maintained as well as expression of key MSC genes; SUSD2, AOC3 (Murakami et al., 2014) and FRZB (Spitzer et al., 2012). We further identified that the signalling pathway blocked by A83-01 was TGF-βR mediated apoptosis via SMAD2/3 phosphorylation. Since multiple pathways work together in regulating MSC fate and TGF-βR pathway signalling is pleiotropic, targeting this pathway may provide an ideal method for maintaining undifferentiated MSC in cell production protocols for clinical use.

We showed culture expansion of eMSC lead to a loss of clonogenicity and expression of SUSD2, CD140b and CD146 surface markers while CD90, a standard ISCT MSC marker does not change. This was also shown by loss of SUSD2$^+$-expressing eMSC when induced by TGF-β1 to differentiate into smooth muscle cells (Su et al., 2014). These properties support the concept that eMSC spontaneously differentiate into fibroblasts lacking the expression of perivascular markers, clonogenicity, and osteogenic and chondrogenic differentiation capacity. Further molecular characterisation of differentiation at the transcript and protein levels is feasible with

qRT-PCR and western blotting respectively. TGF-βR signalling is necessary for chondrogenic differentiation (Ng et al., 2008). However, while the experimental medium for eMSC culture expansion contained A83-01, the chondrogenic differentiation medium contained TGF-β1 without A83-01 to assess chondrogenic differentiation potential of A83-01 treated and untreated cells. One advantage of using small molecules rather than siRNA to modulate receptor activity is that their inhibitory effect is reversed as soon as the small molecules are removed. We were therefore able to show that chondrogenic differentiation was enhanced in A83-01 pre-treated cells. A83-01 not only increased the expression of SUSD2 proteins but also CD140b. In contrast, CD146 gene expression was greater in the untreated group but did not appear to be translated into protein as it was not detected by flow cytometry. Furthermore, expression of CD146 on cultured MSC is regulated by factors such as hypoxia, growth factors, and metalloproteases (Rawdanowicz et al., 1994, Boneberg et al., 2009). Culture expanded MSC are more autofluorescent than the primary cells indicating replicative senescence and loss of proliferative ability (Constantinescu et al., 2007, Wagner et al., 2008) an effect observed in P6 control eMSC which was mitigated by A83-01 treatment.

Endometrial MSC are an attractive source of cells for tissue engineering and cell-based therapies because they can be harvested with minimal discomfort to patients, have standard MSC properties in vitro and in vivo, and they can be cultured in serum free conditions, offering a readily available cell source for allogeneic as well as autologous use. The necessity to expand MSC for clinical use due to their rarity and their subsequent spontaneous differentiation limits the full potential of eMSC and MSC in general.

TGF-β belongs to a superfamily of TGF cytokines which has multiple functions. TGF-β plays a vital role in MSC differentiation along with PDGF and FGF-2 pathways (Ng et al., 2008, Wang et al., 2012). TGF-β is synthesized by endometrial stromal cells under the influence of physiological female hormones, fluctuating during different phases of menstrual cycle. TGF-β production increased in the secretory and menstrual phases but was diminished in the proliferative phase, suggesting that TGF-β promotes differentiation (Chegini et al., 1994). Similarly, TGF-β promoted differentiation of $SUSD2^{+}$ eMSC when cultured on polyamide/gelatin meshes (Su et al., 2014). TGF-β can independently, as well as in association with Wnt and NOTCH signaling pathways, regulates proliferation and differentiation of MSC (Kurpinski et al., 2010, Attisano and Wrana, 2013). Gene profiling of purified eMSC shows that there is an increased fold change in FRZB receptor indicating an interaction with the Wnt signalling pathway. Pluripotency genes are not present in freshly isolated eMSC (Spitzer et al., 2012) and we found that P6 eMSC did not express pluripotency markers nor did A83-01 treatment upregulate their expression.

Apoptosis or programmed cell death mainly results from activation of cellular caspases. The TGF-βR pathway also participates in apoptosis via SMAD activation, in association with Death associated protein 6 and TGF-βR inducible transcription factor (Ribeiro et al., 1999, Black et al., 2007). In epithelial cells and hepatocytes, TGF-βR induces apoptosis and inhibits proliferation (Ribeiro et al., 1999, Buenemann et al., 2001, Black et al., 2007) through the activation of TGFβ-inducible early gene-1 (TIEG1) via phosphorylation of SMAD2/3 and formation of reactive oxygen species. TIEG1 inhibits cell growth and leads to apoptosis (Ribeiro et al., 1999). P6 eMSC treated with 1 μM A83-01 showed significantly increased growth by preventing

apoptosis compared to the untreated cells, however it is not known if TIEG1 is involved.

Human Lgr5$^+$ liver stem cell cultures in mouse culture medium was not supported for more than three weeks and revealed highly active TGF-β signalling. This was also mitigated by blocking with A83-01 which improved cloning efficiency with extended time in culture of the liver stem cells (Huch et al., 2015). A83-01 is a selective inhibitor of TGF-βR type I ALK4/5/7. It has a thiourea group in its structure, conferring copper ion chelation properties. Thioureas prevent copper mediated oxidative cellular damage by chelating copper in the medium (Zhu et al., 2002). One of the potential mechanisms by which A83-01 acts in SUSD2$^+$ eMSC may be the prevention of spontaneous differentiation and apoptosis mediated via the copper chelating thiourea moiety. Our study also demonstrated for the first time that TGF-βR signalling is an essential negative regulator of SUSD2 expression through SMAD2/3 signalling in eMSC. In addition, the TGF-βR pathway is involved in regulating eMSC proliferation, senescence and apoptosis.

2.4.1 Conclusions

In summary, we have shown that TGF-βR signaling is involved in eMSC cell fate in vitro. A83-01, a small molecule TGF-βR inhibitor, enhanced the expression of SUSD2 and CD140b, maintaining eMSC clonogenic phenotype during prolonged culturing, promoting cell proliferation and preventing apoptosis and senescence. Small molecules such as A83-01 that promote eMSC proliferation in the undifferentiated state may provide an approach for the expansion of undifferentiated MSC for use in tissue engineering and cell-based therapies.

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2.6 Appendices

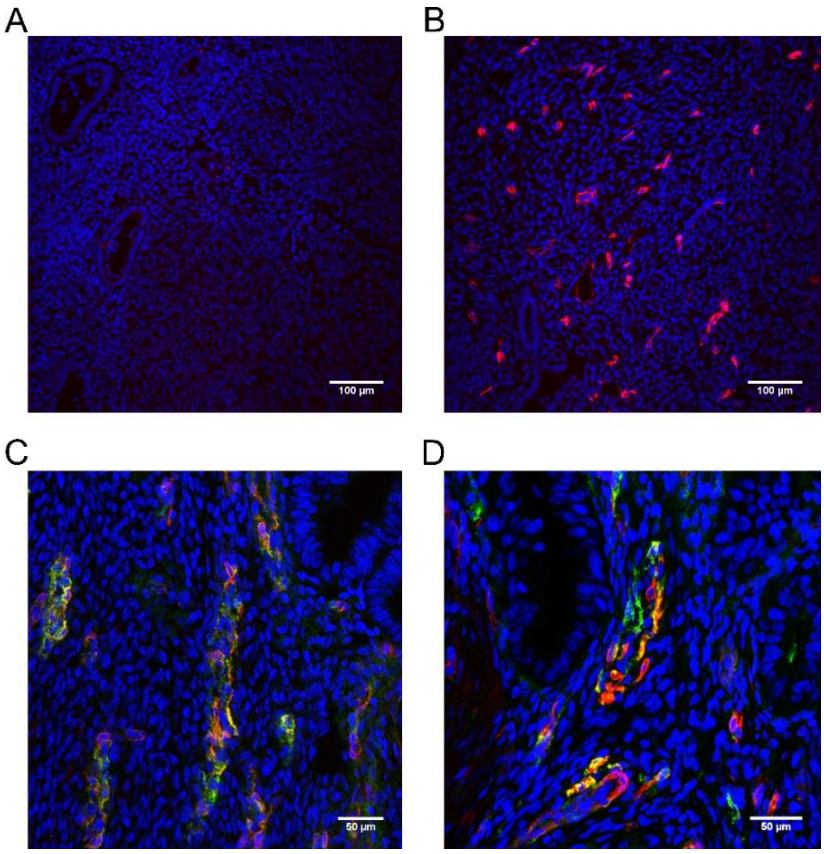

Figure: Localisation of SUSD2 and AOC3 in the human endometrium. Sections of human endometrium were single or double immunostained for AOC3 and SUSD2. **A)** Isotype negative control. **B)** Perivascular cells in the endometrium express AOC3 (red). **(C, D)** Co-localisation of SUSD2 (green) and AOC3 (red) were found in the perivascular region in the endometrium. The epithelial cells do not express AOC3 or SUSD2. Scale bar 100 μm (A, B) and 50 μm **(C, D).**

Chapter 3

In vivo survival of human eMSC cultured in A83-01

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Table of Contents

Declaration

Monash University

Declaration for Thesis Chapter 3

In the case of Chapter 3, my contribution to the work involved the following:

Name	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
Shanti Gurung	Responsible for performing the experiments, collecting all the data, analysing, interpreting and preparing figures and table, and manuscript writing/	80%
James Deane	Assisted with animal surgeries	N
Saeedeh Darzi	Assisted with establishing the mCherry transfection protocols	5%
Jerome A. Werkmeister	Study design, manuscript revision and approved the final manuscript.	N
Caroline E. Gargett	Study design, manuscript revision and approved the final manuscript.	N

Student signature: [redacted] Date: 28/06/2017

Abstract

Introduction: Human endometrial mesenchymal stem cells (eMSCs) are a well characterised adult stem cell type with potential for use in regenerative medicine or cell-therapy. As a proof of principle, we demonstrated that eMSCs promoted wound healing by reducing the inflammatory response through a paracrine action in a subcutaneous rat model of wound repair since the cells only survived a short term but had long term effects *in vivo*. In that study, an efficient protocol for culturing eMSCs in the undifferentiated state and a reliable method of labelling them for cell tracking were lacking. Here, we investigated the use of a lentiviral vector containing the mCherry fluorescent reporter gene to transduce and label eMSCs following *in vitro* culturing in A83-01-containing medium, and different methods of tracing labelled cells following transplantation under sub-renal capsules in NSG mice.

Methods: eMSCs were isolated by enzymatic dissociation from human endometrium and enriched for SUSD2-expressing cells using magnetic bead sorting. eMSCs were transduced by lentiviruses with mCherry fluorescent reporter gene by standard methods, mCherry positive cells were isolated by fluorescence-activated cell sorting (FACS) and cultured until passage six in 5% O_2 in serum free medium with FGF2 and EGF (SFM). The cells were subsequently divided into two flasks and treated with either DMSO (0.01%) or A83-01 (1 μM) for 7 days. Then 5×10^5 cells were encapsulated into a fibrin gel and transplanted under the sub-renal capsules of NSG mice. Tissues were analysed at 7, 14 and 30 days post transplantation.

Results: Human eMSCs were efficiently transduced (40%) with the lentiviral mCherry fluorescent gene. They proliferated and maintained high mCherry expression over 5 passages. When transplanted under the kidney capsule of mice and analysed using PCR, flow cytometry and immunofluorescence, both control and A83-01-treated eMSCs survived for at least 30 days.

Conclusion: The efficient labelling of eMSC using a lentiviral vector and culturing them in an environment maintaining them in an undifferentiated state enables the reliable detection of human eMSC in pre-clinical animal studies and highlights the need for generating a pure population of undifferentiated MSCs for long term survival *in vivo* to prolong their treatment effect.

3.1 Introduction

Cell therapy using mesenchymal stem cells (MSCs) is promising and emerging rapidly. MSCs are used in clinical trials for a wide range of diseases ranging from immunogenic to non-immunogenic disorders (www.clinicaltrials.gov). To date they appear safe for human use and promising benefits have been reported (Trounson and McDonald, 2015, Squillaro et al., 2016). The positive outcomes have been attributed to the ability of MSCs to migrate to injured tissues, differentiate into various cell types, promote angiogenesis and secrete factors that act in a paracrine manner to influence host immune responses as well as modulate macrophages into a wound healing phenotype (Squillaro et al., 2016). However, in majority of studies and trials, MSCs have failed to survive for longer than a month or even engraft into the intended site (Moll et al., 2012, Wang et al., 2012). The quality of MSCs depends upon multiple factors, for example donor age, enrichment process, passage number and contaminating spontaneously differentiated cells. This may explain the short half-life of endometrial MSCs (eMSCs) (Ulrich et al., 2014). While short-term paracrine effects may be beneficial in some circumstances, there is a need to improve the quality of cells to prolong survival and enhance their therapeutic effect *in vivo* (Samsonraj et al., 2015).

Majority of cell therapies use plastic adherent, heterogeneous mesenchymal stromal cells which have been expanded in culture to generate a large population of cells. Enriching these cells using specific surface markers that purify perivascular cells such as CD271 (Battula et al., 2009), CD146 (Tormin et al., 2011), STRO-1 (Simmons and Torok-Storb, 1991) and SUSD2 (4% of endometrial stromal cells and 5% in bone marrow) (Masuda et al., 2012, Sivasubramaniyan et al., 2013) has improved

outcomes. However, significant culture expansion of these rare cells is an unavoidable necessity to generate the large numbers required for cell-based therapies. Unfortunately, this process can lead to their spontaneous differentiation into fibroblasts and generation of heterogeneous cell populations with reduced stem cell properties than their parent cells (Halfon et al., 2011, Gurung et al., 2015, Barragan et al., 2016).

Recently we demonstrated that the small molecule A83-01, a transforming growth factor beta receptor inhibitor, prevented this spontaneous differentiation, maintained stemness and promoted proliferation by blocking senescence and apoptosis in late passage cultures of SUSD2$^+$ eMSCs (Gurung et al., 2015). In a subcutaneous immunodeficient rat model of wound repair, a polyamide mesh/gelatine composite construct seeded with eMSCs was tested for wound healing properties (Ulrich et al., 2014). In this study, the SUSD2$^+$ eMSCs were cultured in 10% FBS in normoxic environment, and following extensive culture, only 10% of the cultured cells were positive for the SUSD2 eMSC surface marker (Ulrich et al., 2014). The cells had been tagged with Vybrant^{TM} DiO reagent (Life Technologies) and almost 99% of them were positive for DiO when implanted *in vivo*., However, few DiO$^+$ cells were detected 14 days later and none at later time points (Ulrich et al., 2014). Nonetheless, the bioengineered construct exerted angiogenic and anti-inflammatory effects and promoted wound healing with minimal fibrosis well beyond the time of cell detection. Lacking in this study was a method to maintain the undifferentiated SUSD2$^+$ eMSC phenotype and an efficient labelling technique to detect them.

In the present study, we labelled SUSD2$^+$ eMSCs with an mCherry fluorescent reporter gene and cultured them in a serum-free medium and low O_2 environment to maintain

them in an undifferentiated state as previously published (Gurung et al., 2015). The aims were to establish a feasible and reliable approach to detect eMSCs *in vivo*, and to address whether culture-expanded eMSCs treated with A83-01 survive longer *in vivo* using an immunocompromised mouse model. We hypothesised that pre-treatment of eMSCs with A83-01 during culture expansion would increase their survival *in vivo* compared to untreated control cells. We used several methods to detect mCherry positive human cells and found that they survived for at least 30 days when transplanted under the kidney capsule of NSG mice. Pre-treatment of eMSCs with A83-01 had no adverse effect on their survival *in vivo*.

3.2 Materials and Methods

3.2.1 Human Endometrial tissue samples

Human ethics approval was obtained from the Monash Health and Monash University Human Research Ethics Committees (CF10/2080, 2010001150). Human endometrial tissue samples were collected from women who were undergoing endometrial curette or hysterectomy for non-endometrial pathologies and who were not on any hormones for at least three months prior to surgery. Informed written consent was obtained from each woman. Six donors provided human endometrium for this study.

3.2.2 Animals

The animal experimental procedure was approved by the Monash Medical Centre Animal Ethics Committee A (MMCA, 2015/29). Non-obese diabetic severe combined immunodeficient and interleukin 2 receptors γ-deficient (NSG) mice were housed in the animal house at Monash Animal Service facilities in compliance with the National Health and Medical Research Council guidelines for the care and use of laboratory animals. NSG is a strain of inbred mice which lacks mature T cells, B cells (adaptive immunity) and natural killer cells.

3.2.3 Isolation of SUSD2^{+} human endometrial MSCs and cell culture

eMSCs were isolated using magnetic beads according to our previously published protocol (Masuda et al., 2012, Gurung et al., 2015). Briefly, endometrial tissue was mechanically minced and dissociated into single cells with 0.5% collagenase type I (Worthington Biochemical Corporation) and 40 μg/ml DNase type I (Worthington

Biochemical Corporation) in DMEM/F-12 in a MACSmix™ for 1 hr at 37ºC. The cell suspension was filtered through a 40 μm cell strainer to obtain the stromal cells in the filtrate. Red blood cells were removed by density gradient using Ficoll-Paque (GE healthcare Bio-science). The interphase containing stromal cells was incubated sequentially with SUSD2-PE antibody (10 μg/ml, BioLegend) and anti-PE magnetic beads (Miltenyi Biotec) for an hour then 30 minutes respectively at 4ºC in dark. The cell suspension was applied to an MS column (Miltenyi Biotec) in a magnetic field and the $SUSD2^+$ eMSCs were eluted and then cultured in DMEM-containing 10% fetal calf serum, 1% antibiotics-antimycotic and 2 mM glutamine, stromal medium (SM). All cultures were maintained in 5% O_2, 5% CO_2, 90% N_2 trigas incubator at 37ºC.

3.2.4 Transduction of eMSCs with mCherry lentivirus and culture

We used 2nd-generation HIV-1 based lentiviral vector with cytomegalovirus immediate early promoter ($P_{CMV\ IE}$) and Woodchuck hepatitis virus posttranscriptional regulatory element in the transfer plasmid that enhances viral titers. The plasmids; plvx-IRES-mCherry (mCherry transfer plasmid; Clontech, # 6312237), pMD2.G (envelop plasmid, Addgene, #12259) and psPax2 (packaging plasmid, Addgene #12260) were kindly donated by Dr Daniel Gough, Centre for Cancer Research, Hudson Institute of Medical Research. The plasmids were amplified in *E. coli* and extracted using PureYield™ Plasmid Maxiprep System protocol (Promega). Briefly, a single colony of bacteria for each plasmid construct was incubated in 250 ml of LB Broth (Sigma) and cultured overnight at 37ºC in a shaking incubator. The cells were pelleted at 5000g for 10 minutes at room temperature, lysed for 5 minutes and mixed with neutralising solution. The lysate was centrifuged at 7000g for 30 minutes at room temperature. DNA was purified using the PureYieldTM Clearing Column/Maxi binding Column. It was washed

and eluted with Nuclease-free water, and the DNA was quantified using Nano drop spectrophotometer.

To generate virus particles for transduction, two to three million 293T cells were seeded in a 100mm dish with SM. The plasmid complex was made by gently mixing 1.5 ml Opti-MEMI Reduced-Serum Medium (Sigma), 20 μg plasmid DNA comprising 10 μg plvx-IRES-mCherry, 1 μg pMD2.G and 9 μg psPax2 plasmids, 40ul TransIT-X2 ® Dynamic Delivery system (MIR 6003, Mirus) and 10 μl Chloroquine. They were incubated for half an hour at room temperature to promote complex formation. The 293T cells were transfected at ~80% confluency and 8.5 ml of SM was added, then TransIT-X2 DNA complexes were added drop-wise to the culture dish. The dish was gently swirled to evenly distribute the complexes. After 6 hours, media was changed back to regular SM. After 48 hours transfection, the supernatant containing the viruses was collected and centrifuged at 1400rpm for 4 minutes, passed through 0.45 μm filter and polybrene (3 μg/ml, Sigma Hexadimethrine Bromide, #107689) was added to promote the transduction process. The virus containing medium was added to passage one (P1) eMSCs. The same process was repeated after six hours. eMSCs were left for 48 hours to promote transduction as well as inactivation of the lentivirus. mCherry positive eMSCs were collected after sorting on a MoFlo flow cytometer using Cyclops SUMMIT software (Version 5.2; Beckman Coulter), and cultured in SM until ~70% confluent. The medium was changed to 5% and 1% FCS SM over 48 hours and then to in-house serum-free medium with FGF2 (10 ng/ml) and EGF (10 ng/ml) (SFM) (Gurung et al., 2015). The cells were then cultured on fibronectin-coated flasks in SFM. At passage 6 (P6), eMSCs were divided into two flasks, one was treated with 1 μm

A83-01 and the second with vehicle control (0.01% DMSO) for seven days with media changed every 48 hours **(Supplementary Figure 3.1)**.

3.2.5 Casting fibrin gel constructs for delivering eMSC

5×10^5 P6 A83-01-treated and control mCherry positive eMSCs were mixed with 10 μl warm fibrinogen (50 mg/ml, #F4753, Sigma) and dispensed onto the lid of a petri-dish. Then 2 μl thrombin (100 units/ml, #T9549, Sigma) was added to it to initiate gel formation. The lid was inverted and the dish incubated at 37^{o}C for ~1 hr to allow the gel to polymerise.

3.2.6 Mouse subrenal capsule Surgery

A total of 108 NSG mice older than 8 weeks were randomly divided into two groups, (54 mice per group) which received control or A83-01 treated P6 eMSCs. A total of six patient cell lines were used for this study. eMSCs from one donor were transplanted into 18 mice in total (9 mice for control cells and 9 mice for A83-01 treated cells, with 3 mice/time point/each group. Three mice were harvested at each time point for each group for the three independent tests performed). Subrenal cell transplantation site was chosen for its high vascularity, and importantly this region provides a confined compartment for cells that allows easy follow up at different time points as shown by various studies

The mice were anesthetised with 100 μl Ketamine/Xylazine (100 mg/kg ketamine and 10 mg/ml Xylazine, Intraperitoneal), and Carprofen (0.3-0.5 mg/100 g body weight, subcutaneous) was given prior to the surgery. The left loin area was shaved, sterilised

with 70% ethanol and bupivacaine (subcutaneous) was given at the site of incision. A 1-2 cm mediolateral dorsal incision was made at the left loin and the left kidney was exteriorised through the posterior wall. A small hole was made in the kidney capsule and the capsule was separated from the underlying cortex. Using a surgical microscope, the eMSC-fibrin gel was inserted under the capsule. The kidney was returned into the body and the muscle was closed with continuous suture and the skin with interrupted suture using resorbable Polyglycolide. The mice were monitored and humanely killed, at day 7, 14 or 30. Left kidney as well as spleen, uterus, liver and incision site-skin/muscle were harvested for analysis to investigate if the cells had migrated from the original site of delivery.

3.2.7 Polymerase chain reaction for detection of human mCherry cells

Freshly harvested tissues were digested in lysis buffer (100 mM Tris pH 7.4, 5 mM EDTA, 0.5% SDS and 200 mm NaCl) and proteinase K (100 μg/ml, #P2308, Sigma) at 50^{o}C overnight. The digests were centrifuged at 14,000 rpm for 10 minutes and the supernatants were transferred to separate tubes. An equal volume of isopropanol was added to the supernatant and the tubes were inverted to precipitate the genomic DNA (gDNA). The gDNA were pelleted by centrifuging at 14,000 rpm for 10 minutes. The supernatant was discarded and gDNA was washed with 70% ethanol. The tubes were centrifuged again at 14,000 rpm for 5 minutes, supernatant discarded and the pellet allowed to dry.

RNase-free water was added and mixed to dissolve the gDNA. The quality and quantity of the DNA were assessed using Nanodrop spectrophotometer. Undiluted gDNA was used to assess the mCherry sequence while 100 ng/μl gDNA was used for

detecting human specific Alu sequences Polymerase chain reaction (PCR) was carried out in 20 μl volume consisting of 10 μl Mytaq (BIOLINE), 1 μl each reverse and forward primers (10 μM, BIONEER), gDNA and milliQ water. The reaction consisted of initial denaturation of 95^{o}C for 2 minutes, followed by 30 cycles of denaturation for 30 seconds, annealing at 55^{o}C for one minute and extension at 72^{o}C for one minute. The PCR products were separated by 1.5% agarose gel electrophoresis. Concurrently, mCherry positive human eMSCs were used as positive control and tails from mice not receiving human cells were used as negative controls. PCR products were visualised in ChemiDoc.

Table 3.1 Primer Sequences

			Product size
Alu	Forward	AGA CCA TCC TGG CTA ACA CG	200
	Reverse	AGA CGG AGT CTC GCT CTG TC	
mCherry	Forward	CCC TCA GTT CAT GTA CGG CT	245
	Reverse	CCC AGC CCA TGG TCT TCT TC	

3.2.8 Fluorescence microscopy to detect mCherry labelled eMSCs in mice tissues

Tissues were harvested and fixed in 4% OCT followed by 30% sucrose for 24 hours each at 4^{o}C and embedded in OCT on dry ice for sectioning at 8-microns. Sections were washed with PBS and the nucleus stained with Hoechst 33258 (1:2000,

Molecular Probes) for 5 minutes. Images were visualised and photographed using a Nikon microscope and analysed using ImageJ software (ImageJ-win32.Ink).

3.2.9 Flow Cytometry

The harvested kidneys were minced and digested with collagenase I for 1 hour in a humidified incubator at 37^{o}C on a rotating MACSmix (Miltenyi Biotech) and filtered through a 40 μm filter to generate single cells. The red blood cells were separated from the dissociated cells by density gradient centrifugation using Ficoll-Paque (GE healthcare Bio-science). Cells were washed with 5% heat-inactivated newborn calf serum in DMEM and incubated with APC-conjugated SUSD2 (1:20, # 327408, eBioscience) antibody in bench medium for 1 hour in the dark on ice. Matched-isotype control IgG-APC was used at the same concentration to set the electronic negative control gate on the flow cytometer. Human mCherry positive/negative cells and cells from non-injected-kidneys were used as controls. The cells were washed and fixed with 4% PFA in 2%FBS/PBS. Samples were analysed using a MoFlo Flow Cytometer and Summit software (version 5.2., Beckman Coulter).

3.2.10 Statistics

Results are reported as mean ± SEM for each experimental group. Friedman test with Bonferroni and Hochberg correction at different time points between the groups was done using GraphPad Prism 7.02 for statistical analysis. Differences between the groups with p-values <0.05 were considered statistically significant.

3.3 Results

3.3.1 Validation of mCherry transduction and retention following extensive culture

eMSCs (passage 1) were cultured for 48 hours following lentiviral mCherry vector transduction to promote plasmid integration and viral inactivation. Flow cytometric analysis revealed that the average transduction efficiency of the lentivector was 20-40 % (n=6). Of the positive cells, we opted to sort the cells with highest mean fluorescence intensity (20%) **(Figure 3.1A)**. The sorted cells were cultured in 5% O_2 environment in SFM. More importantly, fluorescence microscopy and flow cytometric analysis demonstrated that eMSCs expressed mCherry and the intensity was maintained following extensive culture **(Figure 3.1B, C)**. At P6 ~99% of control and A83-01 treated eMSCs were mCherry positive **(Figure 3.1C)**. Propagation of eMSCs was not affected suggesting that lentiviral transduction did not adversely affect cell proliferation.

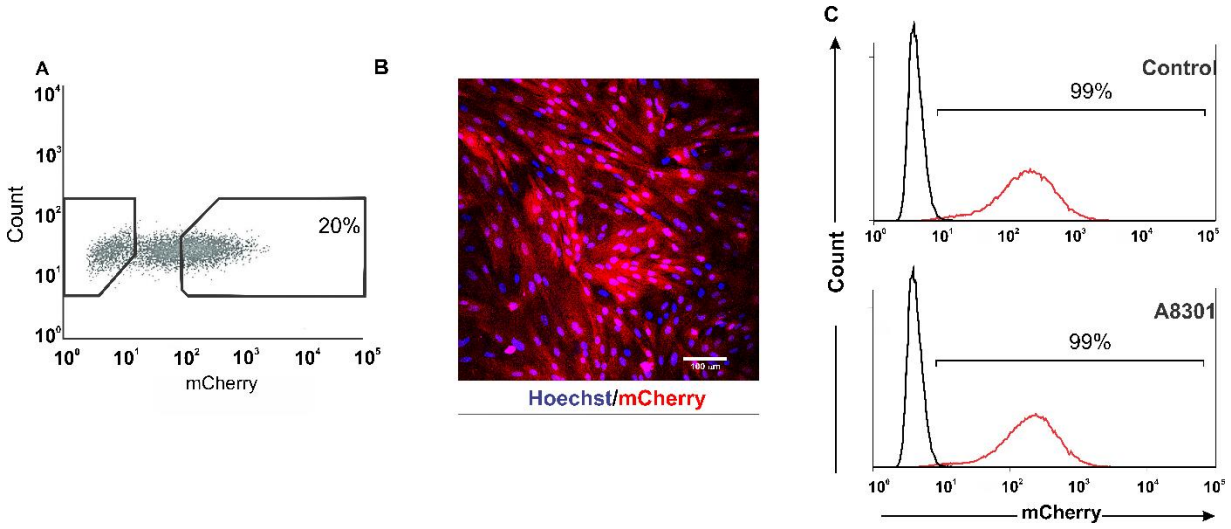


Figure 3.1 Validation of mCherry expression in transduced eMSCs in vitro. **A)** Representative flow cytometry trace illustrating the lentiviral transduction efficiency of mCherry reporter gene in passage 1 eMSCs. Cells with higher mean fluorescence intensity were sorted for further study. **B)** In vitro assessment of mCherry signal following sorting and extensive culture at passage 6 showing a representative image by fluorescence microscopy and C) by flow cytometry where 99% of passage 6 eMSCs were positive for mCherry signal (Top graph - control, Bottom graph – A83-01 treated eMSCs. Black isotype-control and red – mCherry fluorescent intensity). Scale bar in B, 100 μm.

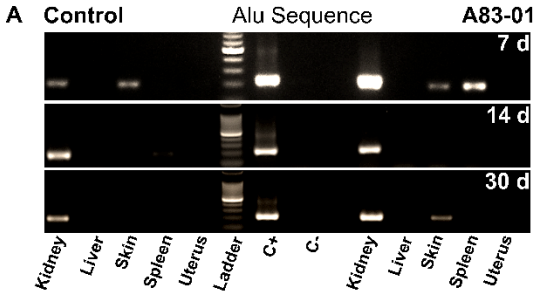
3.3.2 mCherry-labelled eMSCs persist in vivo

All mice had a normal post-operative recovery. Human cells were well tolerated and none of the mice developed any visual or palpable mass at any time point. Human mCherry eMSCs were tracked in NSG mice using three different methods; PCR, immunofluorescence and flow cytometry. Six different human donor eMSCs transduced with mCherry were used in this study. The cells treated with and without

A83-01 were transplanted under the left kidney capsule of NSG mice using fibrin gel as a carrier.

3.3.2.1 Detection of human cells using PCR

To verify the identity of human cells in the mouse kidney, we analysed gDNA samples from mouse tissues by PCR for human Alu sequences **(Table 3.1)**. We used human and mouse gDNA as positive and negative references for Alu sequence respectively. We detected human cells in the kidneys of mice transplanted with and without A83-01 treatment at 7, 14 and 30-day time points **(Figure 3.2A)**. To detect if the transplanted cells migrate from the site of delivery, we also determined the human cell distribution at the time of collection at the incision site (skin/muscle), spleen, uterus and liver. Human cells were detected at the incision site and spleen in some of the mice **(Figure 3.2B)**. Detection of human cells at these sites could be explained by their proximity to the left kidney and the escape of some cells to these sites during the transplantation procedure because the fibrin gel is not completely sealed. We did not detect human cells in the uterus or liver from mice transplanted with the two groups of cells **(Figure 3.2B)**. This shows that the cells do not migrate beyond the site of implantation when fibrin gel is used.



B

	Days	Kidney	Skin	Spleen	Uterus	Liver
Control	7	6/6	3/6	1/6	0	0
	14	6/6	2/6	0	0	0
	30	5/6	2/6	1/6	0	0
A83-01	7	6/6	5/6	3/6	0	0
	14	6/6	3/6	2/6	0	0
	30	6/6	4/6	2/6	0	0

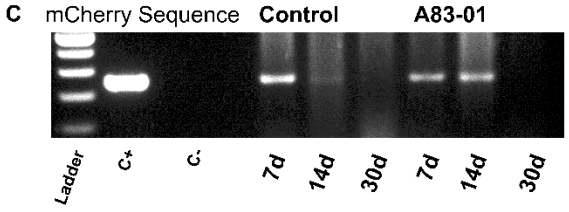


Figure 3.2 PCR amplification products from tissues harvested post-transplantation by electrophoresis on 1.5% agarose gel and stained with Syber Gel Red. A) Representative gel electrophoresis showing 1 kb plus DNA ladder (Invitrogen) and PCR products from Kidney, Liver, Skin, Spleen and uterus from mice transplanted with control and A83-01-treated P6 eMSCs at day 7, 14 and 30. C+ and C– are positive and negative control tissues from human and mouse tissues for the Alu sequence. **B)** Tabulation of the readout of the number of samples showing human Alu sequence from different tissues from six mice in each control and treated group. **C)** 1 kb plus DNA ladder (Invitrogen), C+ (positive) and C– (negative) controls from mCherry-labelled human cells and mouse tissue for mCherry sequence. Representative gel from n=6 samples displaying mCherry product from kidneys of mice transplanted with control or treated cells at day 7, 14 and 30.

To confirm that the detected human cells were from the transplanted mCherry positive cells, we performed PCR with human mCherry sequence **(Table 3.1)** using undiluted gDNA. We showed a mCherry signal at 7 days in control cells and 7 and 14 days in the A83-01-treated groups **(Figure 3.2C)**, further demonstrating that the human cells were from the transduced cells. We were not able to detect mCherry DNA at day 30, presumably because the number of cells had decreased by day 30 and the lower mCherry copy number per cell (in comparison to Alu) was below the threshold of qPCR detection.

3.3.2.2 mCherry^{+} SUSD2^{+} cells were detected in transplanted mice with flow cytometry

We further confirmed the identity of transplanted eMSCs by flow cytometry. We analysed cells harvested from the kidneys at different time points, following tissue dissociation into single cells. Our data shows that the cell suspension included mixtures of human mCherry positive cells and mouse cells. We detected mCherry positive cells at all time points analysed from both groups of mice **(Figure 3.3A)**. However, the total number of mCherry cells detected were less than 20 %, 10 % and 1 % of the total cells transplanted at 7, 14 and 30-day experimental time points. To assess if the transplanted mCherry positive eMSC still expressed the SUSD2 surface marker, cells were stained with APC-SUSD2 antibody. Approximately, 2.5 %, 6 % and 0.3 % in the control group, and 6 %, 11 % and 0.7 % of the mCherry positive cells in the treated group were SUSD2 positive at day 7, 14 and 30 days after transplantations **(Figure 3.3B)**. Along with the decreasing number of mCherry eMSCs, there was also a significant decrease in the percentage of SUSD2 expressing cells during these time point.

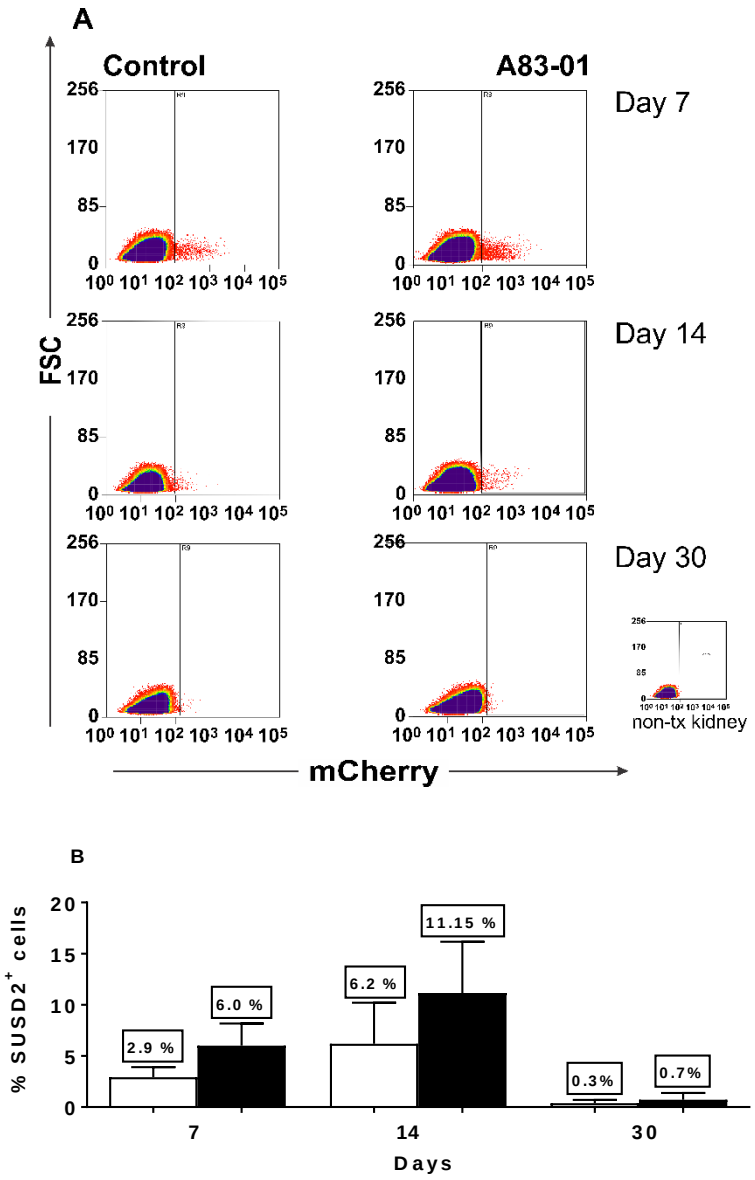


Figure 3.3 Flow-cytometry traces from mice engrafted with human mCherry-labelled SUSD2$^+$ eMSCs analysed at different times post-transplantation. A) Single cells derived from mouse kidneys transplanted with human eMSCs were analysed by flow cytometry to identify human mCherry cells. Traces are from single mice at each time point from control and treatment group, representative of n= 6. The percentage positive cells are shown in the positive gate. The lower right dot-plot represents control kidney not receiving mCherry cells. **B)** % Positive cells for SUSD2 surface marker in mCherry positive cells 7, 14 and 30 days following transplantation in control (white) and treated (black) groups. Data are mean ± SEM of n=6 different patient samples.

3.3.2.3 mCherry fluorescence was present in sections from transplanted mice

We further investigated the presence of mCherry-labelled cells in sections of kidneys by fluorescence microscopy. eMSCs were detected by mCherry fluorescence alone (ie without an mCherry antibody) indicating that the intensity was not diminished following *in vivo* transplantation. As expected from the PCR and flow cytometric results, clusters of cells were detected at 7 days which was diminished at 14 days in both groups and were not detected at 30 days **(Figure 3.4)**. Neither in the control nor in the treated groups did we observe any mCherry positive cells migrating into the kidney cortex. Although endothelial-like cells were observed in the parenchyma of kidneys transplanted with SUSD2 positive cells in the sub-renal capsule of NSG mice (Masuda et al., 2012), we did not observe any migratory cells in any of our murine kidneys.

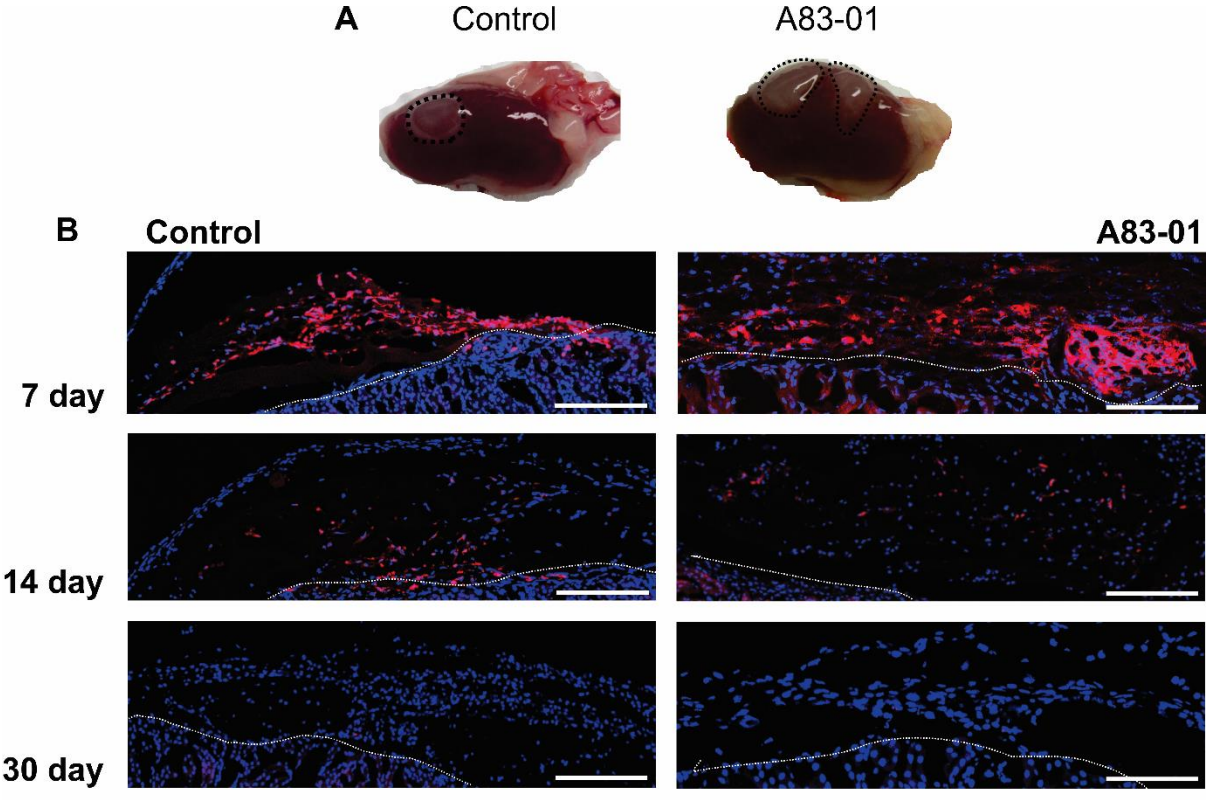


Figure 3.4 Human endometrial SUSD2^{+} mCherry positive cells in vivo. A) Macroscopic view of kidneys 7 days after transplantation with 5×10^{5} mCherry^{+} eMSCs showing the cells encapsulated in the fibrin gel. **B)** Nuclear staining with Hoechst (blue) and mCherry fluorescent protein in human transplanted eMSCs at different time points. Scale bars 100 μm.

3.4 Discussion

This study demonstrated that human endometrial mesenchymal stem cells can be efficiently transduced with lentiviral mCherry vector, and when treated with A83-01, they survive for a longer duration *in vivo* compared to our previous study. Specifically, mCherry reporter gene transduction results in retained expression in long term eMSC-culture (up to 4 weeks *in vitro*). We were also able to demonstrate, using independent methods, that eMSCs survive for at least 30 days in an immunocompromised mouse when cultured in 5% O_2 and in SFM compared to our previous study where very few Dio positive cells were detectable by flowcytometry and they were not confirmed if they were human cells (Ulrich et al., 2014). Our *in vivo* data also show treatment of eMSC with A83-01 *in vitro* has no detrimental effect on their survival *in vivo*.

Culture-expanded MSCs used in research and clinical trials are often a heterogeneous population of cells with reduced functional properties, which may be one of the reasons for the variable outcomes in clinical trials (Ankrum and Karp, 2010, Samsonraj et al., 2015). Producing cells by culturing them in conditions that maintain their potency can improve clinical outcomes (Samsonraj et al., 2015, Darzi et al., 2016). According to our previously published protocol, eMSCs treated with A83-01 at P6 generated a homogeneous population where greater than 95% expressed SUSD2 surface marker (Gurung et al., 2015) and clinical outcomes can be improved by generating cells by culturing in conditions that maintain their potency. However, it is important to demonstrate that A83-01-culture expanded cells have capacity to differentiate in vivo and do not generate tumours.

MSC therapies are proving beneficial (Ankrum and Karp, 2010), however, there is still no clear evidence on how the MSCs function *in vivo*, how long they survive or what cell-type they interact with. Various cell labelling methods have been used to track human cells especially by indirectly labelling them with reporter genes such as luciferase gene, GFP (Kang and Chung, 2008), mCherry (Diana et al., 2013), fluorescent proteins Katushka2S and IRFP (Luker et al., 2015) or directly labelling the cells with nanoparticles such as superparamagnetic iron oxide nanocomposites and NIR815 dye (Guzman et al., 2007, Bossolasco et al., 2012). Indirect methods label cells and their progeny permanently. This is an advantage characteristic over direct labelling, which is lost should the cells undergo proliferation or leak into the surroundings and are taken up by other host cells (Bossolasco et al., 2012, Diana et al., 2013). Lentiviruses are advantageous in cell-labelling with transduction approaches because of their ability to infect both dividing and non-dividing cells, low immunogenicity and high transduction efficiency (Shichinohe et al., 2001, Bossolasco et al., 2012, Diana et al., 2013). Furthermore, the lentiviral mCherry transduction process does not affect the biological characteristics of human MSCs (Wang et al., 2012, Diana et al., 2013, Natarajan et al., 2014). In our study, we showed that mCherry protein expression and intensity were maintained throughout a prolonged (4 weeks) cell culture period following transduction. Although there was a lag in proliferation following sorting of mCherry positive cells- , eMSCs recovered rapidly and propagation was not affected. The lag phase could be due to the Polybrene used to enhance the transduction process which inhibits human MSCs proliferation (Lin et al., 2011). The concentration of Polybrene used here (3 μg/ml) was optimised to balance between high transduction efficiency and maintaining cell proliferation. Therefore, direct labelling of eMSCs using a lentiviral vector with the mCherry gene encoding this

fluorescent protein will help us in understanding not only their survival but also their transformation, differentiation, migration or cellular interaction *in vivo* in preclinical animal models of diseases.

The human genome comprises approximately 20% repetitive DNA sequences, one of which is Alu sequence (Mighell et al., 1997, Allard et al., 2014). It is the most abundant of the transposable elements interspersed throughout the human genome and makes up 13% of the human genomic DNA (approximately one million copies) (Mighell et al., 1997, Miura et al., 2003). By taking advantage of Alu sequence abundance, we could detect human cells in the tissues following transplantation up to day 30 in mice transplanted with eMSCs pre-treated with and without A83-01. They were detected in kidneys, skin and spleen. Since the fibrin gel is not completely sealed, some eMSCs might have escaped to proximal sites such as the incision site and nearby spleen during the procedure. The abundance of Alu sequences enabled eMSC detection even at 30 days at these sites. Although flow cytometry analysis did not show statistical significant, we observed that we could detect eMSCs in these local sites longer in the A83-01-treated eMSC group than the control, implying the possibility of increased survival following the treatment. However, human cells were not detected in tissues distant from the kidney, such as liver and uterus and the control kidney (by flow cytometry), indicating that for future clinical application, local delivery is possible. The identity of transplanted cells was further confirmed with the presence of mCherry transgene. However, we were not able to detect human mCherry eMSCs at later time-points at the same detection level as Alu sequence. This may be because of the decreasing number of total surviving human mCherry positive eMSCs and also due to significantly low mCherry copy number per cell which was below the detectable range

of PCR (Diana et al., 2013). Delivering MSCs to a local site and confining them in a fibrin gel may restrict their distribution from the site of intended use compared to intravenous administration of MSCs where majority of the circulating cells are trapped in the lungs on the first pass and/or are lost in the circulation by an instant blood-mediated inflammatory reaction (Moll et al., 2012).

To test the survivability of control or A83-01-treated eMSCs, further complementary methods were utilised. mCherry positive cells were detected in both cell groups up until 30-day post-transplantation by flow cytometry. Interestingly, there was a significant decrease in the percentage of $SUSD2^+/mCherry^+$ eMSCs in both groups. By the final experimental time point, less than 1% of human cells were detectable. This indicates that although majority of the cells were not positive for SUSD2, they might have undergone differentiation. Even though they were detected using immunofluorescence staining, it was difficult to detect them at the 30-day time point. No $mCherry^+$ migratory human cells were observed in our study, unlike those observed when primary $SUSD2^+$ eMSCs were transplanted in the sub-renal capsule of NSG mice (Masuda et al., 2012). This could be because Masuda et al (2012) used primary $SUSD2^+$ eMSCs which were delivered in DMEM, as opposed to the current study using A83-01-cultured eMSCs in a fibrin gel. In addition, mice in the Masuda et al study were hormonally regulated by ovariectomising and treating with exogenous estrogen and progesterone which could have promoted their differentiation into endometrial cell types.

A limitation of this study is the choice of immunocompromised animal model in assessing the survivability of the two groups of cells as it does not mimic the

pathophysiological immune environment in patients who need cell-therapy or regenerative medicine. However, to understand the role of MSCs alone and their interaction with immune cells NSG mice are good model as their lack of NK cells enables longer persistence compared to other immunocompromised mouse models. Further study using an immunocompetent animal model or disease model will provide further insight into whether pre-treating eMSCs with A83-01 provides any added benefits for healing and repairing processes imperative for a cell-therapy and clinical translation.

In conclusion, this study demonstrates the ability to efficiently and safely tag eMSCs and shows their ability to survive longer than our previous study with our improved eMSCs culturing protocol using a small molecule inhibitor. In fact, the persistence of transplanted eMSCs demonstrated in this study is the longest that has been shown for their survival *in vivo*. Although there were no significant differences in the survivability of eMSCs pre-treated with or without A83-01, there was a trend as shown by their longer detection in local sites. Likewise, the decrease percentage of SUSD2 population indicates *in vivo* differentiation. Using A83-01-treated eMSCs in models may shed a light in their applicability for clinical translation.

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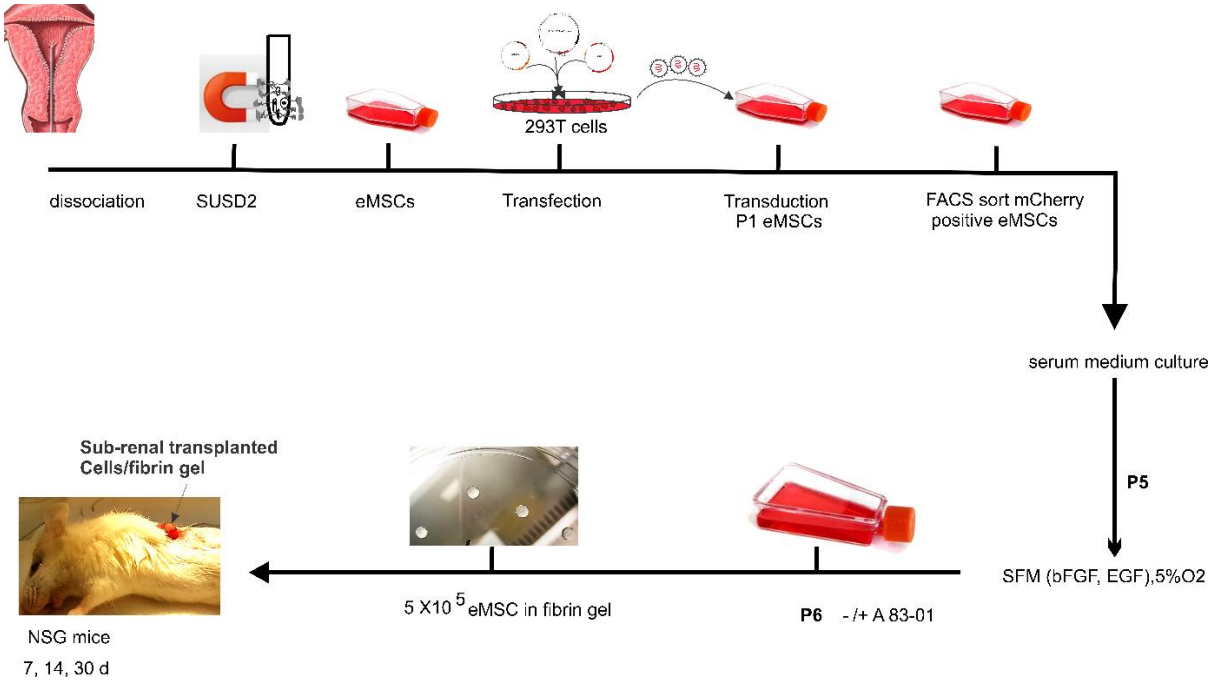

Supplementary Figure 3.1 Experimental Protocol. This flow chart shows the steps from SUSD2^{+} eMSCs isolation, lentiviral transduction, propagation and subrenal transplantation in NSG mice till 7, 14 and 30-day experimental time points before analysis by PCR, flow cytometry and immunofluorescent staining.

Chapter 4

Transcriptome sequencing of human endometrial mesenchymal stem cells cultured under TGF-βR inhibition

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Table of Contents

Declaration

Monash University

Declaration for Thesis Chapter 4

In the case of Chapter 4, my contribution to the work involved the following:

Name	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
Shanti Gurung	Responsible for performing the experiments, collecting all the data, analysing, interpreting and preparing figures and tables, and manuscript writing	80%
Sarah Williams	Assisted with RNA sequencing data analysis	N
Jerome Werkmeister	Study design, manuscript revision and approved the final manuscript.	N
Caroline Gargett	Study design, manuscript revision and approved the final manuscript.	N

Student signature: [redacted] Date: 28/06/2017

Abstract

Human mesenchymal stem cells (MSCs) are multipotent cells with immunomodulatory properties due to their ability to secrete soluble factors. They have been used in the clinical trials for a wide range of disorders. However, MSCs are a rare population of cells in tissues, which require extensive expansion *in vitro* to generate a large number of cells for clinically relevant doses. Despite extensive proliferative ability, MSCs undergo spontaneous differentiation, replicative senescence and cell death during this process. We previously demonstrated that A83-01, a TGF-βR inhibitor increased the percentage of SUSD2$^+$ endometrial mesenchymal stem cells (eMSCs) and promoted proliferation, prevented apoptosis and maintained their MSC functions *in vitro* in long-term cultures. Here we undertook transcriptional profiling of passage 6 SUSD2$^+$ eMSCs treated with and without A83-01 for 7 days from six women to identify differentially regulated genes to provide insight into the genes and pathways associated with the undifferentiated eMSC state and identify genes activated during spontaneous differentiation. We identified 1206 differentially expressed genes using a false discovery rate cut-off at 0.01 and fold change >2. Using DAVID functional annotation and KEGG pathway database, we confirmed that TGF-βR signalling pathway was downregulated following A83-01 treatment of eMSCs. Genes upregulated were cytokines and chemokines involved in chemotaxis and homing, angiogenesis, immune response, response to interferon-γ, positive regulation of cell proliferation, extracellular matrix organisation and negative regulation of smooth muscle cell proliferation. Genes downregulated were involved in the TGF-β and Wnt signalling pathways and positive regulation of fibroblast proliferation. In addition, anti-fibrotic, anti-apoptotic and cell proliferation genes were induced. Overall, the data

suggests that TGF-βR signalling regulates multiple pathways in eMSCs and treatment with A83-01 may prime the cells to an immuno-modulatory and healing phenotype, whilst maintaining stemness, proliferative capacity and prolonging their survival, ideal properties for an MSC-based cell therapy.

4.1. Introduction

Human endometrium is a dynamic tissue which undergoes monthly cycles of proliferation and differentiation under the influence of estrogen and progesterone respectively (Jabbour et al., 2006). These changes prepare the endometrium to be receptive for embryo implantation. In the absence of pregnancy circulating progesterone levels drop and the functional layer of the endometrium is shed as menses. Remarkably, 4-10 mm of a new functional layer is regenerated in the first half of the subsequent cycle in response to increasing estrogen levels (Ettinger et al., 1997). This efficient and regeneration process of the endometrium is scarless healing and continues every month for 400-500 cycles during a woman's reproductive life (Jabbour et al., 2006).

MSCs have been isolated from almost all postnatal adult tissues now (Crisan et al., 2008, Armstrong et al., 2012, Lv et al., 2014). Endometrial mesenchymal stem cell (eMSC) population has been identified and characterised in the human endometrium (Schwab and Gargett, 2007, Masuda et al., 2012). First identified as clonogenic plastic adherent stromal cells (Chan et al., 2004), recently they have been enriched using surface markers CD140b/CD146 and SUSD2 (Schwab and Gargett, 2007, Masuda et al., 2012). eMSCs expressing these markers are clonogenic, highly proliferative and multipotent *in vitro* and generate stromal tissue *in vivo*. Discovery of these markers added to their identification in the perivascular region of functional and basalis layer of the endometrium (Schwab and Gargett, 2007, Masuda et al., 2012, Spitzer et al., 2012). MSCs are known to maintain tissue homeostasis so it is likely that eMSCs are responsible for initiation of the receptive endometrium and endometrial tissue regeneration during reproductive cycles in women.

MSCs are rare in tissues and need culture expansion to generate a sufficient number of cells for clinical use (Pittenger et al., 1999). This is especially important for use in adult patients, for repeated use or when delivered via systemic route (von Bahr et al., 2012). In fact, a majority of mesenchymal stem cell clinical trials use culture-expanded MSCs (Trounson and McDonald, 2015). However, during the culture process, MSCs undergo replicative senescence and spontaneous differentiation (Gurung et al., 2015, Samsonraj et al., 2015, Barragan et al., 2016). This may lead to a reduction in the population of potent cells, diminishing their efficacy when used *in vivo* (Samsonraj et al., 2015). We identified that long-term-cultured eMSCs treated with a small molecule, A83-01, targeting the transforming growth factor beta receptor (TGF-βR), retain their MSC properties such as clonogenicity, differentiation, phenotype, and capacity to proliferate as well as block senescence and apoptosis (Gurung et al., 2015).

TGF-β is a pleiotropic cytokine with important roles in various biological functions, including differentiation, extracellular matrix generation, angiogenesis, fibrosis, immune surveillance, apoptosis, tissue homeostasis and repair (Massague, 2012). Upon TGF binding, TGF-βR signals through SMAD proteins in a canonical pathway but also crosstalks with a wide range of intracellular signalling pathways including MAPK, NOTCH and JNK independently of SMAD (Heldin et al., 1997, Zhang, 2017). We wanted to understand the mechanism by which A83-01 acts on eMSCs to regulate their functional properties. To this end, we used the power of RNA-seq for transcriptome analysis, evaluating two groups of six paired passage 6 human $SUSD2^{+}$ eMSCs treated with and without A83-01 for 7 days. The differential expression profile between the two groups confirmed that A83-01 targets the TGF-βR pathway as well as various non-canonical pathways such as Wnt pathway. The results reveal that A83-

01 treated eMSCs have angiogenic, anti-inflammatory, anti-apoptotic and anti-fibrotic properties compared with the control group, thus enhancing the desired properties of eMSCs for potential use in the treatment of a wide range of clinical conditions.

4.2. Materials and Methods

4.2.1. Human endometrial tissue samples

Human endometrial tissue samples were obtained from pre-menopausal women undergoing endometrial biopsy for non-endometrial pathologies e.g. fibroids, heavy menstrual bleeding and endocervical polyp, and who were not taking any exogenous hormones for three months prior to the surgery. Human ethics approval was obtained from Monash Health and Monash University Human Research Ethics Committees (CF10/2080, 2010001150), and patients gave written informed consent. The experimental protocols were conducted under the ethical guidelines of the National Health and Medical Council (NHMRC) of Australia's National Statement on Ethical Conduct in Human Research.

4.2.2. Isolation and culture of SUSD2$^+$ eMSCs with A83-01

eMSCs were isolated following published protocols (Masuda et al., 2012, Gurung et al., 2015) (Chapter 2). In brief, endometrial tissue was mechanically and enzymatically dissociated with 0.5% (wt/ vol) collagenase I (Worthington Biochemical Corporation) and 40 μg/ml deoxyribonuclease type I (Worthington Biochemical Corporation) for one hour at 37^{o}C. The epithelial glands and undigested tissue were removed by filtration and red blood cells by Ficoll-Paque density gradient centrifugation. SUSD2$^+$ stromal cells were purified by magnetic bead sorting using Phycoerythrin (PE)-conjugated SUSD2 antibody and anti-PE microbeads (MACS, Miltenyi) followed by column separation in a magnetic field. The eluted SUSD2$^+$ eMSCs were then washed and counted.

$SUSD2^+$ eMSCs were cultured in 10% FCS, 0.1% Primocin and 2 mM glutamine, and gradually changed to 5% to 1% FCS media and to serum-free DMEM-based medium with basic fibroblast growth factor (FGF2, 10 ng/ml) and epidermal growth factor (EGF, 10 ng/ml) (SFM) at 37ºC in 5%O_2/5%CO_2/90%N_2 humidified incubator, as described previously (Rajaraman et al., 2013, Gurung et al., 2015) (Chapter 2). At subsequent passaging, the cells were seeded at 5000 cells/cm^2. At passage 6 (P6), the cells were separated into two groups, one group was treated with 1 μM A83-01 and the control group with (0.01% DMSO) vehicle for 7 days with media changed every 48 hours.

4.2.3. RNA Isolation and RNA sequencing

P6 A83-01 treated and control eMSCs cells were trypsinised and pelleted. The pelleted cells were lysed and total RNA was extracted using the PureLink® RNA mini Kit (Ambion, Invitrogen) following the manufacture's protocol, including treatment with RNase-free DNase (Qiagen) to obtain genomic DNA-free total RNA. The integrity and quantity of the RNA from individual samples (n=12, 2 groups of 6 individual donor cells) were determined using the Agilent 2100 Bioanalyzer to meet quality standards, integrity and yields. All samples had RNA integrity number greater than 9 indicating high integrity.

RNA-seq was undertaken at Monash Health Translation Precinct, Melbourne, Australia Genomics Facility. Total RNA was depleted of ribosomal RNA and 1 μg of RNA was processed using the Illumina TruSeq Poly-A mRNA Library Pro Kit protocol 15031047 RevD (Illumina, Hayward, CA) to generate indexed cDNA libraries for each RNA sample. Libraries were verified by Bioanalyzer and quantified by qPCR. A single equimolar pool was prepared and sequenced with 50 base pair single-end reads

(approximately 50-70 million reads/sample) with the Illumina HiSeq3000. Image analysis, raw nucleotide base calling, conversion from bcl to fastq format, and quality filtering were conducted using Illumina CASAVA v1.8.2 software (Illumina, Hayward, CA) with >97% of reads having quality >Q30.

4.2.4. Bioinformatic analysis of RNA sequencing data

The raw fastq data from each RNA-seq sample was further cleaned to remove any low-quality residues and adaptor sequences trimmed using standard Illumina TruSeq. The cleaned/trimmed RNA sequenced reads were mapped against the Human Reference Genome GRCh38 and Ensembl 87 gene annotation using STAR (v2.5.2) with the RNAsik pipeline and manipulated with samtools (v1.3.1) and picard tools (v2.8.2). From the mapped reads, raw read counts/gene were quantified using htseq-count (v0.6.1p1). The aligned read counts were assembled for subsequent analysis and differential expression was calculated using voom and limma (v3.26.9) via the degust interface. The differentially expressed genes were listed and gene transcript abundance between the control and treated groups were analysed using a paired test with false discovery rate at a significance threshold of $p<0.01$ and absolute log change of 1 cutoff (> 2-fold change). Functional annotation of the differentially expressed genes was performed by KEGG (Kyoto Encyclopedia of Genes and Genomics) and gene ontology analysis using DAVID (Database for Annotation, Visualisation and Integrated Discovery), version 6.8.

4.2.5. Transcriptional validation using Fluidigm PCR

Differentially expressed candidate genes were selected for validation based on the findings from KEGG and Gene Ontology analysis and by selecting from the literature for being involved in various pathways. Human TaqMan primer-assays, designed across exon-exon junctions that do not detect homologs and specific for the candidate genes were purchased from Life Technologies. 95 differentially expressed genes from the RNA-seq data analysis and a housekeeping gene (β-actin) **(Supplementary Table 4.1, Supplementary Table 4.2)** were selected for qRT-PCR validation using the Fluidigm BioMark system (Fluidigm). First-stranded cDNA was synthesised using SuperScript III first-strand synthesis system (Invitrogen). Reverse transcriptase negative samples were also prepared from 4 random samples to rule out genomic DNA contamination in the cDNA samples. All the samples were quality checked by qRT-PCR on ABI 7900HT (Applied Biosystems) using β-actin with SYBR Green chemistry before loading to pre-primed 96.96 Dynamic Array IFC (Fluidigm). cDNA (50 ng/μl) was amplified and detected using Taqman assay. Each reaction was run with 18 μM optimised primers and 4 μM probe. The PCR amplification reaction included initial Taq activation at 95^{o}C for 60 seconds, followed by 35 cycles of denaturation at 95^{o}C for 5 seconds and annealing/polymerisation at 60^{o}C for 20 seconds. Target genes expression were normalised to *β-actin* and relative gene expression and fold change was calculated using the $2^{-\Delta\Delta CT}$ method (Gurung et al., 2015).

4.2.6. Statistical analyses

The RNA-sequence data were analysed using stranded paired t-test with multiple hypotheses corrected p-value or false discovery rate (FDR) of <0.01. Biological pathways were considered significant with Benjamini-Hochberg test $p<0.05$. qRT-PCR validation data were analysed using Wilcoxon matched-pair signed rank test between the A83-01 treated and control eMSCs group in Graph Pad Prism 6.07. Data are presented as mean ± standard error of mean.

4.3. Results

We have previously published that inhibiting the TGF-βR pathway with a small molecule A83-01 maintained SUSD2$^+$ eMSC stemness, promoted proliferation, and blocked senescence and apoptosis in late passage cultures (Gurung et al., 2015). The TGF-βR signalling pathway regulates a wide range of cellular processes through both SMAD-dependent and independent pathways (Massague, 2012). Therefore, to gain further insight into the molecular mechanisms involved in maintaining stemness of eMSCs when treated with A83-01, purified mRNA isolated from A83-01 treated and control P6 eMSCs from six different donors was sequenced using Illumina TruSeq RNA-sequencing platform.

4.3.1. Transcriptome analysis of A83-01 treated P6 eMSCs

Global genomic profiling and differential gene expression between the control and A83-01 treated passage 6 eMSCs from six donors were investigated using RNA sequencing analysis. Principal Component Analysis clustered the samples primarily into two distinct groups based on whether they were control or treated with A83-01 (Clustering was defined based on the first and second % variance including all detected transcripts) **(Figure 4.1A)**. A total of 21,830 transcripts were detected in the P6 eMSCs. Raw and processed data are available in https://figshare.com/s/077080c0157e77e3b0b7. We identified 1206 genes which were differentially regulated after only 7 days of A83-01 treatment compared to the control eMSCs (>2-fold change, $p<0.01$). Among these, there were 609 genes upregulated >2 fold ($p<0.01$) and 597 genes downregulated >2 fold ($p<0.01$) in the treated compared to control eMSCs.

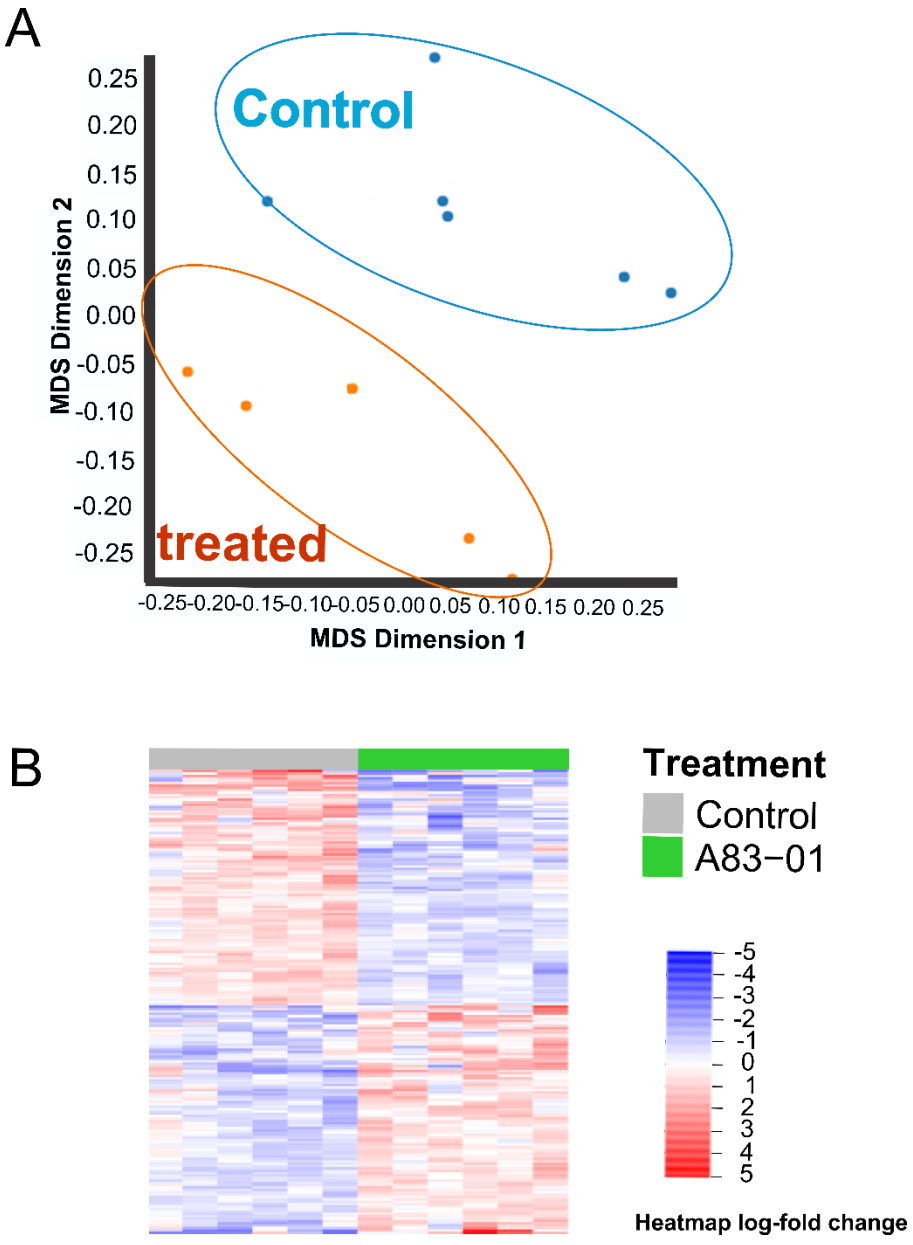

Figure 4.1 Transcriptome analysis of A83-01 treated and control P6 eMSCs. **A)** Principal component analysis (PCA) plot shows the distribution of individual samples defined by the percentage of variance by Multiple-dimension scaling. The samples clustered into two distinct groups according to whether they were A83-01-treated (orange) or control (blue). **B)** Hierarchy clustering of the top 200 significantly differentially expressed genes ($p<0.01$, >2-fold change) after 7 days' treatment with A83-01. Each row represents a gene and each column represent a patient sample.

4.3.2. Gene network and biological functional annotation of A83-01 treated eMSCs

The 609 significantly upregulated genes (p<0.01) with >2-fold change in A83-01 treated vs control populations are available in https://figshare.com/s/077080c0157e77e3b0b7.Functional enrichment analysis of the differentially upregulated genes was performed using DAVID and KEGG pathway bioinformatics databases. KEGG analysis arranged 226 genes into 36 different pathways. Similarly, Gene Ontology classification was also used to identify biological pathways associated with these genes. Some of the relevant pathways (Benjamini p<0.05) are given in **Table 4.1** (KEGG pathway) and **Table 4.2** (Gene Ontology/GO TERM BP DIRECT). The enriched pathways included complement and coagulation cascades, TNF signalling, inflammatory response, positive regulator of cell proliferation, immune response, angiogenesis and negative regulation of smooth muscle cell proliferation. These pathways play important role in immune regulation, cell proliferation, angiogenesis, cell migration and homing properties of MSCs.

Table 4.1 Biological pathways associated with upregulated genes in A83-01 treated eMSC vs control.

	Term	Count	Gene Description	Fold Enrichment	Benjamini-Hochberg p<0.05
KEGG PATHWAY	Pathways in cancer	29	NFKBIA, MAPK10, RAC2, DAPK2, SMAD3, PTGS2, NTRK1, BIRC7, LPAR3, GNG11, FGF7, FGF20, PPARG, RASGRP2, BDKRB2, BDKRB1, RARB, PTCH2, STAT5A, FGF13, WNT2B, HGF, CSF3R, PGF, ADCY4, NKX3-1, FGFR3, LAMA4, EPAS1	2.26	0.015
	Complement & coagulation cascades	11	PLAT, BDKRB2, BDKRB1, C2, CFB, CFD, C1R, TFPI, MASP1, CFI, C3	4.87	0.008
	TNF signalling pathway	13	MAPK10, NFKBIA, MAP3K5, PTGS2, JUNB, IL15, CX3CL1, SOCS3, CXCL3, VCAM1, CSF1, CXCL2, TNFAIP3	3.75	0.012

Table 4.2 Biological pathways associated with upregulated genes in A83-01 treated eMSC vs control.

	Term	Count	Gene Description	Fold Enrichment	Benjamini-Hochberg p<0.05
GO TERM BP DIRECT	Inflammatory response	27	NOD1, PTGS2, CD14, TNFRSF21, HRH1, CD40, LXN, CSF1, PTX3, TNFAIP3, BDKRB2, BDKRB1, CCL8, TLR3, HDAC9, C3, IL15, CXCL3, AOX1, APOL3, HYAL1, CCRL2, EPHX2, GGT5, XCR1, CXCL2, LY75	2.61	0.041
	Positive regulation of cell proliferation	30	AKR1C3, RAC2, EFNB2, NAMPT, TNFSF13B, IL6ST, SLC25A27, ST8SIA1, FGF7, CSF1, CDC25B, FGF20, LIFR, ADRA2A, NRG1, SFRP1, RARB, IL6R, DPP4, CSF3, ID4, HGF, ADRA1D, IL15, PGF, NKX3-1, FGFR3, PTH1R, CLDN7, OSR2	2.36	0.041
	Response to interferon-gamma	7	IFITM3, IFITM2, CXCL16, SNCA, KYNU, IFITM1, GCH1	10.69	0.030
	Signal transduction	56	ROR2, RAC2, NOD1, PTGES, S100A6, NAMPT, ARHGAP20, CD38, TNFSF13B, IGFBP6, GNG11, FGF7, SP110, IGFBP2, PPARG, ACVRL1, TLR3, IGFBP1, CSF3R, IL15, PGF, STAC, LY75, GRB7, EPAS1, SIGIRR, MAPK10, IL15RA, ASIC1, SAV1, TNFRSF21, ANK1, CRABP2, PDE3B, CLIC2, CD83, ARHGAP15, FGF20, ADRA2A, RASGRP2, CCL8, RARB, NDP, PTCH2, ARHGAP6, FGF13, ARHGAP44, C3, ARRDC2, FYB, STARD8, SDC4, SECTM1, SORL1, GRP, APOL3	1.77	0.026
	Ureteric bud development	8	SDC4, SFRP1, SMAD3, RARB, BMPER, SLIT2, OSR1, WT1	7.72	0.033
	Immune response	27	CD36, SMAD3, TINAGL1, MBP, TNFSF13B, TNFRSF21, CX3CL1, IFI6, CD40, IFITM3, IL1R1, HLA-DPA1, C1R, CCL8, ENPP2, CSF3, C3, OAS1, IL15, FYB, TGFBR3, CXCL3, SECTM1, IFITM2, NFIL3, CXCL2, LY75	2.35	0.040
	Extracellular matrix organisation	17	HAPLN1, VWA1, NDNF, FBLN1, ADAMTSL4, VIT, HPSE, ABI3BP, EGFLAM, ITGB8, ITGB4, ICAM4, CSGALNACT1, LAMA4, VCAM1, CRISPLD2, FOXF2	3.18	0.035
	Negative regulation of smooth muscle cell proliferation	7	IL15, NPR1, APOD, KLF4, PPARGC1A, TNFAIP3, PPARG	8.85	0.035
	Angiogenesis	18	EMCN, ACVRL1, PTGS2, NDNF, NOV, PGF, APOD, PDE3B, TNFAIP2, TMEM100, CEACAM1, HS6ST1, PLXND1, JAG1, HIF3A, ENPEP, HAND2, EPAS1	2.96	0.038

Similarly, 597 downregulated genes ($p<0.01$) with >2-fold change in A83-01 treated samples compared to the control groups are available in https://figshare.com/s/077080c0157e77e3b0b7. 191 genes were arranged into 27 different categories using the KEGG pathway database. Gene Ontology analysis identified additional relevant biological pathways. Pathways with Benjamini-Hochberg correction ($p<0.05$) are shown in **Table 4.3** and **Table 4.4**. Some of the pathways involved were Wnt signalling, PI3K-Akt, protein digestion and absorption, differentiation, collagen metabolism and positive regulation of fibroblast proliferation pathways. Moreover, as can be expected 9 genes involved in TGF-βR signalling pathway were also downregulated which confirmed that A83-01 has actively targeted this pathway in our experimental setup. Similarly, 16 Wnt signalling promoting genes were downregulated while 3 Wnt-inhibitory genes were upregulated indicating significant inhibition of Wnt pathway by A83-01 in a non-canonical manner.

Table 4.3 Biological pathways associated with downregulated genes in A83-01 treated eMSC vs control.

	Term	Count	Gene Description	Fold Enrichment	Benjamini-Hochberg p<0.05
KEGG PATHWAY	Dilated cardiomyopathy	14	TNNT2, ACTC1, ADCY2, SGCD, TGFB2, PLN, ITGA4, ITGB5, TPM1, DES, ITGB3, ITGB5, CACNA2D3, ITGA1	6.03	0.00008
	ECM-receptor interaction	14	FN1, TNN, THBS1, ITGB5, LAMA1, COL4A2, COL4A1, ITGB3, COL11A1	5.82	0.00006
	Focal adhesion	20	COMP, COL1A1, ITGA4, TNC, ITGA1	3.51	0.00022
	Wnt signalling pathway	16	CTNNBIP1, LEF1, WNT11, WNT7B, WNT2, NKD2, DAAM1, PRICKLE1, MMP7, BAMBI, FZD7, ANGL2, SFRP4, DKK2, GPC4, WNT5A	4.19	0.00025
	PI3K-Akt signalling pathway	26	TNN, MET, COL4A1, FN1, PDGFC, PDGFB, THBS1, ITGB5, LAMA1, ITGB3, COL11A1, ITGA1, TNC, FGF1, IRS1, COL4A2, COMP, TEK, COL1A1, ITGA4, IL7R, ANGPT1, CDK6, VEGFD, IGF1, FGF10	2.73	0.00029
	Hypertrophic cardiomyopathy (HCM)	12	TNNT2, ACTC1, ITGB5, DES, TPM1, IGF1, ITGB3, SGCD, TGFB2, CACNA2D3, ITGA4, ITGA1	5.57	0.00027
	Pathways in cancer	27	LI1, LEF1, MET, WNT11, COL4A1, TGFB2, MMP2, APOBEC3G, FN1, PDGFB, FZD7, LAMA1, DKN2B, FGF1, RASGRP3, MAPK8, PLCB4, COL4A2, ADCY2, CDK6, RASGRP1, CXCR4, MECOM, EGFD, IGF1, FGF10, WNT5A	2.49	0.00068
	Proteoglycans in cancer	17	IGF2, ANK3, ESR1, MET, WNT11, WNT2, TGFB2, MMP2, MRAS, FN1, FZD7, THBS1, ITGB5, TGB3, IGF1, HBEGF, WNT5A	3.08	0.00285
	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	10	LEF1, ITGB5, DSP, DES, ITGB3, SGCD, CACNA2D3, ITGA4, ITGA1, CDH2	5.10	0.00283
	TGF-beta signalling pathway	9	ACVR1C, BAMBI, SMURF2, THBS1, CDKN2B, GDF6, INHBA, TGFB2, TGIF2	3.88	0.04060
	Protein digestion and absorption	9	COL10A1, ELN, SLC8A1, COL21A1, COL4A2, COL12A1, COL4A1, COL11A1, COL1A1	3.70	0.04936

Table 4.4 Biological pathways associated with downregulated genes in A83-01 treated eMSC vs control.

	Term	Count	Gene Description	Fold Enrichment	Benjamini-Hochberg p<0.05
GO TERM BP DIRECT	Cell adhesion	38	PCDHB2, CDH6, NEDD9, LOXL2, NUAK1, DSC3, ARVCF, SORBS1, PCDHB15, FN1, SPON2, THBS1, LAMA1, CTGF, COL8A1, ITGB5, ITGB3, ADAM12, TNC, PCDHB12, CDH3, ASK, COL12A1, PCDHA4, OLR1, COMP, COL1A1, ITGA4, SPOCK1, NRP2, S1PR1, PPFIBP1, NCAM1, ASS4, VCAN, SORBS2, CD24, CDH2	3.10	4.8E-06
	Collagen catabolic process	14	COL10A1, MMP11, COL4A2, ADAMTS14, COL12A1, COL4A1, MMP15, COL1A1, MMP10, MMP2, MMP7, COL8A1, COL11A1, MMP3	8.20	1.2E-05
	Endodermal cell differentiation	10	MMP2, FN1, ITGB5, COL8A1, COL4A2, COL12A1, INHBA, COL11A1, MMP15, ITGA4	13.88	1.4E-05
	Homophilic cell adhesion via plasma membrane adhesion molecules	20	PCDHB2, PCDHB12, CDH6, AMIGO2, DSC3, PCDHB10, FAT4, CDH3, PCDHA4, CADM1, DCHS1, CDHB15, FAT3, PCDHB5, CELSR2, TENM3, PCDHGB1, CLSTN2, PCDHB7, CDH2	4.74	2.4E-05
	Extracellular matrix organization	22	COL10A1, PXDN, FBLN5, COL4A2, COL4A1, COMP MFAP5, COL1A1, TGA4, FN1, ELN, PDGFB, LOX, THBS1, ITGB5, LAMA1, COL8A1, VCAN, ITGB3, COL11A1, ITGA1, NC	4.21	2.9E-05
	Collagen fibril organization	10	GREM1, MMP11, LOXL2, LOX, SCX, ADAMTS14, COL12A1, COL11A1, TGFB2, COL1A1	9.61	2.4E-04
	Positive regulation of cell migration	19	GLI1, FGF1, LEF1, WNT11, F3, COL1A1, NTF3, SEMA7A, CD274, S1PR1, F2RL1, PDGFC, PDGFB, HBS1, SEMA3F, IGF1, HBEGF, EDN1, SEMA3D	3.87	6.8E-04
	Skeletal system development	16	COL10A1, IGF2, EXTL1, PTHLH, ALPL, COL12A1, COMP, TGFB2, COL1A1, NKX3-2, VDR , FGFRL1, SOX4, VCAN, IGF1, DLX5	4.38	1.1E-03
	Wound healing	12	FN1, LOX, SCARB1, CDH3, VANGL2, DSP, TPM1, ITGB3, FGF10, TGFB2, WNT5A, TNC	5.62	2.1E-03
	Positive regulation of MAP kinase activity	10	FGF1, PDGFC, PDGFB, RASGRP1, NEK10, TPD52L1, NOX4, CD24, DIRAS1, EDN1	6.35	5.3E-03

	Positive regulation of endothelial cell migration	9	FGF1, SCARB1, THBS1, ITGB3, TEK, ANGPT1, NRP2, EDN1, WNT5A	7.33	5.2E-03
	Cell morphogenesis	10	PDPN, GREM1, VDR, SHROOM2, FRY, NOX4, TENM3, SHROOM3, TGFB2, IL7R	6.05	6.7E-03
	Positive regulation of fibroblast proliferation	9	FN1, CDK6, PDGFC, PDGFB, ESR1, PLCB4, IGF1, FGF10, WNT5A	6.25	1.4E-02
	Extracellular matrix disassembly	10	MMP2, MMP10, MMP11, MMP7, FN1, ELN, HTRA1, CAPNS2, MMP15, MMP3	4.93	2.9E-02
	Positive regulation of gene expression	19	LEF1, ANK3, SCX, ADM2, WNT11, CDH3, ACTC1, WNT7B, TGFB2, IL7R, FOXD1, VDR, FN1, CDK6, PDGFB, CTGF, SFRP4, INHBA, TNC	2.72	3.5E-02
	Angiogenesis	17	FGF1, NRXN3, SRPX2, ADM2, COL4A2, TEK, TGFB2, NRP2, ANGPT1, MMP2, S1PR1, FN1, CTGF, ARHGAP22, COL8A1, VEGFD, FGF10	2.86	4.4E-02
	Positive regulation of cell proliferation	27	GLI1, IGF2, LEF1, TGFB2, EDNRB, FN1, PDGFC, PDGFB, CRLF1, THBS1, CTGF, SOX4, EDN1, TNC, FGF1, SCX, IRS1, PTHLH, PLCB4, IL31RA, NTF3, GREM1, BAMBI, VEGFD, IGF1, TNFSF4, HBEGF	2.17	4.2E-02
	Axon guidance	14	ANK3, NRXN3, KIF5C, KIF26B, TGFB2, ENAH, NRP2, FOXD1, NCAM1, SPON2, SEMA3F, SPTBN2, DLX5, WNT5A	3.30	4.1E-02

4.3.3. Additional differential gene expression profile of eMSCs treated with A83-01

Several cytokines, their receptors and chemokines were among the differentially upregulated genes in the A83-01 treated eMSCs. Transcript levels of *IL-15* and its receptor *IL-15RA*, *IL-33* and its receptor *IL-1R1*, *IL-6* and *IL-6R*, and *IL-18BP* were upregulated. Chemokines such as *CX3CL1*, *CXCL2*, *CXCL3*, *CXCL12* and *CXCL16* were also upregulated. Furthermore, 11 genes involved in the complement cascade were upregulated, including *PLAT*, *BDKRB2*, *BDKRB1*, *C2*, *CFB*, *CFD*, *C1R*, *TFPI*, *MASP1*, *CFI*, *C3,* which are known to have anti-inflammatory activity (Kolev et al., 2014).

In addition to cytokines and their receptors, we observed that *SFRP1* and *SOD3* were the most significantly upregulated genes in the A83-01 treated eMSCs. SFRP1 is a Wnt pathway inhibitor while SOD3 is an anti-oxidant enzyme. Similarly, other highly upregulated genes were *PLA2G4A*, *PTGS2* (COX-2) and *PTGES*, all of which are vital for PGE2 synthesis. Interestingly, *Toll-like receptors* (TLR) *2 and 3*, macrophage recruiting factor *CSF1* were also induced.

We also found that anti-apoptotic factors such as *FAIM2*, *ENPP2*, *TNFAIP3*, *NFKBIA*, *IGFBP2* were enhanced while pro-apoptotic factors such as *ROS1* and *TP53I3* were significantly downregulated. Cell proliferation factors such as *ISG20*, *PGF*, *NDP* and *TACSTD* were upregulated, confirming functional differences between A83-01-treated and control eMSCs we found in our previous study (Gurung et al., 2015). Additionally, pro-angiogenic factors such as *VCAM1*, *HGF*, *NGF* and HA6ST1were also

upregulated. We also found upregulation of anti-fibrotic factor *HGF* and downregulation of fibrosis-related factors such as *CTGF*, *COL1A* and *ACTA2*.

4.3.4. Standard MSC markers were not different

The expression of MSC marker genes in the two groups is shown in **Table 4.5.** Consistent with our previous study, *CD146* mRNA level was downregulated upon A83-01 treatment. We then examined the International Society for Cellular Therapy (ISCT) MSC markers. Transcripts of *CD90, CD73 and CD105* were highly expressed in both A83-01-treated and control eMSC groups but not statistically different. We also looked into MSC-defining markers, and transcripts of molecules previously suggested as markers for purifying MSCs from bone marrow and other sources (Lv et al., 2014). Transcripts of *CD49f*, *CD166* (*ALCAM*), *CD56, CD44* and Nestin were highly expressed in both A83-01-treated and control eMSCs but there was no statistically significant difference. Transcripts of *CD271*, *SSEA3/4* and *FZD9* were either not expressed or expressed at very low levels. Mesodermal differentiation marker for osteogenesis (*RUNX1*) and chondrogenesis (*COL10A1*) were significantly downregulated in the treatment group but there was no difference in the adipogenic marker *FABP4*. Pluripotency genes *OCT4*, *NANOG* or *SOX2* were not expressed in either group and there was also no upregulation of mesodermal marker (*SNAIL*, *SOX17*) or endodermal marker (E-cadherin).

Table 4.5 List of MSC defining markers between control and A83-101 treated P6 eMSCs. FDR/false discovery rate (multiple hypotheses corrected p-value) and N/D (not detected)

	A83-01 treated Vs Control	
MSC Gene Symbol	**Log_2 Fold change**	**FDR**
CD90	0.054	0.759
CD73	0.646	0.016
CD105	0.217	0.137
CD166	-0.604	0.015
CD44	-0.114	0.471
CD49f	0.672	0.057
NESTIN	-0.439	0.054
FZD9	-0.703	0.200
CD271	N/D	
SSEA-4	N/D	
MSC negative markers		
CD34	-0.985	0.158
CD45	-0.003	0.998
HLA-DRB1	0.289	0.752
HLA-DRB5	0.867	0.356
HLA-DRB6	1.254	0.292
HLA-DRB3	N/D	
HLA-DRB4	N/D	

4.3.5. Validation of differentially regulated genes by Fluidigm qPCR

95 genes involved in regulating immunoregulation, angiogenesis, fibrosis, apoptosis, MSC potency and differentiated state of MSCs were validated by Fluidigm qPCR **(Supplementary Table 4.1** and **Supplementary Table 4.2)**. Consistent with the RNA-seq results, genes involved in TGF-βR signalling were significantly downregulated **(Figure 4.2A)** while the cytokines, its receptors and chemokines were significantly increased in A83-01 treated eMSCs compared to the controls **(Figure 4.2B and C)**. Assessment of cell-proliferative genes also supported our RNA-seq data, except for those of *TACSTD2*, *STAT5AI*, *PPARG* and *MECOM* **(Figure 4.3)**. Furthermore, genes involved in angiogenesis, fibrosis, apoptosis and Wnt signalling were also validated to be differentially regulated **(Figure 4.4)**. Similarly, all the MSC potency gene and fibroblast-associated genes (except *ADCY2* and *VCAN*) were validated **(Figure 4.5).**

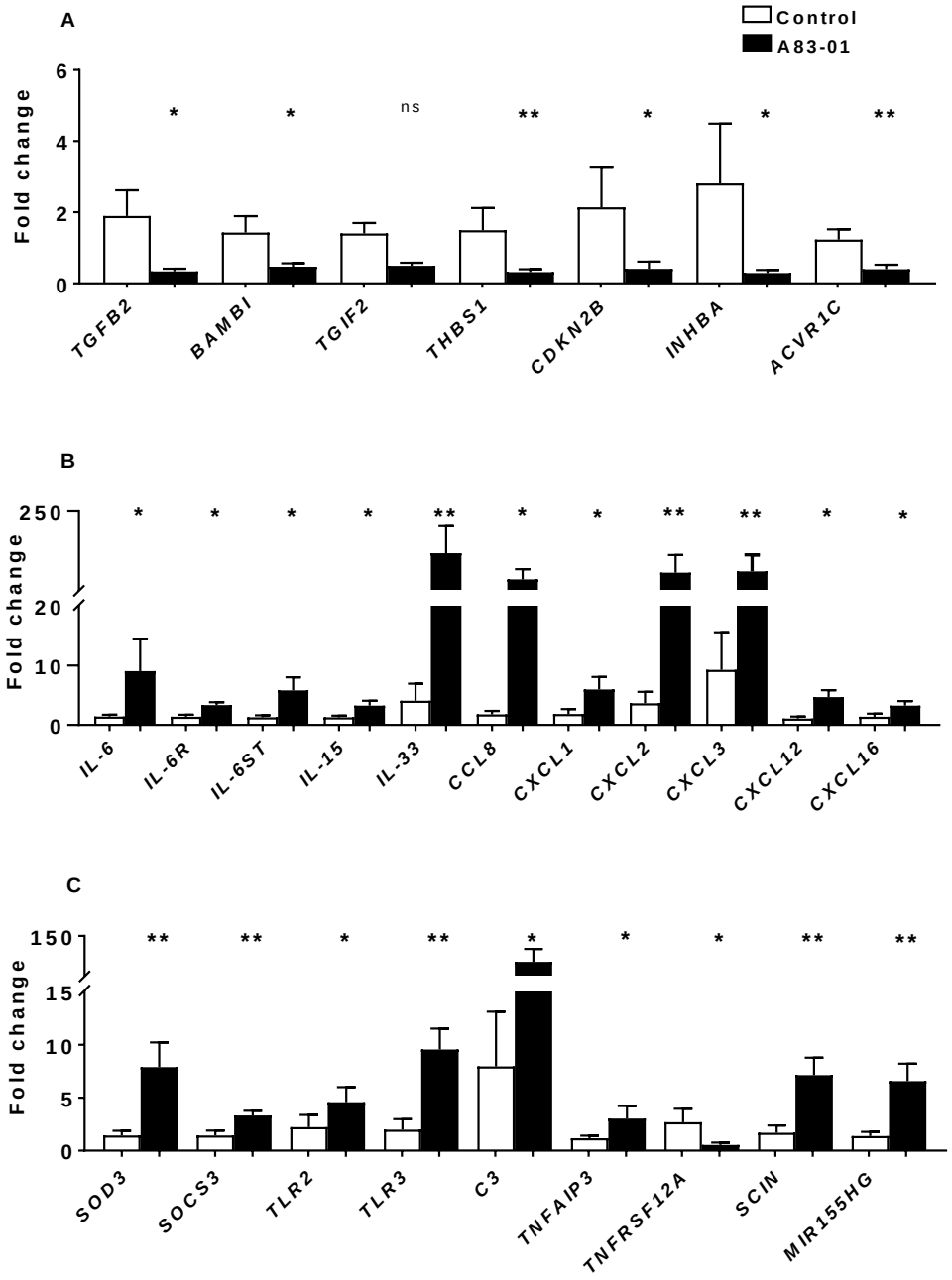

Figure 4.2 Validation of TGF-βR pathway and immune-related genes in control and A83-01 treated P6 eMSCs by Fluidigm qPCR. Genes related to A) TGF-βR signalling pathway, **B)** Cytokines and cytokine receptors, and **C)** Immuno-regulation assessed for validation were statistically significant between control (white bar) and A83-01 treated (black bar) eSMCs. The graphs show the mean ± SEM from 6 independent biological samples (*=p<0.03 and **=p<0.002).

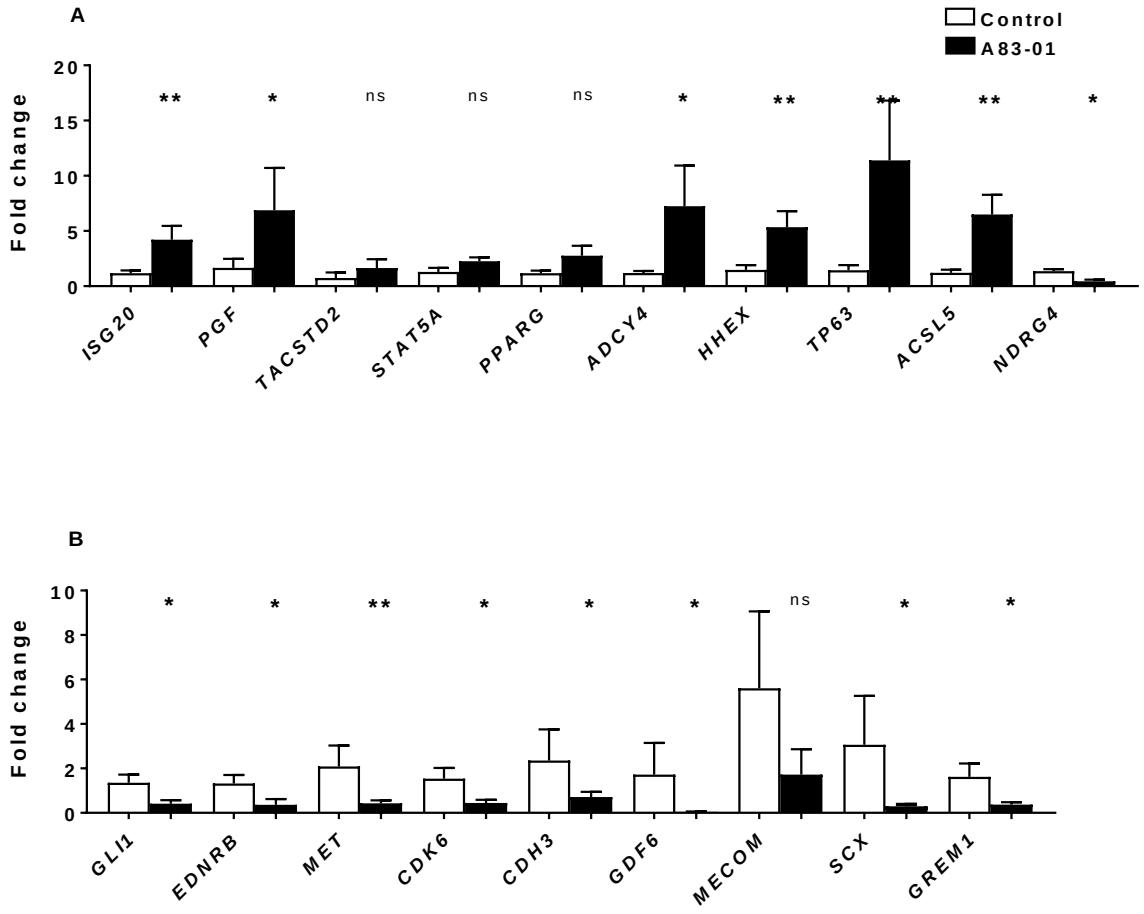

Figure 4.3 Cell proliferation-related genes in P6 eMSCs treated with and without A83-01. Total cell proliferation-related transcript levels were measured in P6 eMSCs following a week of treatment with and without A83-01. The graphs show the mean ± SEM from 6 independent biological samples (*=p<0.03 and **=p<0.002).

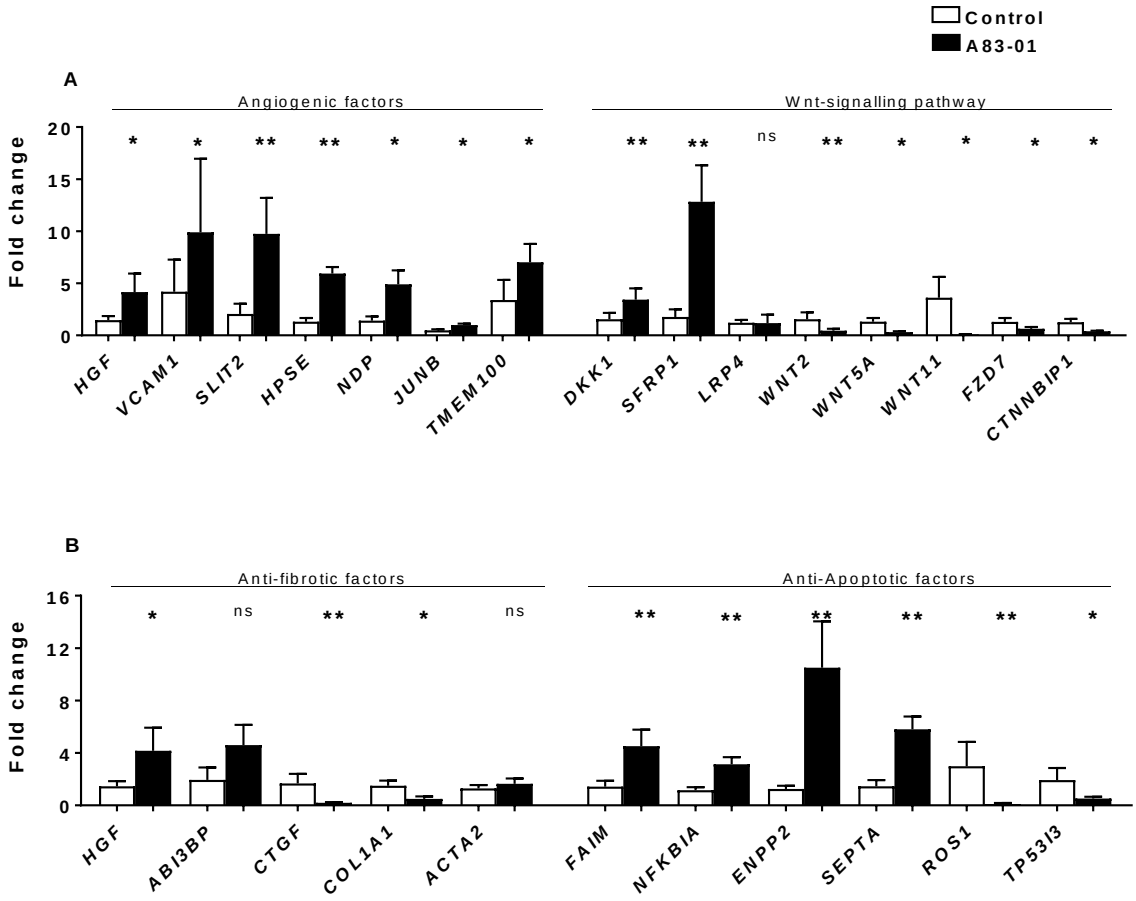

Figure 4.4 Differentially regulated wound healing transcripts in A83-01 treated eMSCs. Assessment of **A)** Pro-angiogenic and Wnt inhibitory pathway, and **B)** anti-fibrotic and anti-apoptotic transcripts in eMSC cultures treated either with A83-10 or control. The graphs show the mean ± SEM from 6 independent biological samples (*=p<0.03 and **=p<0.002).

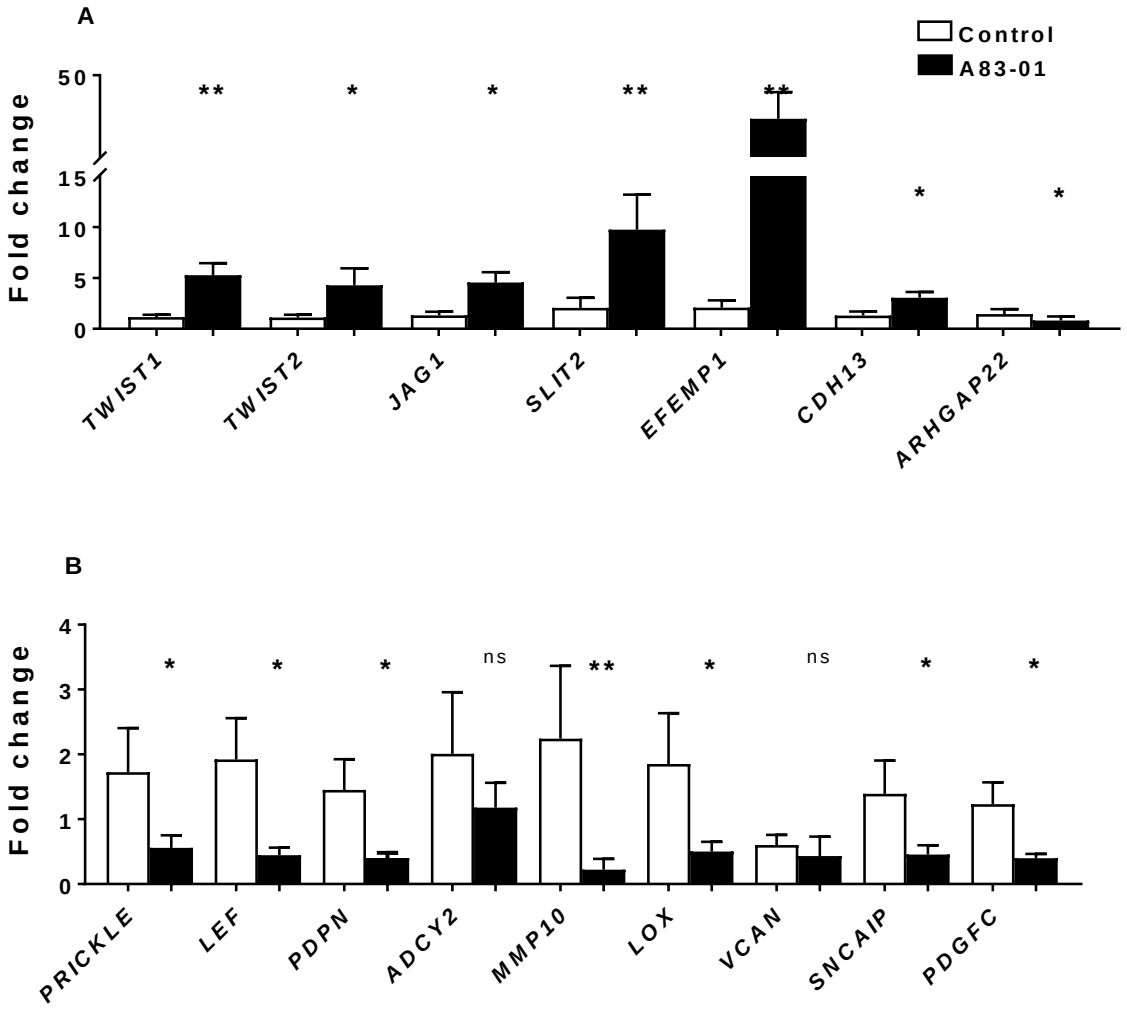

Figure 4.5 Validation of MSC- and fibroblast-related genes in P6 eMSCs treated with and without A83-01 by Fluidigm qPCR. A) MSC-potency and **B)** Fibroblast-specific genes between control (white bar) and A83-01 treated (black bar) eMSCs were assessed for validation. The graphs show the mean ± SEM from 6 independent biological samples (*=p<0.03 and **=p<0.002).

4.4. Discussion

Our RNA-sequencing analysis of late passage eMSCs treated with A83-01, a TGF-βR inhibitor, for just 7 days, revealed a unique gene profile of upregulated genes associated with stemness, chemotaxis, anti-inflammatory response, and angiogenesis compared with control eMSCs. In our previous study, we identified treating eMSCs with A83-01, increased the colony forming efficiency, prevented replicative senescence and apoptosis (Gurung et al., 2015). The additional information from RNA seq analysis and Fluidigm qPCR validation suggest that TGF-βR signalling regulates multiple pathways in eMSCs and treatment with A83-01 may prime them to an immuno-modulatory and healing phenotype for example by promoting angiogenesis and decreasing fibrosis, whilst retaining their undifferentiated state and proliferative capacity, ideal properties for an MSC-based cell therapy.

We found that treating with A83-01 significantly promoted the expression of cytokines and its receptors in eMSCs. Cytokines are key regulators of migration and maturation of various cell types (Bachmann et al., 2006). MSCs are known to secrete a wide range of cytokines and chemokines such as IL-6, IL-8, IL-10, CXCL2, CXCL3 and CXCL12 (Schinkothe et al., 2008, Hoogduijn et al., 2010). Although cytokines have been classified into pro-inflammatory (e.g. Interleukin-1 and tumor necrosis factor (TNF)) and anti-inflammatory categories (e.g. IL-10 and interferon-alpha), they have dual functions depending upon the target cells, cytokine concentration and the environment (Cavaillon, 2001). Human umbilical cord blood-MSCs transduced with murine IL-15 ($MSC^{IL\text{-}15}$) significantly inhibited pancreatic tumor growth and prolonged the survival of tumor-bearing mice via accumulation of natural killer and T-cells (Jing et al., 2014). IL-15 can also stimulate neovascularisation through activation of endothelial cells,

promote microglial growth and survival as well as induce PGE2 synthesis (Angiolillo et al., 1997, Hanisch et al., 1997, Agarwal et al., 2001). IL-33 is constitutively expressed in cells indicating its role in maintaining homeostasis (Cayrol and Girard, 2014). Widely known as an alarmin, IL-33 not only alerts the immune system of tissue damage but also activates endothelial cells and induces angiogenesis (Choi et al., 2009). Furthermore, in the endometrium, it signals temporal expression of receptive genes during the mid-secretory phase in preparation for embryo implantation (Salker et al., 2012). For instance, embryo implantation rate dropped from 90% to 14% in mice treated with condition media from transduced decidualised human endometrial stromal cells with IL-33 and IL-33^{siRNA} respectively due to failure of formation of the functional decidual-placental interface in latter (Salker et al., 2012). Similarly, endogenous IL-6 secreted by MSCs suppressed apoptosis of Oxygen and Glucose Deprived-injured neurones *in vitro* and reduced neurological impairment caused by hypoxic-ischemic brain damage in a rat model of brain injury (Gu et al., 2016). Our eMSCs treated with A83-01 strongly transcribed these genes indicating their unique anti-inflammatory and angiogenic properties compared to the control.

Chemokines are cytokines known for mediating chemotaxis. In our current study, we demonstrated significant upregulation of genes involved in chemotaxis, such as CXC chemokine genes *CXCL1*, *CXCL2*, *CXCL3*, *CXCL8*, *CXCL12 (SDF-1)* and *CXCL16*, and *CCL2* in A83-01 treated eMSCs. Chemokines are known for their potent migratory effects on neutrophils, monocytes and have pro-angiogenic properties (Owen and Mohamadzadeh, 2013, Bodnar, 2015, Kim et al., 2016). CXCL8 promoted human MSCs migration and mediated angiogenesis *in vitro* (Ringe et al., 2007, Martin et al., 2009) while IL-6 also induced T cell migration to the site of inflammation (Weissenbach

et al., 2004). MSCs$^{SDF\text{-}1}$ prevented cardiac myocyte death and improved the cardiac function in a rat model of acute myocardial infarction (Zhang et al., 2007). Similarly, MSC^{CCL2} also ameliorated Systemic lupus erythematosus (SLE) development in MRL*Fas*^{lpr} mice. (Lee et al., 2017). This suggests that A83-01 enhances chemotactic profile of eMSCs and prime them toward immuno-modulatory phenotype.

Toll-like receptors are proteins of innate immune cells that recognise pathogens and have also been identified to be expressed by MSCs (Delarosa et al., 2012, O'Neill et al., 2013, Sangiorgi and Panepucci, 2016). Interestingly but not surprisingly, A83-01 enhanced *TLR2* and *TLR3* expression while *TLR4* was not affected in eMSCs. Stimulation of MSCs with TLR-ligands trigger release of immunomodulatory cytokines, chemokines and TLR-related genes (Tomchuck et al., 2008). In fact, depending upon the TLR activation, MSCs can be pro- or anti-inflammatory phenotype for example activation of MSCs through TLR2 and 3 ligands increase PGE2 and IDO synthesis priming them to an anti-inflammatory phenotype while TLR4-primed MSCs are involved in pro-inflammatory mediation (Waterman et al., 2010, Wang et al., 2014b). In addition to enhanced *TLR2* and *TLR3*, we could identify critical enzymes-related genes required for PGE2 synthesis such as *PLA2G4A*, *PTGS2* (*COX-2*) and *PTGES*. This agrees with our cytokines and chemokines transcription profiles suggesting that A83-01 treatment portrait eMSCs as immunomodulatory and regenerative cell source.

Treatment of eMSCs with A83-01 also activated other immune-regulatory factors such as *SOCS3* and *SOD3*. SOCS3 is an intracellular key regulatory protein of the immune system (Liu et al., 2015). A previous study reported intracellular signalling of SOCS3 in MSCs inhibited Th17 differentiation (Liu et al., 2015). This was mediated via

activation of interferon gamma and STAT3 inhibition (Liu et al., 2015). MSCs secreted IL-6 has also been shown to activate STAT3/SOCS3 pathway leading to inhibition of NFκB signalling and polarisation of alveolar macrophages into anti-inflammatory phenotype (Zhang et al., 2016). Although our data was consistent with *SCOS3* and *NFKBIA* (NFκB inhibitor) activation there was no change in IFN-γ and STAT3 indicating an alternate pathway for its regulation. SOD3 protected survival of nitric oxide-induced damaged of neurones and axons (Kemp et al., 2010). In this study, activated microglial cells promoted SOD3 secretion of MSCs which was responsible for the anti-oxidant effect on the neurones *in vitro* and *in vivo*. Therefore, these transcriptional landscapes further reinforce the anti-inflammatory, antioxidant and immunomodulatory phenotype of A83-01 treated eMSCs.

In recent years, the regenerative and anti-inflammatory properties of MSCs are contributed to their ability to secrete a wide range of biological soluble factors (Bassi et al., 2012, Mastri et al., 2014, Wang et al., 2014b). These properties have gained more momentum than their role in directly differentiating *in vivo* (Mastri et al., 2014). A simple way to enhance anti-inflammatory property of eMSCs and other MSCs can be by treating them with A83-01 for the final passage before clinical use. Cumulative data suggest the secretion of anti-inflammatory factors by MSCs is significantly upregulated in an inflammatory environment (Regulski, 2017). For example, pro-inflammatory cytokine TNF-α initiated activation of PGE2 synthesis pathway in MSCs which in turn inhibited activated-T cells and altered the cytokine secretion profile of immune cells to anti-inflammatory type (Aggarwal and Pittenger, 2005, Prockop and Youn Oh, 2012). eMSCs delivered on a polyamide and gelatin mesh (P/A) initiated an acute inflammatory reaction which was followed by its reduction and deposition of a

more organised collagen in rat (Ulrich et al., 2014). In a physiological environment, perivascular MSCs (pericytes) are quiescent and they are activated when blood vessels are injured or inflamed to maintain homeostasis. These collective evidence support therapeutic benefit of A83-01 treated eMSCs when used to treat patients with inflammatory diseases.

VEGF is the key regulator of angiogenesis. However, no difference in its expression was identified between eMSCs treated with and without A83-01 in our study. Nonetheless, other angiogenic factors upregulated in our data were *HGF, VCAM1, HPSE* and *SLIT2*. HGF is recognised not only as a potent mitogen for hepatocytes but also as a potent angiogenic growth factor (Bussolino et al., 1992, Ha et al., 2017) while VCAM1, a cell adhesion molecule facilitating vessel growth, increased in bmMSCs when pre-treated with cytokines such as TNF-α and IL-1β leading to improvement of ischemic parameters in rat (Wang et al., 2014a). MSC^{VCAM1} promoted proliferation, migration of endothelial cells *in vitro* and significantly improved the functional outcome in an ischemic hindlimb of BLAB/c nude mice compare to MSC^{null} treated group (Du et al., 2016). Additionally, MSC^{HPSE} significantly increased blood flow by enhancing angiogenesis and capillary density compared to MSC^{null} treatment in a rat model of hindlimb ischemia (Hu et al., 2015). Similarly, rats treated with eMSCs on P/A mesh also had significantly higher number of α-SMA-positive vessels around the mesh promoting angiogenesis compared to mesh alone (Ulrich et al., 2014). Therefore, angiogenic phenotype of A83-01 treated eMSCs also highlights its benefit in using for regenerative therapy.

Another striking feature of our RNA seq analysis is that we identified significantly differentially regulated genes regulating fibrosis between A83-01 treated and control eMSCs. TGF-βR signalling is known to activate myofibroblasts and stimulate deposition of extracellular matrix proteins leading to fibrosis (Leask and Abraham, 2004, Lipson et al., 2012) while MSCs are known to heal damaged tissue and repair without fibrosis. For example, eMSCs on P/A meshes promoted wound repair with minimal fibrosis (Ulrich et al., 2014). In addition, they promoted deposition of matrix that was well organised physiological fine fibrils (Edwards et al., 2015). Similarly, antifibrotic factors such as HGF and PGE2 induced by human adipose MSCs also reduced fibrosis and preserved the lung architecture in a radiation-induced rat model of lung fibrosis (Dong et al., 2015) while human MSC^{HGF} prevented transplantation induced fibrosis and increased the survival in rat (Yu et al., 2010). In addition to inhibiting one of the potent fibrotic factors TGF-βR, A83-01 further suppressed pro-fibrotic factors such as *CTGF*, *ACTA2* and *COL1A* and activated anti-fibrotic factors such as HGF and PGE2, suggesting A83-01-treated eMSCs' beneficial role in regenerative therapy.

The TGF-βR pathway also participates in apoptosis and cell proliferation depending upon cell type (Black et al., 2007). In this study, we also identified genes regulating apoptosis and cell proliferation whose expression sheds insight into enhanced functional properties we showed in our previous study (Gurung et al., 2015). In hepatocytes, TGF-β mediated generation of ROS1 and induced apoptosis (Black et al., 2007). Similarly, another study reported increased activation of Wnt signalling in MSCs led to increased molecular markers of DNA damage (gamma-H2AX) and senescence (p16^{Ink4a}, p53, ROS and p21) (Zhang et al., 2011). Undifferentiated MSCs

are more proliferative than more committed cells due to their high expression of cell proliferation genes (Liu et al., 2009) such as *TACSTD, IL-6*, *ISG20, PGF* and *NDP* which is also our findings in present study. These data indicate that treating eMSCs with A83-01 makes them survive longer during culture expansion without losing their proliferative potential.

In addition to the functional phenotype of A83-01 treated eMSCs, we also looked into gene expression level of ISCT surface markers as well as additional surface markers that have been suggested to be expressed by MSCs with high potency (Prockop et al., 2014, Samsonraj et al., 2015). Our results revealed increased expression of such genes; *TWIST1, TWIST2*, *JAG1*, *SLIT2*, *EFEMP1* and *TNFRSF21* inferring improved stemness which we showed functionally by increased colony forming efficiency in our previous study (Gurung et al., 2015). Not surprisingly, transcripts of ISCT surface markers; CD90, CD73 and CD105 were highly expressed but not differentially regulated between the treated and control eMSCs. This further attests to their lack of correlation to their expression and possible potency *in vivo*. Unlike upregulation of pluripotency factors such as OCT4 and NANOG upon inhibition of PDGFR signalling with PDGFR inhibitor-IV (#521233, Merck, Merck Chemicals, Nottingham, UK) (Ball et al., 2012), we did not detect any pluripotency-related genes, inferring the unlikeliness of transformation into an immortal state and their safety for clinical use.

The most exciting findings in our study were the downregulation of the fibroblast-related genes following treatment of eMSCs with A83-01. A recent Microarray study comparing endometrial fibroblasts lineage-associated genes between freshly isolated $CD140b^{+}/CD146^{+}$ eMSCs and their long-term cultures further highlighted spontaneous

differentiation of eMSCs on culture (Barragan et al., 2016, Gargett and Gurung, 2016). Extensive culture of enriched eMSCs led to downregulation of 81% of eMSCs genes while upregulation of 55% of fibroblasts genes inferring fewer differences between the culture eMSCs and the fibroblast cultures (Barragan et al., 2016, Gargett and Gurung, 2016). Furthermore, comparing the list of upregulated fibroblasts-associated genes in eMSCs cultures identified in Barragan et al study with our study, we found common genes that were downregulated in our treated eMSCs as shown in **Supplementary Table 4.3**. This key comparison between our study and that of Fatima Barragan further support the hypothesis that eMSCs undergo spontaneous differentiation into stromal fibroblasts and that A83-01 prevent this process and maintain them in an undifferentiated state.

Overall, our RNA-sequencing data provided valuable information into in-depth transcriptional landscape of A83-01 treated eMSCs. This gene profiling analysis also offers an understanding of the endometrial-related pathophysiology regarding TGF-βR signalling and on future studies such as potential gene target as well as on their potential use in treating immune-related as well as non-immune-related diseases.

The limitation of this study is that it is descriptive, however, our transcriptome analysis and validation study of long-term culture of eMSCs with and without A83-01 provides a future direction for *in vitro* studies such as tube formation analysis, secretome analysis, migration assays using wound scratch assay, immune cell proliferation assay and matrigel-based tube formation assay to assess vasculogenesis. In human endometrium, there is a transient window of implantation when the endometrium is receptive for embryo implantation (Salker et al., 2012). This period is also a transient pro-inflammatory phase where a lot of cytokines are secreted by the human endometrial stromal/stem cells (HESCs) before settling for anti-inflammatory phase. Further, it may be worthwhile examining *in vitro* decidualisation experiments, preparation for receptive endometrium and studying on its use in animal model of female infertility.

4.5. Conclusions

Our RNA-seq data analysis comprehensively showed differential gene profiles of A83-01 treated eMSCs compared to control cells. It supports the pleiotropic nature of the TGF-Rβ signalling pathway acting via canonical as well as non-canonical signalling pathways. The differentially expressed genes in the A83-01 treated P6 eMSCs suggest that A83-01 not only maintains eMSCs stemness, prevents apoptosis and

promotes proliferation but also changes the phenotypic properties to an anti-inflammatory or healing phenotype. *In vitro* and *in vivo* studies with culture expanded eMSCs, and perhaps other MSCs, in the presence of A83-01 would expect to provide further insight into the molecular mechanisms of MSC functions. Our study also demonstrates the possibility of applicability of A83-01 treated eMSCs for the treatment of a variety of diseases.

4.6. References

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Supplementary Table 4.1 List of upregulated genes for Fluidigm validation

Gene Symbol	Gene Description	Gene Symbol	Gene Description
CXCL2	C-X3-C motif chemokine ligand 2	ADCY4	adenylate cyclase 4
CX3CL1	C-X3-C motif chemokine ligand 1	CXCL3	C-X-C motif chemokine ligand 3
CXCL12	C-X-C motif chemokine ligand 12	CSF1	colony stimulating factor 1
CXCL16	C-X-C motif chemokine ligand 16	C3	complement C3
JAG1	jagged 1	CCL8	C-C motif chemokine ligand 8
ISG20	interferon stimulated exonuclease gene 20	CYP3A5	cytochrome P450 family 3 subfamily A member 5
TNFAIP3	TNF alpha induced protein 3	CD38	CD38 molecule
HGF	hepatocyte growth factor	CDH13	cadherin 13
TMEM100	transmembrane protein 100	HPSE	heparanase
SFRP1	secreted frizzled related protein 1	IL15	interleukin 15
TWIST1	twist family bHLH transcription factor 1	IL6ST	interleukin 6 signal transducer
SOD3	suppressor of cytokine signaling 3	IL-6R	interleukin 6 receptor
TACSTD2	tumor-associated calcium signal transducer 2	IL-6	interleukin 6
FAIM2	Fas apoptotic inhibitory molecule 2	STAT5A	signal transducer and activator of transcription 5A
ABI3BP	ABI family member 3 binding protein	SLIT2	slit guidance ligand 2
NFKBIA	NFKB inhibitor alpha	SCIN	scinderin
DKK1	dickkopf WNT signaling pathway inhibitor 1	SEPT4	septin 4
JUNB	JunB proto-oncogene, AP-1 transcription factor subunit	TP63	tumor protein p63
EFEMP1	EGF containing fibulin like extracellular matrix protein 1	TLR2	toll like receptor 2
PTGS2	prostaglandin-endoperoxide synthase 2	TWIST2	twist family bHLH transcription factor 2
SOCS3	superoxide dismutase 3, extracellular	MIR155HG	MIR155 host gene
TLR3	toll like receptor 3	MAP3K5	mitogen-activated protein kinase kinase kinase 5
VCAM1	vascular cell adhesion molecule 1	NDP	NDP, norrin cystine knot growth factor
ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2	PGF	placental growth factor
PPARG	peroxisome proliferator activated receptor gamma	PPARA	Peroxisome proliferator-activated receptor alpha
HHEX	hematopoietically expressed homeobox	RARB	retinoic acid receptor beta
ACSL5	acyl-CoA synthetase long-chain family member 5		

Supplementary Table 4.2 List of Downregulated genes for Fluidigm validation

Gene symbol	Gene Description	Gene symbol	Gene Description
CTGF	connective tissue growth factor	EDNRB	endothelin receptor type B
COL1A1	collagen type I alpha 1 chain	MET	MET proto-oncogene, receptor tyrosine kinase
CTNNBIP1	catenin beta interacting protein 1	VCAN	versican
ROS1	ROS proto-oncogene 1, receptor tyrosine kinase	ARHGAP22	Rho GTPase activating protein 22
WNT11	Wnt family member 11	CDK6	cyclin dependent kinase 6
GLI1	GLI family zinc finger 1	CCIN	calicin
MMP10	matrix metallopeptidase 10	CDH3	cadherin 3
TP53I3	tumor protein p53 inducible protein 3	FZD7	frizzled class receptor 7
TGFB2	transforming growth factor beta 2	GDF6	growth differentiation factor 6
BAMBI	BMP and activin membrane bound inhibitor	LOX	lysyl oxidase
WNT5A	Wnt family member 5A	LRP4	LDL receptor related protein 4
TNFRSF12A	TNF receptor superfamily member 12A	MECOM	MDS1 and EVI1 complex locus
CDKN2B	cyclin dependent kinase inhibitor 2B	SCX	scleraxis bHLH transcription factor
SNCAIP	synuclein alpha interacting protein	SOX4	SRY-box 4
PDGFC	platelet derived growth factor C	PDPN	podoplanin
WNT2	Wnt family member 2	GREM1	gremlin 1, DAN family BMP antagonist
PRICKLE1	prickle planar cell polarity protein 1	NDRG4	NDRG family member 4
ADCY2	adenylate cyclase 2	TGIF2	TGFB induced factor homeobox 2
INHBA	inhibin beta A subunit	TGFB2	transforming growth factor beta 2
ACVR1C	activin A receptor type 1C	THBS1	thrombospondin 1
LEF1	lymphoid enhancer binding factor 1	ACTA2	actin, alpha 2, smooth muscle, aorta
	Housekeeping gene		
	ACTB	actin beta	

Supplementary Table 4.3 Comparative analysis of differentially expressed fibroblast genes common between this study and that of Barragan et al. 2016.

Gene Description	Gene symbol	Fold change Our study		Barragan et al. 2016, Fold Change in eMSC vs eSF	
				FACS sorted	Late culture
adenylate cyclase 2 (brain)	ADCY2	-1.09	Down	Down	NS
calbindin 2	CALB2	0.02	NS	Down	NS
carboxymethylenebutenolidase	CMBL	1.01	UP	Down	NS
calsyntenin 2	CLSTN2	-3.05	Down	Down	NS
	CD24	-3.87	Down	Down	Down
deiodinase, iodothyronine, type II	DIO2	-2.47	Down	Down	NS
DEAD (Asp-Glu-Ala-Asp) box polypeptide 60-like	DDX60L	0.32	NS	Down	NS
fibroblast growth factor 7	FGF7	1.66	UP	Down	NS
fibroblast growth factor 9	FGF9	-0.50	NS	Down	NS
fibroblast growth factor 10	FGF10	-1.57	Down	Down	Down
Fanconi anemia, complementation	FANCL	0.41	NS	Down	NS
gap junction protein, alpha 1, 43kDa	GJA1	-0.04	NS	Down	NS
hedgehog interacting protein	HHIP	-2.34	Down	Down	Down
integrin, beta-like 1 (with EGF-like	ITGBL1	0.46	NS	Down	NS
insulin-like growth factor 1 (somatomedin C)	IGF1	-1.53	Down	Down	Down
insulin-like growth factor 2	IGF2	-2.18	Down	Down	NS
phospholipase A2 receptor 1, 180kDa	JAZF1	0.17	NS	Down	NS
lysyl oxidase	LOX	-1.89	Down	Down	Up
leucine rich repeat containing 17	LRRC17	-2.85	Down	Down	NS
lymphocyte cytosolic protein 1 (L-plastin)	LCP1	0.99	NS	Down	NS
leucine carboxyl methyltransferase 1	LCMT1	0.01	NS	Down	NS
melanoma associated antigen	MUM1L1	-0.85	NS	Down	NS
matrix metallopeptidase 10 (stromelysin 2)	MMP10	-4.80	Down	Down	Up
microfibrillar-associated protein 4	MFAP4	0.33	NS	Down	Up
membrane metalloendopeptidase	MME	1.01	UP	Down	Up
5'-nucleotidase, ecto (CD73)	NT5E	0.65	NS	Down	NS
podoplanin	PDPN	-1.41	Down	Down	NS
platelet derived growth factor C	PDGFC	-1.83	Down	Down	Up
pregnancy-associated plasma protein A	PAPPA	-0.75	NS	Down	NS
platelet derived growth factor D	PDGFD	-0.09	NS	Down	NS
roundabout, axon guidance receptor, homolog 2 (Drosophila)	ROBO2	-1.19	Down	Down	NS
secreted frizzled-related protein 4	SFRP4	-1.69	Down	Down	Down
synuclein, alpha interacting protein	SNCAIP	-1.95	Down	Down	NS
solute carrier family 27 member 6	SLC27A6	-1.48	Down	Down	Down
transmembrane protein 45A	TMEM45A	-0.08	NS	Down	NS
wingless-type MMTV integration site family member 2	WNT2	-2.32	Down	Down	NS
wingless-type MMTV integration site family, member 5A	WNT5A	-1.96	Down	Down	Up

Chapter 5

Effect of A83-01 on mesenchymal stem cells from different tissues

Table of Contents

Declaration

Monash University

Declaration for Thesis Chapter 5

In the case of Chapter 5, my contribution to the work involved the following:

Name	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
Shanti Gurung	Responsible for experimental design, performing the experiments, collecting all the data, analysing, interpreting and preparing figures and table, and manuscript writing	90%
Daniela Ulrich	Provided pmMSCs from her previous studies	N
Tommy Supit	Provided two pMSCs from his previous studies	N
Jerome Werkmeister	Study design, manuscript revision and approved the final manuscript.	N
Caroline Gargett	Study design, manuscript revision and approved the final manuscript.	N

Student signature:  Date: 28/06/2017

Introduction

Mesenchymal stem/stromal cells (MSCs) are a rare population of cells that maintain homeostasis of the tissues in which they reside (Caplan, 1991, Bianco, 2014). MSCs self-renew and differentiate into cells of mesodermal lineages; osteoblasts, adipocytes, chondrocytes and myoblasts (Pittenger et al., 1999). Recently, MSCs were demonstrated to have additional properties including immunomodulation, exerted in a paracrine manner by their secretory function, adding to the repertoire of MSC actions. They are being used in pre-clinical and clinical studies for the treatment of immune-related disorders, for example, graft-versus-host diseases (GVHD) (Ringden et al., 2006, Le Blanc et al., 2008) and tissue regenerative ailments for repair of bone and cartilage defects (Goldberg et al., 2017).

The therapeutic properties of MSCs have been shown whether used from allogeneic (allo-) or autologous (auto-) sources. MSCs secrete soluble factors through which they modulate both the innate and specific immune systems. They express a low level of the major histocompatibility complex (MHC) class I surface molecules and lack expression of MHC class II, as well as the costimulatory molecules CD80, CD86 or CD40 (Menard et al., 2013). However, there is still concern about the possibility of acute anti-donor immune responses against infused MSCs following repeated allo-MSCs administration (Eliopoulos et al., 2005, Huang et al., 2010, Griffin et al., 2013, Joswig et al., 2017). In fact anti-donor antibodies have been detected following allo-MSCs administration leading to worsening of graft rejection (Eliopoulos et al., 2005, Huang et al., 2010). Likewise, there are concerns regarding allo-MSCs differentiating *in vivo* and becoming more immunogenic, thereby activating the host immune system

(Huang et al., 2010). From clinical trial outcomes to date, there is still no consensus regarding the advantages of using auto-MSCs or allo-MSCs. However, for business models or a pragmatic approach, it would be preferable to use cultured-cryopreserved allogeneic cells as an “off-the-shelf” therapeutic agent, avoiding the need for cell harvesting, isolation and culture delay before treatment (Hare et al., 2012). Nonetheless, the exciting discovery of MSCs in almost all tissues of the body has reinvigorated the discussion on using MSCs as an autologous cell source (Lv et al., 2014).

First recognised as colony-forming unit fibroblasts (CFU-F) in bone marrow in 1970s, MSCs have since been identified in other tissues such as adipose tissue (Zuk et al., 2002), umbilical cord blood (Romanov et al., 2003), placenta (In 't Anker et al., 2004), menstrual blood (Hida et al., 2008) and endometrium (pre and post-menopausal women) (Chan et al., 2004, da Silva Meirelles et al., 2006, Schwab and Gargett, 2007, Ulrich et al., 2014). Women have the option of sourcing MSCs from their own tissues as an autologous source of reparative cells. Various surface markers that can isolate highly clonogenic MSCs from tissues have now been identified (Lv et al., 2014, Darzi et al., 2016). SUSD2 is a recently identified surface antigen that recognises highly clonogenic bone marrow MSCs (bmMSCs) (Buhring et al., 2007, Sivasubramaniyan et al., 2013). Recently, SUSD2 was also identified as a single marker capable of purifying MSCs from pre- and post-menopausal endometrium (eMSCs) (Masuda et al., 2012, Ulrich et al., 2014). Menstrual blood is shed body waste which contains fragments of the functional layer of the endometrium where eMSCs reside. Similarly, decidua basalis, the maternal part of the placenta is also a part of the basal layer of the endometrium **(Figure 1.5)**. Therefore, the specific eMSC surface markers can be

used to isolate menstrual blood MSCs (mbMSCs) and placental MSCs (pMSCs). Although the International Society for Cellular Therapy (ISCT) guidelines indicate CD34 as a negative marker for MSCs, this has been recently challenged (Lin et al., 2012, Bourin et al., 2013). There are reports of multipotent perivascular progenitor populations in human $CD34^+$ cells from bone marrow (Simmons and Torok-Storb, 1991, Akiyama et al., 2012), adipose tissue (Bourin et al., 2013, Busser et al., 2015) and skeletal muscle (satellite cells) (Pasut et al., 2012). Recently, ISCT and the International Federation for Adipose Therapeutics and Science (IFATS) published a joint statement defining CD34 as a marker of adipose tissue MSCs (adMSCs) (Bourin et al., 2013).

Although autologous MSCs may be the preferred choice for cell-based therapies, it is well known that MSC numbers in tissues not only decrease with increasing age but also the quality of MSCs decreases (Choudhery et al., 2014). Furthermore, the extensive culturing required to generate large number of MSCs required for clinical use is detrimental to the quality of cells due to spontaneous differentiation and increased senescence of the cells. In chapter 2 we showed that the small molecule A83-01, an Alk4/5/7 inhibitor (1 μM) significantly improved the stemness of culture expanded pre-menopausal eMSCs by promoting proliferation and preventing replicative senescence and apoptosis (Gurung et al., 2015). In this study, we aim to investigate if A83-01 has a similar effect in promoting proliferation, and blocking apoptosis and senescence of MSCs from the post-menopausal endometrium, placenta, menstrual blood, bone marrow and adipose tissue.

5.1 Materials and Methods

5.1.1 Human tissue samples

Post-menopausal MSCs were obtained from a previous study in our laboratory (Ulrich et al., 2014). The post-menopausal women had been given estrogen replacement therapy (Progynova 2mg daily for 6-8 weeks) to regenerate their endometrium in order to obtain higher cell yields. Menstrual blood samples were collected from healthy women who were <40 years and not taking any hormones. Placentas were collected from women undergoing elective caesarean section at term pregnancy. Adipose tissues were obtained from healthy patients who were undergoing abdominal liposuction for cosmetic purpose (Ethics # CF15/379-2015000185). All human tissues were collected following ethical approval from Monash Health and Monash University Human Research Ethics Committees. Bone marrow stromal cells from healthy donors were kindly donated by A/Prof Marian Sturm from Centre for Cell Therapy and Regenerative Medicine, University of Western Australia. All patients gave written informed consent. Six samples were collected for each tissue type for this comparative study.

5.1.2 Isolation of MSCs

Two sets of surface markers, SUSD2 and CD34 were used to isolate MSCs with magnetic beads, $CD45^{-}CD31^{-}CD34^{+}$ for ad tissue MSCs and $SUSD^{+}$ve for MSCs from post-menopausal endometrium, placenta, menstrual blood and bone marrow cells.

5.1.2.1 Post-menopausal MSCs

Cryopreserved passage 1 (P1) pmMSCs enriched with SUSD2 were obtained from frozen stocks stored from a previous study (Ulrich et al., 2014). The cells were thawed quickly at 37ºC in a water bath and diluted slowly with 10 ml 5% heat-inactivated newborn calf serum in DMEM (bench medium) (BM). The cells were pelleted and washed again with BM and viable cells counted in haemocytometer using trypan blue exclusion.

5.1.2.2 Menstrual blood, Placental and Bone marrow MSCs

Menstrual blood was collected in a menstrual cup on the second day of the menstrual cycle and transferred to a 50 ml tube. It was diluted with equal volume of BM and manually mixed gently with pasture pipette for 2-3 minutes without creating any bubbles to separate any mucous, blood clots and clumps. Then it was filtered through 40 μm sieve (BD Biosciences). The filtrate was centrifuged for 5 minutes at 1100 rpm 4ºC. The supernatant was removed and the cell pellet was resuspended in 5 ml of BM in a 5 ml tube.

Freshly collected placentas were used in this study except for two placental MSCs, which were sourced from a previous study. Decidual placental tissues were collected 5 mm deep from the maternal surface of the central cotyledon. The tissues were mechanically minced into 1 mm pieces and digested with 0.5% collagenase type I and 40 μg/ml deoxyribonuclease type I (Worthington Biochemical Corporation) in 10% fetal calf serum DMEM medium (SM) for 60 minutes in a humidified incubator at 37°C on a rotating MACSmix (Miltenyi Biotech). The tissue digest was filtered through a 40 μm

cell strainer (BD Biosciences) to separate any undigested tissues. The filtrate was centrifuged for 5 minutes at 1100 rpm at 4^{o}C. The supernatant was removed and the cell pellet was resuspended in 5 ml of BM in a 5 ml tube.

Red blood cells (RBCs) were subsequently removed from the menstrual and placental filtrate by density gradient centrifugation above 3 ml of Ficoll-Paque (GE healthcare Bio-science) at 1500 rpm for 15 minutes at room temperature (RT) without braking. The cellular stromal interphase was collected, diluted with 5 ml BM and centrifuged to collect the stromal cells. The supernatant was removed and cell counts were performed using a haemocytometer.

Bone marrow stromal cells had previously been selected as plastic adherent cells and were at passage 2 and 3 when we received them in cryovials which were stored in liquid nitrogen until required. The cells were thawed quickly at 37^{o}C in a water bath and washed with 10 ml BM. They were pelleted and washed again with BM and cells counted. The cells were further purified as SUSD2^{+} cells by magnetic bead sorting (Masuda et al., 2012) before culturing them further.

5.1.2.2.1 Magnetic Bead Sorting

The stromal cell pellet was incubated in 100 μl Phycoerythrin (PE)-conjugated anti-human SUSD2 (10 μg/ml, BioLegend)) in 0.5% FCS/PBS (bead medium) for an hour in the dark on ice. The cell suspension was washed with 2 ml bead medium and further incubated with anti-PE magnetic-activated cell sorting microbeads (Miltenyi Biotec) for one hour in the dark on ice. The conjugated cell pellet was washed, resuspended in 500 μl bead medium and applied to a Miltenyi column (Miltenyi Biotec, #130-042-201)

in a magnetic field. The column was washed three times with 500 μl bead medium without creating bubbles. The separated cells, containing the $SUSD2^+$ MSCs in the column were eluted in 1 ml bead medium and the cells counted. Then they were cultured in 5 ml of SM in a T25 flask in the $5\%O_2/5\%CO_2/90\%N_2$ (trigas) incubator.

5.1.2.3 Adipose Tissue MSCs

200 ml of adipose tissue was collected. The tissue was washed thoroughly twice with PBS. Then the tissue was digested with 200 ml collagenase II (Worthington 330 U/mg dry weight, 1 mg/ml in PBS) in a shaking water bath for 1 hour at 37ºC. The digested tissue was transferred to 50 ml tubes for centrifugation at 800*g* for 10 min at RT. Any floating undigested tissue, fatty oily layer and supernatant were removed leaving the stromal vascular fraction (SVF). The SVF was washed with BM and passed through 40 μm cell strainer. RBCs were removed similarly with Ficoll-Paque as above at 300g for 30 minutes at RT without braking. The cellular interphase was collected, washed and cells were counted in trypan blue using a haemocytometer.

$CD45^+$ leucocytes and $CD31^+$ endothelial cells were negatively selected from the stromal fraction. Cells were incubated with CD45 Dynabeads (4 beads/cell, Invitrogen) in a rotator for 30 minutes at 4ºC and applied on the magnetic stand for 2 minutes. Then the unbound cell suspension was collected, washed and pelleted. Cells were resuspended in 60 μl of 2% FBS/PBS and 20 μl FcR blocker (Miltenyi Biotec) then incubated with 20 μl CD31 Microbeads (Miltenyi Biotec) at 4ºC for 15 minutes. The pellet was washed and passed through a column in a magnetic field. The filtrate was passed through the column once more to collect the pure $CD31^-$ population. This $CD45^-/CD31^-$ cell population was washed and pelleted at 1100 rpm/5 min 4ºC. Finally,

the cells were incubated with 10 μl CD34-PE antibody (BD Pharmingen) in 100 μl 2% FBS/PBS followed by 20 μl anti-PE beads (Miltenyi Biotec) and 80 μl 2% FBS/PBS for 30 min each at 4ºC in dark with a wash in between. Then 500 μl of bead medium was added and passed through a column in a magnetic field. The column was washed twice with 500 μl bead medium and the RBC-free $CD45^{-}CD31^{-}CD34^{+}$ adMSCs were collected by eluting the column with 1 ml of bead medium and cells were then counted.

5.1.3 Propagation of MSCs from different tissues

MSCs from different tissues were cultured in SM in a trigas humidified incubator with media change every 2-3 days. Once ~70 % confluent, media was changed stepwise to 5% and 1% FCS medium and then to SFM (serum-free medium with 10 ng/ml each of FGF2 and EGF) every 24 hours. Following the second passage, cells were seeded at 5000 cells/cm^2 in fibronectin-coated plates with media changed every 2-3 days. Since bmMSCs could not be cultured in our in-house SFM, they were cultured in αMEM with 10% FCS and 10 ng/ml FGF2 in uncoated plates. At P6, MSCs from each tissue were divided into two groups, which were treated with either 1 μM A83-01 or 0.01% DMSO vehicle control for 7 days with media changed every 48 hours.

5.1.4 Immunophenotyping MSCs

Following culture in control or A83-01 medium for 7 days, MSCs were trypsinised with TrypLE™ (Life Technologies) and resuspended at 10^5 cells/tube. The cells were washed with BM and incubated with PE-, APC- or FITC-conjugated primary antibodies and matched isotype controls in BM for 30 minutes in the dark on ice. Primary antibodies used were CD146, CD73, CD105; PE-conjugated antibodies were

CD140b, CD34, CD31, and CD271 while APC-conjugated antibodies were SUSD2, CD45, CD44 and CD90. Details of antibodies used are given in **Table 5.1**. Isotype control antibodies at the same concentration as the primary antibody were included for each run and were used to set the electronic negative control gate to <1% positive cells on the flow cytometer. Finally, cells were washed with BM and fixed with 4% paraformaldehyde (PFA) in 2% FBS/PBS. Samples were analysed using a MoFlo Flow Cytometer (Beckman Coulter).

Table 5.1 Antibodies used for flow cytometry

Primary antibodies					Secondary antibodies		
Antigen	Clone	Isotype	Conc	Supplier		Clone	Supplier
CD73	AD2	Mouse IgG2a	10 μl/ml	BD Pharmingen	PE rat anti-mouse IgG2a (5 μg/ml)	A85-1	BD Pharmingen
CD105	266	Mouse IgG1	10 μg/ml	BD Pharmingen	FITC rat anti-mouse IgG2a (5 μg/ml)	R19-15	BD Pharmingen
CD146	CC9	Mouse IgG2a	1:1	A kind gift from Prof David Haylock CSIRO			
CD140b	PR7212	Mouse IgG1	25 μg/ml	R&D systems	-		
CD34	581	Mouse IgG1	200 μl/ml	BD Pharmingen	-		
CD31	M89D3	Mouse IgG2a	10 μg/ml	BD Pharmingen	-		
CD271	ME20.4-1.H4	Mouse IgG1	100 μl/ml	Miltenyi Biotec	-		
SUSD2	W5C5	Mouse IgG1	50 μg/ml	Biolegend	-		
CD45	HI30	Mouse IgG1	10 μg/ml	Invitrogen	-		
CD44	IM7	Rat IgG2b	10 μg/ml	eBioscience	-		
CD90	5E10	Mouse IgG1	25 μg/ml	BD Pharmingen	-		

5.1.5 Colony forming assay

Following treatment with and without A83-01, cells were seeded at 50-100 cells/cm^2 in a fibronectin-coated culture plate in SFM for three weeks in a trigas humidified incubator (5%O_2/5%CO_2/90%N_2) with weekly media changes. The clones on the plates were fixed in 4% PFA for 10 minutes. They were stained with haematoxylin (Amber Scientific) for 5 minutes after washing with PBS and transferred to Scott's tap water to develop blue colour. Colonies with cells more than 50 cells were considered as a colony (Chan et al., 2004). Colony efficiency was calculated by counting the number of colonies and dividing by the total number of cells seeded and calculating the percentage (Chan et al., 2004).

5.1.6 Cell cycle and apoptosis analyses by flow cytometry

P6 MSCs treated with and without A83-01 were trypsinised, pelleted and fixed in ice-cold 70% ethanol for 30 minutes at 4°C. The cells were centrifuged at 2000 rpm for 5 minutes at 4°C and washed with PBS. The pelleted cells were incubated with 50 μl RNase (100 μg/ml, Sigma) at RT for 15 min to remove any RNA contamination. 200 μl propidium iodide (PI) (50 μg/ml, Sigma) was added and samples analysed immediately by flow cytometry using BD FACS CantoTM II using linear mode for PI. The data were analysed using Flow Jo 7.6.3 as previously reported (Gurung et al., 2015).

To assess apoptosis, P6 MSCs treated with and without A83-01 were trypsinised and stained with Annexin V-APC/PI kit following the manufacturer's protocol (#88–8007-72, eBioscience). The trypsinised cells were pelleted and washed in 2% FBS/PBS

followed by washing with binding buffer at 1100 rpm for 5 min at 4^{o}C. Then the pellet was incubated with 5 μl Annexin-V-APC in 100 μl binding buffer 10 minutes at RT protected from light. This was followed by washing with binding buffer and resuspending with 5 μl of PI in 200 μl binding buffer. Events were acquired immediately by flow cytometry using BD FACS CantoTM II and analysed with Flow Jo 7.6.3 (Gurung et al., 2015).

5.1.7 Polymerase chain reaction (PCR) for SRY gene.

This protocol was performed with passage 4 placental MSCs. The cells were digested with lysis buffer (100 mM Tris pH 7.4, 5 mM EDTA, 0.5% SDS and 200 mm NaCl) and proteinase K (100 μg/ml, #P2308, Sigma) at 50^{o}C for one hour in a shaker. The suspension was centrifuged at 14,000 rpm for 10 min at RT. The supernatant was collected and genomic DNA (gDNA) was precipitated with equal volume of isopropanol. This was followed by washing with 70% ethanol and drying any residue before resuspending in RNase-free water. Polymerase chain reaction (PCR) was carried out in a 20 μl volume consisting of 10 μl Mytaq (Bioline), 1 μl each of forward (5'-tcagcaagcagctgggatac-3') and reverse (5'-aactgcaattcttcggcagc-3') primers (10 μM SRY, Bioneer), gDNA and milliQ water. The reaction consisted of initial denaturation at 95^{o}C for 2 minutes, followed by 30 cycles of denaturation for 30 seconds, annealing at 55^{o}C and extension at 72^{o}C for one minute each. The PCR products were separated by 1.5% agarose gel electrophoresis. Concurrently, human eMSCs were used as female negative control while cells from male donor were positive control. PCR products were visualised by ChemiDoc XRS+ system (BioRad).

5.1.8 Immunofluorescence microscopy

Human term placental tissue from the central cotyledon was fixed in 4% PFA for 24 hours, washed with PBS and equilibrated with 30% sucrose at 4^{o}C for 24 hours. The tissue was then embedded in Optimal cutting temperature compound (OCT). 8 μm tissue sections were cut with a cryotome and washed with PBS and blocked by protein block (Dako, X0909) for one hour. This was followed by incubation with SUSD2-PE conjugated antibody (5 μg/ml, clone W5C5, Biolegend) in 2% FCS/PBS for 1 hour at RT in dark. The Isotype control IgG_1 antibody was used at the same concentration as a negative control. Nuclei were visualised following 5 minutes incubation with Hoechst 33258 (0.5 μg/ml, Molecular Probes). Images were visualised and photographed using a Nikon fluorescent microscope and analysed using ImageJ software (ImageJ/NIH) as described in Chapter 2.

5.1.9 Detection of cell senescence by β–galactosidase activity

Adipose tissue MSCs were cultured in SFM with and without A83-01 on fibronectin-coated coverslips as described above. Then the cells were fixed with 4% PFA for 10 minutes and washed with PBS. This was followed by staining with freshly prepared X-Gal (1 mg/ml in DMSO) staining reagent (5 mM $K_3[Fe(CN)_6]$, 5 mM $K_4[Fe(CN)_6]$, 2 mM $MgCl_2$, 150 mM NaCl) in sodium citrate buffer at pH 6 for 24 hours at 37°C in a non-humidified incubator. The slides were wrapped in foil to prevent drying during the incubation. Following this, the cells were washed twice with PBS and counterstained with nuclear fast red (Sigma-Aldrich, 0.1% w/v) for 10 minutes. Images were taken with 4X and 10X objective lens using the DP25 digital camera (Olympus).

5.1.10 Statistics

The data were not normally distributed using D'Agostino & Pearson normality test using GraphPad Prism 7. Therefore, nonparametric Wilcoxon matched-pairs tests was used to test for statistical significance between control and A83-01 treatment groups. Data are presented as mean ± standard error of mean. Differences were considered statistically significant at $p < 0.05$.

5.2 Results

5.2.1 A83-01 promotes MSC proliferation

In our previous study, we identified that the treatment of P6 pre-menopausal eMSCs with A83-01 promoted proliferation compared to the control (Gurung et al., 2015). MSCs from post-menopausal endometrium, term placenta and menstrual blood were isolated using SUSD2 antibody while CD34 antibody was used to isolate MSCs from adipose tissue. To examine the effect of A83-01 on MSCs from these sources, cultured MSCs were separated into two groups and treated with and without 1μM A83-01 for 7 days. Similar to the effect on pre-menopausal eMSCs, A83-01 significantly promoted proliferation of MSCs from the above-mentioned tissues compared to the control **(Figure 5.1)** ($p<0.05$). Bone marrow MSCs isolated with SUSD2 antibody at P2-P3 were cultured in 10% serum-containing medium with and without A83-01. However, their proliferation was not promoted in the presence of A83-01 **(Figure 5.1)**.

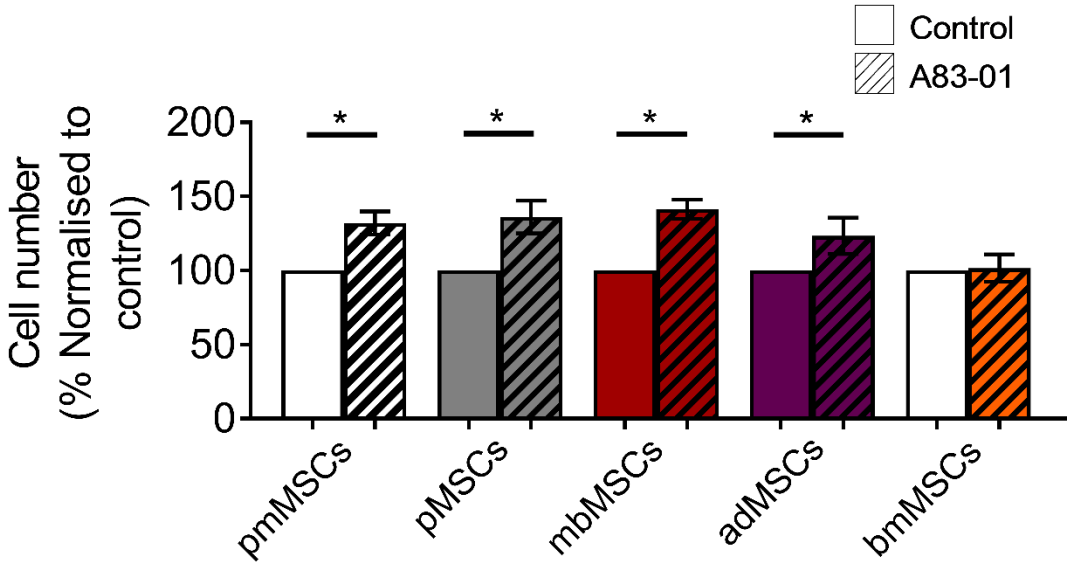

Figure 5.1 A83-01 promote MSC proliferation. Late cultured P6 MSCs from post-menopausal endometrium (pmMSCs), term placenta (pMSCs), menstrual blood (mbMSCs) and bone marrow (bmMSCs), and P4 MSCs from adipose tissue (adMSCs) were treated with and without A83-01 in 5%O_2/5%CO_2/90%N_2 for 7 days. The cell number was counted and normalised to vehicle control DMSO (set to 100%) and plotted as mean ± SEM for n=6 for each tissue type. *p<0.05.

5.2.2 Localisation and selection of maternal pMSCs

SUSD2$^+$ eMSCs reside in a perivascular region in both functional and basal layer of the endometrium (Schwab and Gargett, 2007, Masuda et al., 2012). We first looked for presence of perivascular SUSD2 positive cells in the middle cotyledon of decidua basalis because decidua basalis is derived from the basal layer of the endometrium. Indeed, SUSD2 positive cells are present around the maternal vessels in decidua basalis **(Figure 5.2A-B)**. No positive staining was detected in the chorionic villi and

any vessels towards the chorionic plate **(Figure 5.2C**), indicating specificity for maternal MSCs.

Of the six women who had donated term placenta, three had given birth to healthy boys and one a healthy girl (gender of babies from two donors were not known). Therefore, to confirm non-contamination of fetal cells in our four pMSC lines, I performed a PCR assay to detect the male-specific SRY gene in the pMSC-cultures. None of the pMSCs showed a positive band for SRY **(Figure 5.2E)** confirming our simple method allowed isolating and propagation of pure maternal origin pMSCs.

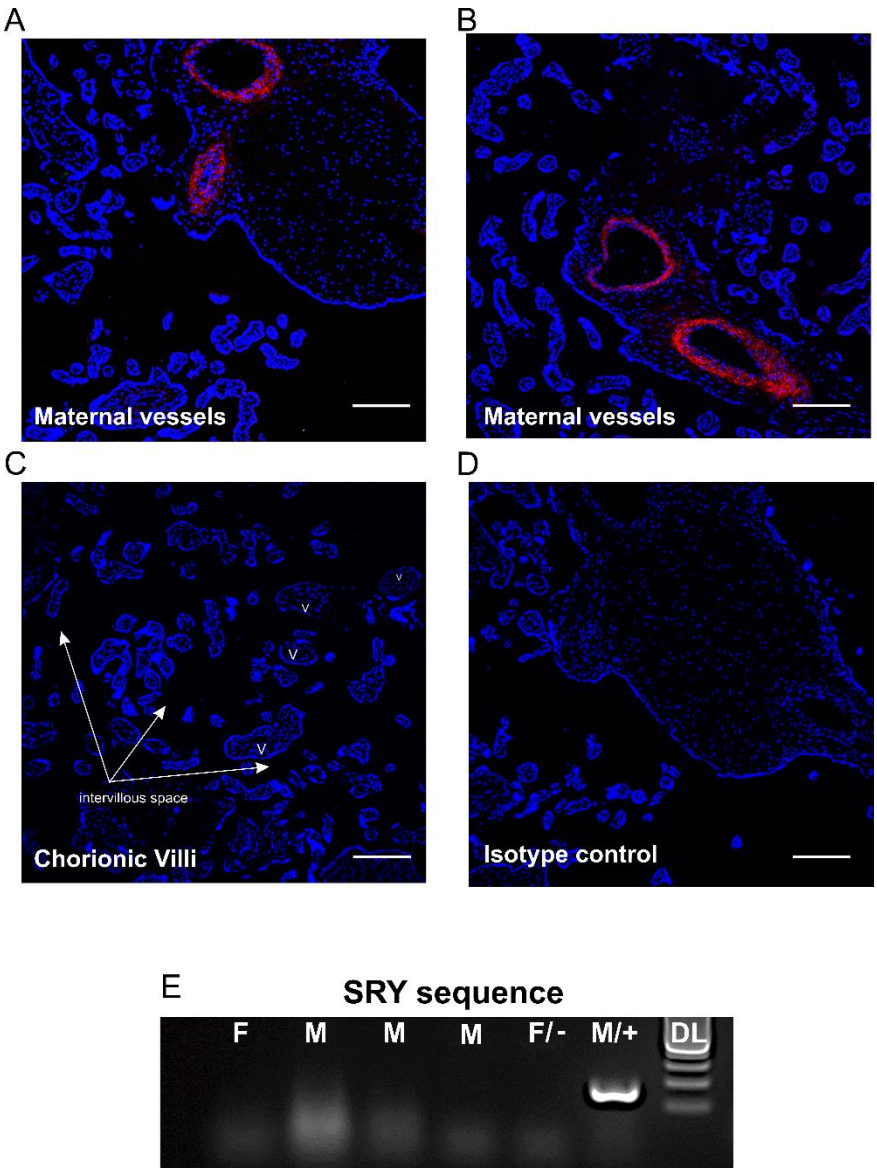

Figure 5.2 Localisation of SUSD2 MSCs in human term placenta. Middle cotyledon from human term placental sections were stained with SUSD2 antibody. Maternal vessels immunostained with SUSD2 (red) in a perivascular location **(A, B)** while there was no perivascular immunostaining in the chorionic villi. v, villi and showing inter villous space with white arrows **(C)**. Panel **D)** is the isotype control. Scale bars 200 μm. **E)** PCR gel image showing no bands in 4 pMSCs lines shown in the first four columns indicating absence of male fetal cells in the placental MSC cultures. F, donor delivered female baby, M, donor delivered male baby, F/-, female negative control cells, M/+, male positive control cells for SRY primers. DL, 100 bp DNA ladder.

5.2.3 Surface Phenotype of A83-01 treated MSC

We next examined the phenotype of MSCs treated with and without A83-01. Single colour flow cytometric analysis revealed all MSCs were >95% positive for CD90, one of the ISCT markers **(Figure 5.3, Figure 5.4)**. The percentage of MSCs positive for CD90 did not change in placental, menstrual blood, post-menopausal endometrium, bone marrow or adipose tissue MSCs following A83-01 treatment suggesting its ubiquitous presence in stromal cells, fibroblasts and MSC. However, there was a significant decrease in the percentage of cells expressing SUSD2, CD140b and CD146 upon extensive culture of all MSC types apart from adMSCs **(Figure 5.3, Figure 5.4)**. Following A83-01 treatment, % $SUSD2^+$ cells increased in MSCs from post-menopausal endometrium, placenta and menstrual blood. However, % $SUSD2^+$ cells did not increase following A83-01 treatment of bmMSCs. We observed that A83-01 increased the % SUSD2+ cells from 29% to 60% in three bmMSCs while there was a decrease in the other three, from 15% to 11% **(Supplementary Figure 5.1)**. Similar to pre-menopausal eMSCs, the percentage of $CD140b^+$ cells were increased in placental and menstrual blood MSCs following A83-01 treatment while there were no differences in the MSCs from pmMSCs and bmMSCs. The most significant change observed was in the percentage of CD146 positive cells for all MSC types. It was very low in cultured MSCs, except for bmMSC cultures. Compared to the others, bmMSCs had a higher percentage of CD146 positive cells **(Figure 5.3)**. However, in all MSC types, there was an overall trend towards a further decrease in $\%CD146^+$ cells following A83-01 treatment.

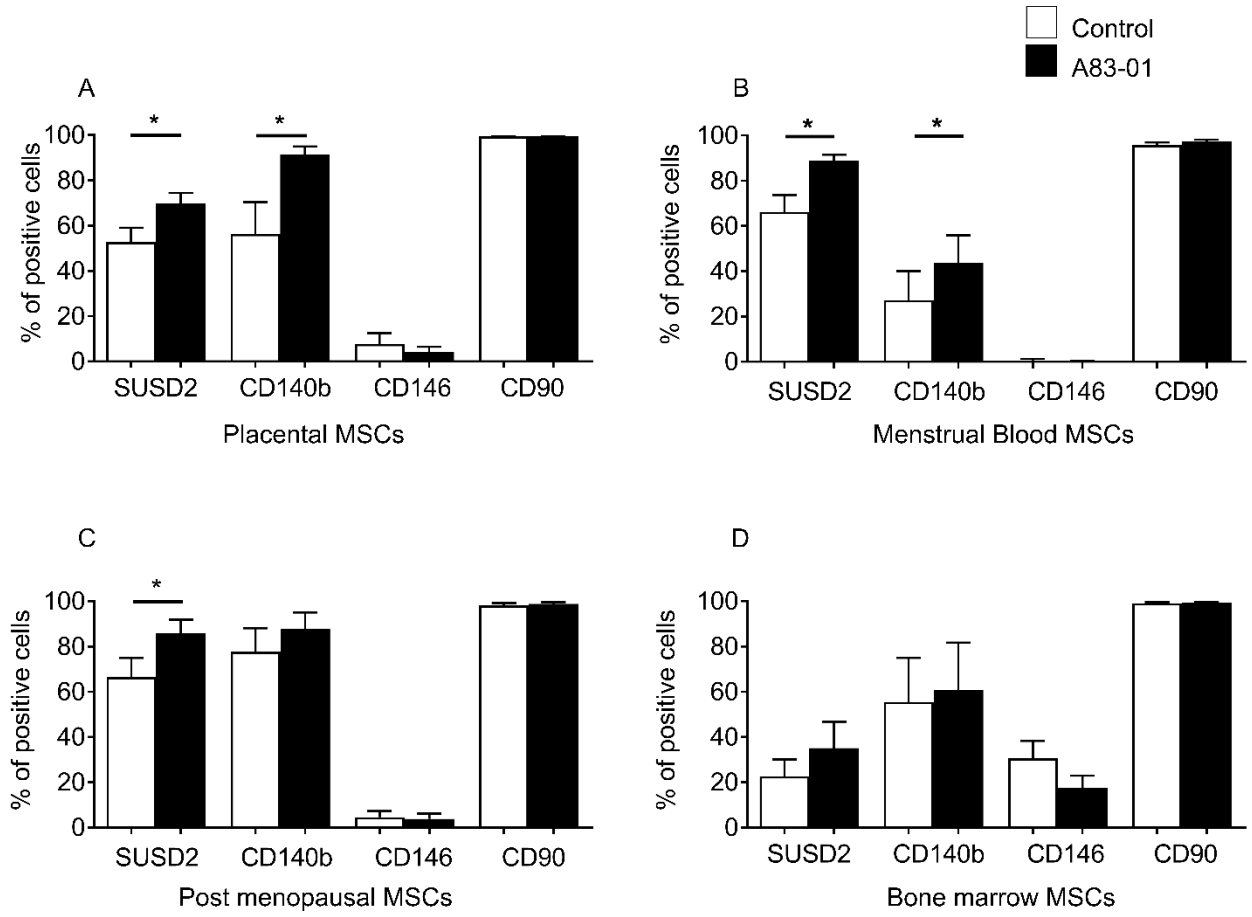

Figure 5.3 Phenotype of P6 MSCs cultured with and without A83-01 in 5%O_2. Percentage of positive cells for MSC surface markers cultured with 1 μM A83-01 (black bars) and 0.01% DMSO (white bars) for 7 days from **A)** Placental, **B)** Menstrual Blood, **C)** Post-menopausal and **D)** Bone marrow MSCs and assessed by single colour flow cytometry. Data are presented as mean ± SEM of n=6 for each tissue type. *p<0.05

A different isolation process was carried out for adipose tissue MSCs based on the surface profile of adipose tissue-derived MSCs from the literature (Bourin et al., 2013, Busser et al., 2015). First, we studied the adipose tissue SVF. Adipose tissue SVF consisted of 4. 7% SUSD2, 12.3% CD34, 8.3% CD31, 19.5% CD45 cells. In addition, 3.53% of cells were both CD31 and CD34 positive, while 0.75% were both CD34 and SUSD2 positive **(Figure 5.4A)**. Surface marker profiles of adMSCs were also assessed following culture of $CD31^{-}CD45^{-}CD34^{+}$ adMSCs at P0 and P3. The percentage of CD90 adMSCs was high (100 % at P0 and 99% at P3), like other MSCs and it did not change even at P4. Cultured $CD34^{+}$ adMSCs were negative for CD31, CD45, CD146 and CD271 surface markers. At P0 58.5% and 96.5% of adMSCs were positive for SUSD2 and CD140b surface markers, respectively, which decreased to 13% and 65.5% by P3 **(Figure 5.4B)**. Similarly, 66.9% of P0 cultured adMSCs were CD34 positive. Passaging had a negative effect on the surface profile of adMSCs and by P3 none were positive for CD34. At P4, 20.5% of adMSCs were positive for SUSD2, 80% for CD140b, 99.8% for CD90, 100% for CD44, 1% for CD34 and 0.8% for CD271 and there was no change in the percent positive cells for all these surface markers following treatment with A83-01 **(Figure 5.4C)**.

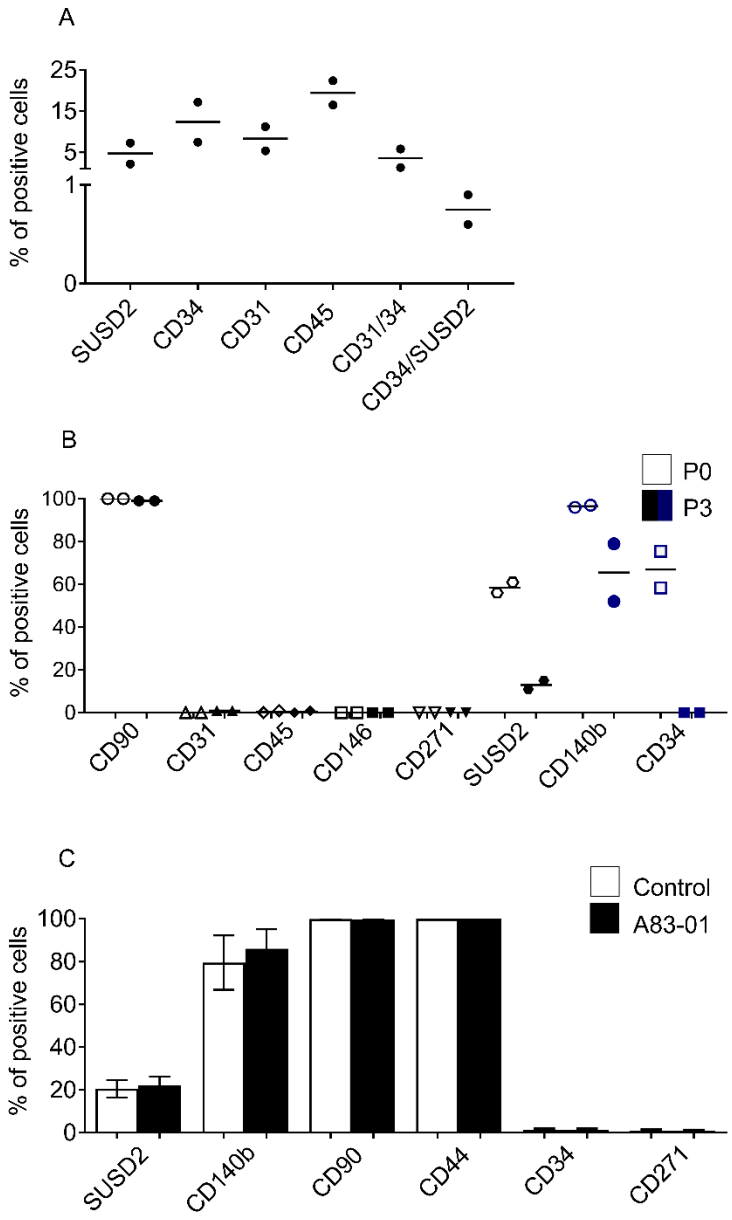

Figure 5.4 Phenotypic profile of freshly isolated adMSCs and cultured to various passages, and cultured with and without A83-01 at P4 in 5%O_2. A) Percentage of positive cells for surface markers from freshly isolated adipose tissue stromal vascular fraction by flow cytometry. Graphs show individual results of n=2 and the mean (bar). **B)** Surface markers of P0 (open pattern) and P3 (solid pattern) adMSCs by single colour flow cytometry. Graphs show individual results of n=2 and the mean (bar). **C)** Phenotypic profile of surface markers on P4 adMSCs cultured with 1 μM A83-01 (black bars) and control (white bars) for 7 days from adMSCs assessed by single colour flow cytometry. Data are presented as mean ± SEM of n=6.

5.2.4 Effect of A83-01 in MSC Colony Forming Ability

Colony-forming assay was used to assess the enrichment of MSCs in the A83-01-treated and vehicle control groups. There was a significant increase in the colony forming efficiency in pmMSCs treated with A83-01 (1.4% control and 2.6% A83-01 treated, $P<0.05$). Surprisingly, there were no differences in colony forming ability in the placental, menstrual blood and adipose tissue MSCs treated with and without the small molecule A83-01 **(Figure 5.5E)**.

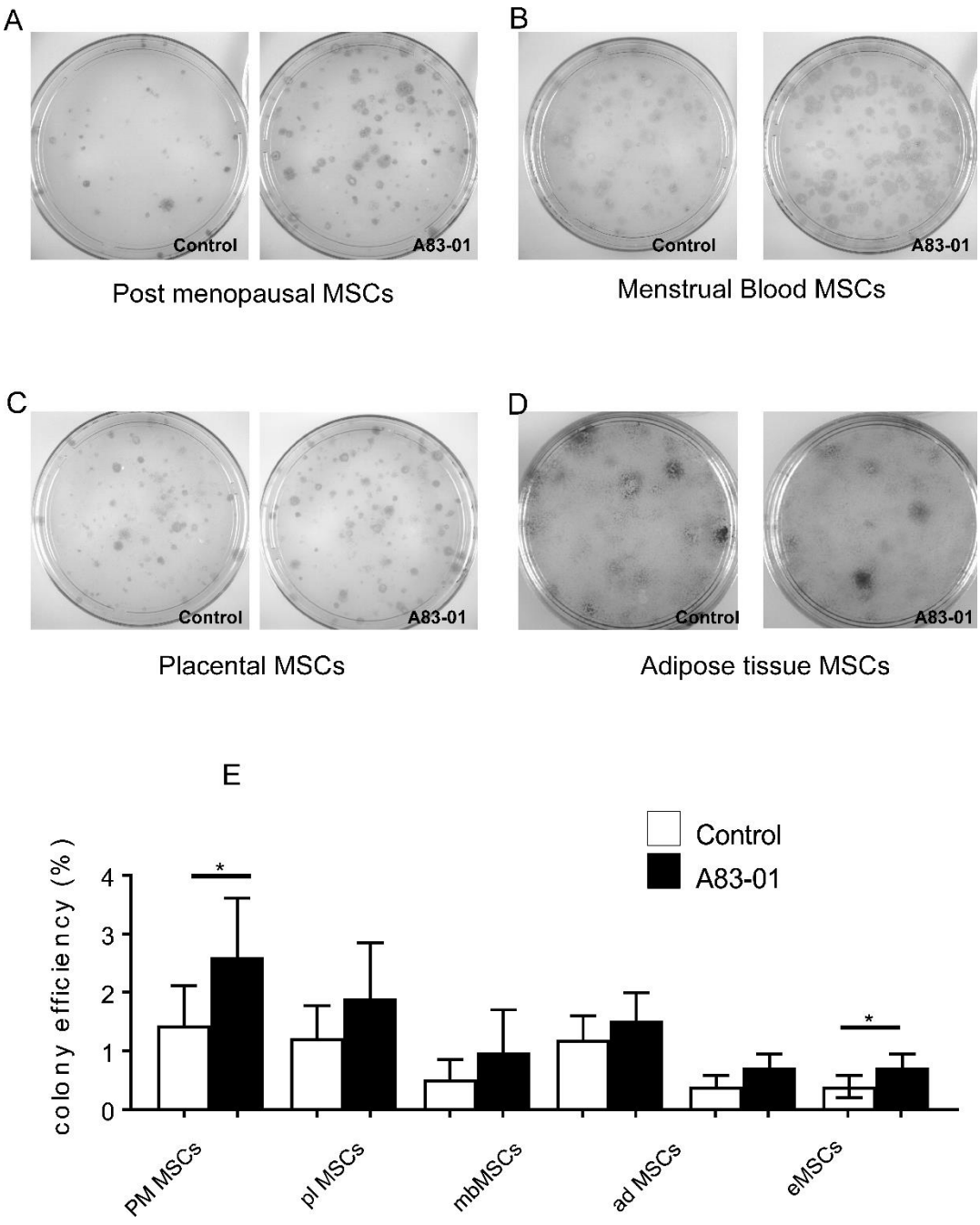

Figure 5.5 Colony-forming efficiency of MSCs treated with and without A83-01 in SFM. A-D) Representative culture plates of post-menopausal, menstrual blood, placental and adipose tissue MSCs seeded at clonal density. **E)** Graph showing colony forming efficiency of MSCs (including eMSCs for comparison) pretreated with and without A83-01 for 7 days followed by low-density clonal culture in SFM for 3 weeks. Data are mean ± SEM of n=6 different patient samples, *p<0.05.

5.2.5 Effect of A83-01 on MSC Cell Cycle and Apoptosis

To investigate the cell cycle profiles of MSCs treated with and without A83-01, I assessed PI-stained cells following 7 days of A83-01 treatment by flow cytometry to assess their cell cycle status. Treatment with A83-01 decreased the percentage of apoptotic cells (sub G1/G0 phase) in MSCs from post-menopausal endometrium and term placenta **(Figure 5.6A and C)**. There was also a significant increase in G2/M phase in the placental MSCs treated with A83-01. However, there was no change in any phases in mbMSCs **(Figure 5.6B)**. Similarly, there were no differences in any cell cycle phase in bmMSCs, although 63% of cells were in the sub G1/G0 phase in both A83-01-treated and control indicating apoptosis in a substantial proportion **(Figure 5.6D)**.

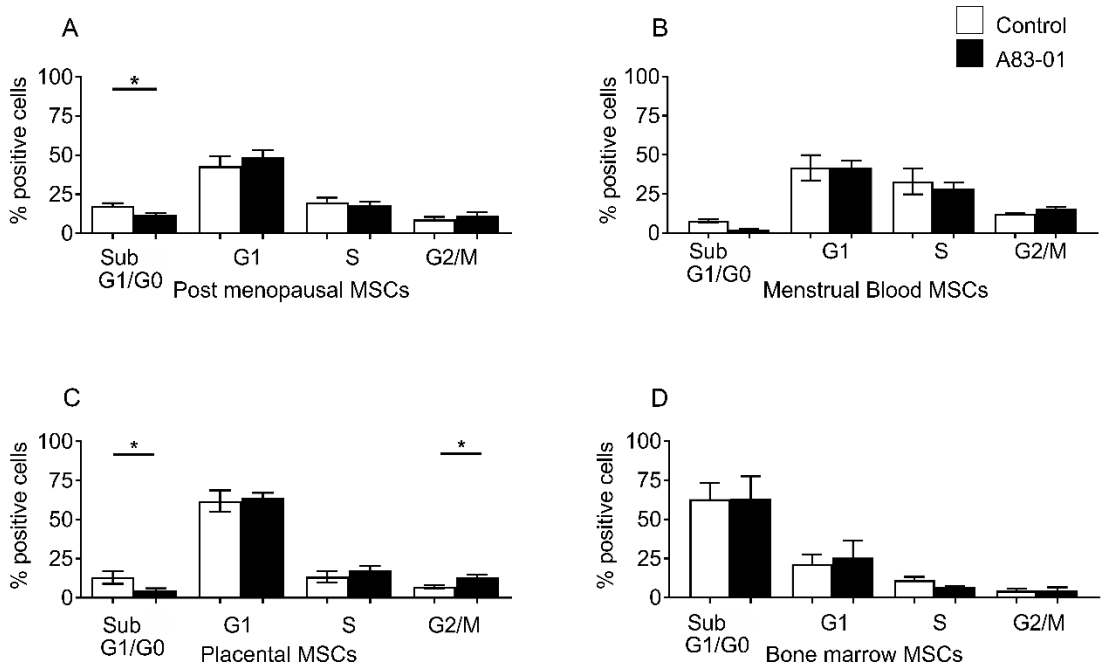

Figure 5.6 Flow cytometric analysis of the cell cycle in A83-01 treated and untreated pmMSCs, mbMSCs, pMSCs and bmMSCs. The Graph shows the percentage of cells in SubG1/G0, G1, S and G2/M phases of the cell cycle (while bar control; black bar A83-01). Data are presented as mean ± SEM of n=6 different patient samples, *p<0.05.

Flow cytometry analysis was also used to assess cell apoptosis by double staining with Annexin-V and propidium iodide. Live, early apoptotic, late apoptotic and necrotic cells in the cultures were quantified. Live cells comprised 88% and 67% of cells in pmMSC culture and pMSCs respectively **(Figure 5.7A** and **B)**. However, there were no significant differences in either the live cells or the apoptotic cells following A83-01 treatment **(Figure 5.7A** and **B)**. There was, however, a significant increase in live cells (89.7% in control, 94.2% in A83-01-treated, p< 0.05) and decrease in the late apoptotic cells (3.5% in control, 1% in A83-01-treated, P < 0.05) in mbMSCs treated with A83-01 compared with the control **(Figure 5.7C)**. Surprisingly 95% bmMSCs were live cells but there were no significant differences between the 2 groups **(Figure 5.7D)**.

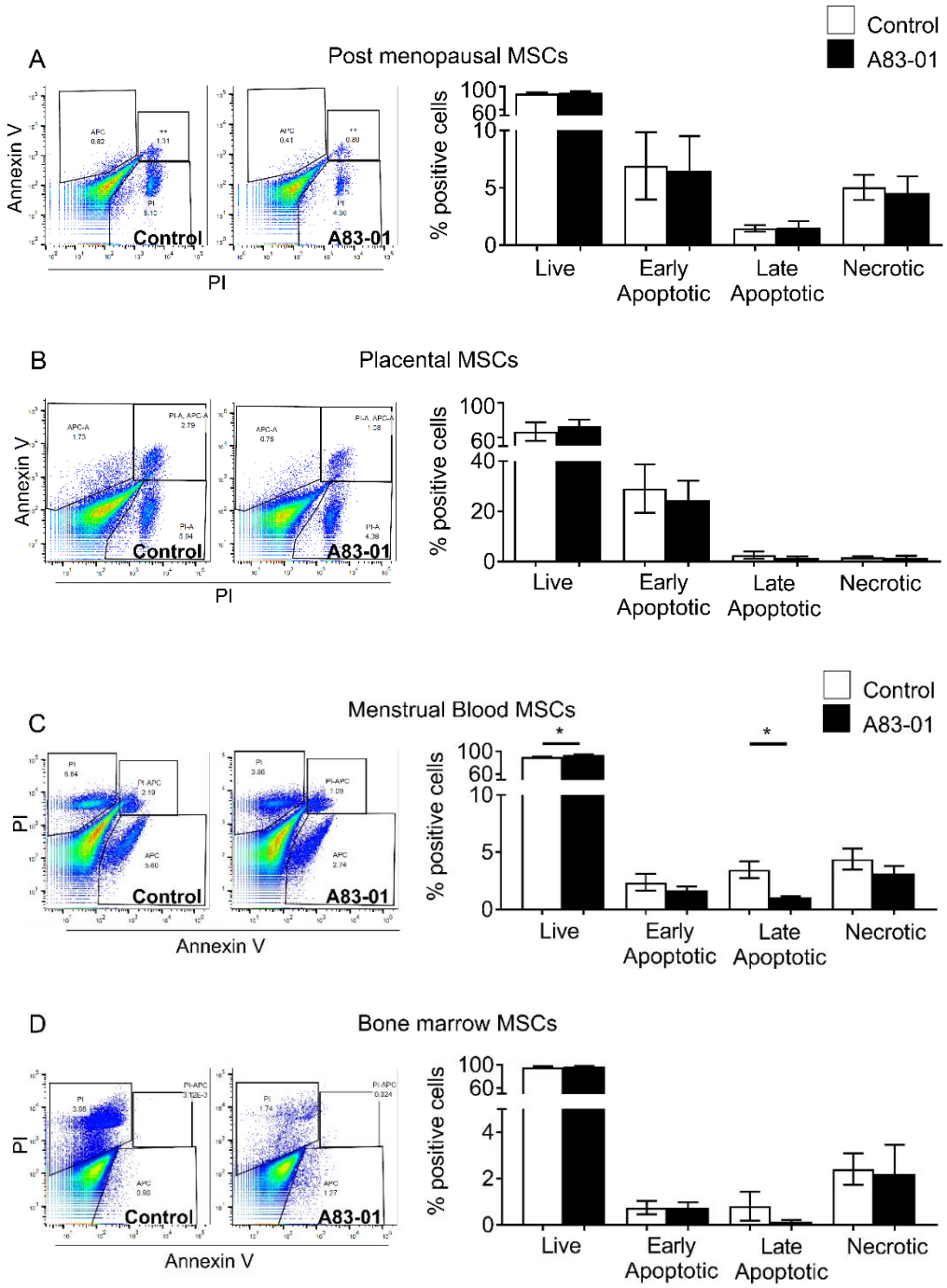

Figure 5.7 Effect of A83-01 on MSC apoptosis. The graphs show the percentage live, early and late apoptotic, and necrotic cells in A83-01-treated and untreated MSCs from **A)** pmMSCs, **B)** pMSCs, **C)** mbMSCs and **D)** bmMSCs (while bar control and black bar A83-01). Left panels show representative dot plots for control and A83-01 cells for each cell type. Data are presented as mean ± SEM of n=6 different patient samples, *p<0.05.

5.2.6 Effect of A83-01 on MSC senescence

Initially, the experimental design was to perform the treatment regime with A83-01 at passage 6 for the five MSC types, similar to the protocol used for pre-menopausal eMSCs. Since the adMSCs could not be cultured beyond P5, we performed the experiments at P4. Given the decreased propagation ability of adMSCs beyond P5, I stained for senescence-associated lysosomal β–galactosidase (SAβ-Gal) rather than undertaking the apoptosis and cell cycle assays, by incubating the cells with X-Gal reagent (Sigma) at pH6. As shown in **Figure 5.8**, P4 control adMSCs displayed blue staining, indicative of senescent cells, while the A83-01-treated cells showed a decrease in blue senescence cells.

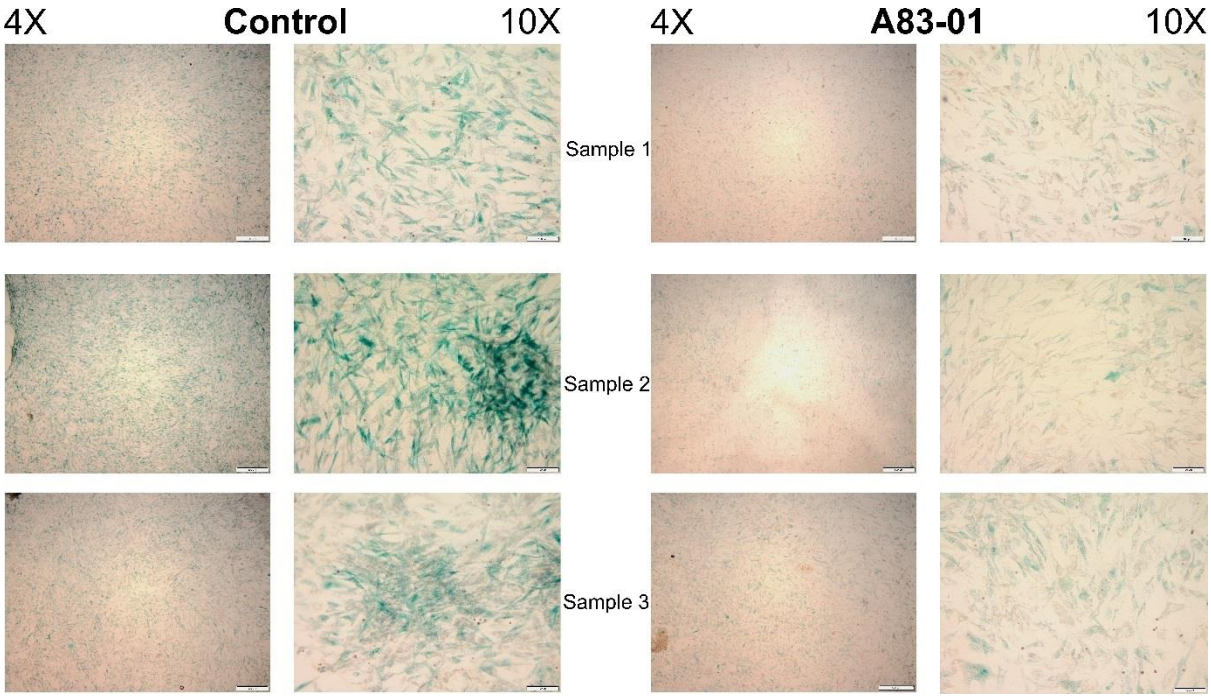

Figure 5.8 A83-01 reduces SAβ-Gal activity in P4 adMSCs. Representative images of three control and three A83-01-treated adMSCs. adMSCs undergo replicative senescence with increasing lysosomal β-galactosidase activity demonstrated by blue staining which was markedly decreased with A83-01 treatment.

5.3 Discussion

This study demonstrates that A83-01 promoted MSC proliferation and significantly increased the percentage of SUSD2 expressing MSCs especially in those derived from reproductive tissues; post-menopausal endometrium, placenta and menstrual blood. However, the effect of A83-01 on cell cycle status and apoptosis varied between the MSC types and were generally not consistent between the two assays used. A83-01 prevented apoptosis in reproductive tissue MSCs in at least one of the assays. adMSCs had limited ability to proliferate and exaggerated replicative senescence.

TGF-β is a common cytokine secreted by MSCs from bone marrow, adipose tissue and human endometrium (Baglio et al., 2015) (chapter 4). It is a potent inhibitor of cell proliferation for epithelial cells, keratinocytes, $Lgr5^+$ liver and intestinal epithelial stem cells, lymphocytes and chronic lymphocytic leukemia B cells (Pietenpol et al., 1990, Lotz et al., 1994, Xu et al., 2009, Sato et al., 2011, Huch et al., 2015). $Lgr5^+$ intestinal epithelial cells cultured in A83-01-containing medium had a significantly increased lifespan, colony forming efficiency and percentage of $Lgr5^+$ cells (Sato et al., 2011, Huch et al., 2015). A83-01 treatment also increased proliferation of MSCs from post-menopausal endometrium, menstrual blood, placenta and adipose tissue in a similar manner to pre-menopausal eMSCs (Gurung et al) (Chapter 2), indicating that endogenous synthesis of TGF-β had a negative effect on their growth during culture expansion in serum-free medium. However, this was not the case for bmMSCs. The concentration of A83-01 was optimised in serum-free medium to inhibit the autocrine TGF-β secreted by the cells, however, in this case the concentration may not have been enough to block autocrine as well the TGF-β in serum. In contrast, a significant increase in the colony forming efficiency was only evident in pmMSCs pre-treated with

A83-01. The effect of A83-01 appears to have variable efficacy in retaining undifferentiated MSC from different tissues.

In the present study, we chose one of the ISCT surface markers, CD90, as a representative MSC marker. As demonstrated in five different MSC types and in chapter 2, CD90 is a ubiquitous protein in stromal cells. CD90 does not change during MSC propagation and its presence does not reflect differences in morphology and functional ability (Busser et al., 2015, Li et al., 2015). SUSD2 is a recently identified surface marker for bone marrow MSCs. In addition, SUSD2 has now identified clonogenic MSCs from endometrium and other tissues, however, how its expression is regulated was not identified until our previous study where we showed that SUSD2 mRNA and protein is regulated through TGF-β/SMAD2 pathway (Gurung et al., 2015).Here, we showed that culture expansion of MSCs leads to a decrease in the percentage of SUSD2-expressing cells, particularly in bone marrow and adipose tissue MSC, confirming spontaneous differentiation upon culturing. The percentage of $SUSD2^+$ cells increased following A83-01 treatment of MSCs from reproductive tissues but not in adipose tissue and bone marrow. In the 6 bone marrow stromal cells we received, only 62% of the cells were viable following cell count with trypan blue. In addition, only 10% of these stromal cells were $SUSD2^+$ cells. The reduced numbers and metabolic state of our starting bone marrow stromal cells may have compromised the beneficial effect of A83-01 on bmMSCs. The functional properties of bmMSCs also decrease significantly with increasing age (Stolzing et al., 2008) which may have contributed to the variability of our bmMSC data.

Recently, MSCs isolated from adipose tissue using the CD34 antibody were found to be more clonogenic than those using the SUSD2 antibody (Busser et al., 2015). However, these adMSCs isolated using CD34 had significant contamination with $CD31^+$ endothelial cells and $CD45^+$ leucocytes. In our study, we removed red blood cells by Ficoll density gradient as well as endothelial cells and leucocytes by negative selection with their respective antibodies before purifying the $CD34^+$ adMSCs population according to the recently published joint statement by the ISCT and IFATS on the isolation of pure adMSCs (Bourin et al., 2013). Despite this selection process as has been shown in other study (Akiyama et al., 2012), CD34 expression significantly decreased as early as first passage and none of the cells was positive by third passage. This indicates that despite due care in selecting pure adMSCs, they undergo spontaneous differentiation early on in culture. However, surface profile of cultured MSCs is a part of the assessment of potency of MSCs and adMSCs alike. Therefore, looking only at the surface marker expression doesn't advocate for or against adMSCs. We were also not able to propagate adMSCs beyond P5, unlike other groups (da Silva Meirelles et al., 2015). Furthermore, we cultured adMSCs in 5% O_2 in in-house SFM while others have either enriched them with only CD34 antibody and cultured them with 10% serum medium in 20% O_2 or cultured the SVF in serum and xeno-free medium (Traktuev et al., 2008, Lindroos et al., 2009, Busser et al., 2015). Therefore, these differences in enriching and culturing adMSCs could have impacted on the inconsistent results in this and other studies. Nonetheless, they underwent senescence quite early in culture which could also be due to no serum as well as replicative senescence.

TGF-β is a pleiotropic cytokine which mediates stem cell differentiation especially into smooth muscle cells (Kurpinski et al., 2010, Su et al., 2014) and also senescence in several cell types (Senturk et al., 2010, Wu et al., 2014, Legzdina et al., 2016). TGF-β also induces apoptosis via upregulating the death-associated protein and its kinase, and TGF-βR inducible transcription factor (Ribeiro et al., 1999, Jang et al., 2002, Black et al., 2007). Using two approaches, we demonstrated that A83-01 prevented apoptosis, especially in MSCs from reproductive tissues and senescence of adMSCs. Interestingly, cell cycle analysis of bmMSCs showed 63% of Sub G1/G0 apoptotic cells in contrast to 95% of live cells using Annexin-V/PI analysis. The cell cycle analysis protocol uses ethanol fixation to permeabilise the cell/nuclear membranes enabling PI to access and bind the DNA. In contrast, the Annexin-V/PI protocol relies on the integrity of the cell membrane and detects earlier stages of apoptosis. Therefore, the permeabilization process may have allowed increased PI binding to the DNA in cell cycle analysis giving the true picture of the loss of viability of bmMSCs.

One of the limitations of this study was that the concentration of A83-01 used was optimised for pre-menopausal eMSCs. No further optimisation was done in MSCs from the above-studied tissues. The post-menopausal endometrial, placental and menstrual blood MSCs are derived from the endometrium. This may be one of the reasons A83-01 has similar effect on these MSCs and lesser effect on MSCs from the bone marrow and adipose tissue.

In future studies, culture expansion of MSCs continuously with A83-01 from the initial cultures may improve its effects on both reproductive, bone marrow and adipose MSCs. Given that we identified additional signalling pathways involved in spontaneous

differentiation of eMSCs in Chapter 4, small molecules targeting these in addition to A83-01 could be tried in future to generate homogeneous population of undifferentiation MSCs. Nevertheless, this study is also the first to demonstrate the presence and feasibility of isolating maternal $SUSD2^{+}$ perivascular MSCs in term placenta. Furthermore, women can use MSCs from different tissues as autologous sources and culture expansion with A83-01 may provide an approach for generating enough undifferentiated cells for therapeutic applications.

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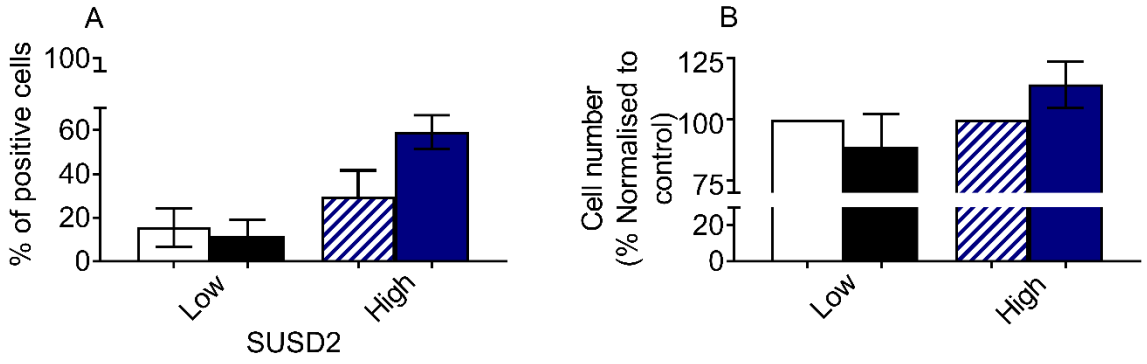

Supplementary Figure 5.1 Differences between bmMSC donors. A) Three bmMSCs samples had low percentages of SUSD2$^+$ cells which showed a decreasing trend even following A83-01 treatment while the other three samples had much higher percentage of SUSD2$^+$ cells and showed increased trend with A83-01 treatment. **B)** The two groups showed similar trend in their proliferative ability respectively. Variation in the percentage of SUSD2$^+$ cells and total number in the two groups may bedue to their age, as a decreasing trend was observed in patients who are older than that the second group. However, the transport process and the how the cells responded could have affected the cells as well.

Chapter 6

Development of high content screening assay of A83-01 analogues for eMSCs culture expansion

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Table of Contents

Declaration

Monash University

Declaration for Thesis Chapter 6

In the case of Chapter 6, my contribution to the work involved the following:

Name	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
Shanti Gurung	Responsible for performing the experiments, collecting all the data, analysing, interpreting and preparing figures and table and manuscript writing	90%
Jerome Werkmeister	Study design, manuscript revision and approved the final manuscript.	N
Caroline Gargett	Study design, manuscript revision and approved the final manuscript.	N

Student signature: [redacted] Date: 28/06/2017

6.1. Introduction

Mesenchymal stem/stromal cells (MSCs) were first identified in bone marrow as colony forming fibroblast-units (Friedenstein et al., 1974). They were initially thought to augment and support bone marrow homeostasis (Majumdar et al., 1998). Therefore, they were primarily used in bone marrow transplantation to support the bone marrow cells for cancer patients (Lazarus et al., 1995). At present, MSCs have been isolated from multiple vascularised tissues, including adipose tissue (Zuk et al., 2002) and endometrium (Schwab and Gargett, 2007, Masuda et al., 2012). With increasing knowledge about their differentiation potential and secretory profiles responsible for their immunomodulatory and wound healing properties, MSCs are currently in use in many pre-clinical and clinical trials. MSCs used in various human cell-based therapies are culture-expanded cells. To date, no serious adverse events have been documented for therapies which use MSCs. Successful outcomes from some trials leading to the approval to treat specific paediatric cases of graft-versus-host disease (GVHD) in Canada and New Zealand is encouraging (Cyranoski, 2012). Similarly, cryopreserved allogeneic MSCs are now routinely used in medical practice in European jurisdictions (Ringden et al., 2006, Le Blanc et al., 2008, Galipeau, 2013) since promising results were seen from phase II clinical trials for treatment of steroid-resistance GVHD (Le Blanc et al., 2004, Le Blanc et al., 2008, Bernardo et al., 2011). A USA company (Prochymal; Osiris Therapeutics, Inc., Columbia, MD, USA) conducted a similar phase III clinical trial demonstrated an improvement in response rate in patients with steroid-refractory-acute GVHD, however, overall it did not achieve a complete response rate compared to the placebo control ($p=0.12$) (Martin et al., 2010). A majority of clinical trials using MSCs have not shown clear and significant

clinical benefits, therefore, have not progressed beyond phase I/II studies (Galderisi et al., 2014, Trounson and McDonald, 2015), indicting the need for improvement.

The low frequency of MSCs obtainable from tissues means extensive passaging *in vitro* is unfortunately required to generate clinically optimal doses. Although extensive proliferation is a prominent feature of MSCs, it has a negative impact on MSC properties leading to replicative senescence, clonal impoverishment, telomere shortening, loss of potency and cell death (Parsch et al., 2004, Bernardo et al., 2007, Bork et al., 2010, Bigildeev et al., 2012, Moll et al., 2012, Bertolo et al., 2017). Moreover, the imperative immunomodulatory property of MSCs is substantially diminished with increasing expansion (Moll et al., 2012). Patients with GVHD who were given minimally expanded MSCs survived significantly longer than those who were given culture-expanded late passage MSCs (von Bahr et al., 2012). Similarly, the negative outcome of the above-mentioned phase III clinical trial conducted by the USA company was self-evident in retrospect because astoundingly 10,000 MSC doses (2 million cells per dose and mostly likely senescence cells) were produced from one donor compared to five to ten doses in the European studies (Galipeau, 2013). Certainly, the culture expansion process has negative consequences for MSCs and therapeutic outcomes.

Various approaches have been used to counteract the problem of loss of potency from culture expansion. These include, selecting MSCs with specific surface markers known to be expressed by clonogenic stromal cells with enhanced properties (Lv et al., 2014), smaller cell-size, longer telomere length and certain cytokines expression (Samsonraj et al., 2015). Despite these measures, MSCs undergo genetic

reprogramming in an *in vitro* environment generating a heterogeneous population with a variable potency which influences the therapeutic outcome (Samsonraj et al., 2015). We identified a small molecule inhibitor A83-01 that targets the TGF-βR signalling pathway, and promotes endometrial MSC (eMSC) proliferation, maintains their stemness and blocks apoptosis and senescence (Gurung et al., 2015). Furthermore, analysis of flow cytometry data demonstrated that A83-01 increased not only the percentage of $SUSD2^{+}$ eMSCs in cultures but also the number of SUSD2 molecules per cell suggesting these cells form a relatively homogeneous population of undifferentiated MSCs.

TGF-β superfamily members are multifunctional cytokines which regulate a wide range of biological functions such as cell proliferation, differentiation, tumor promotion and apoptosis (Derynck et al., 2001, Tojo et al., 2005). A83-01 is a potent TGF-βR inhibitor (Tojo et al., 2005). It inhibits activity induced by TGF-β type I receptor ALK-5, activin type IB receptor ALK-4 and nodal type I receptor ALK-7 (Tojo et al., 2005). In addition to this activity, A83-01 is an organosulfur compound containing a thiourea ($SC(NH_2)_2$) moiety in its molecular structure **(Figure 1.9)**. Copper inhibits MSCs proliferation, promotes spontaneous cellular differentiation and oxidative stress-induced cellular damage (Rodriguez et al., 2002, Peled et al., 2004). Copper chelators prevent copper-medicated negative effects on MSCs (Hafizi et al., 2015). Thioureas are known to have copper chelating properties and protect against copper-induced damage (Zhu et al., 2002).

High content screening (HCS) is a new technology that utilises biological assays for drug discovery involving standard immunofluorescent staining combined with

automated fluorescence microscopy. It has the advantage of allowing the simultaneous screening of a large number of chemicals with qualitative and quantitative readouts at the cellular level. To fully exploit the potential of the TGF-βR inhibitor in generating a homogeneous population of eMSCs with high potency, we wanted to screen a set of analogues of A83-01 using high content screening. The hypothesis was that the structure function relationship between A83-01 and its analogues will identify the active moiety of the molecule and determine its mechanism of action, whether it be copper chelation and/or block of senescence and apoptosis. The aim was to optimise a high content screening assay for a small molecule library of A83-01 analogues and employ eMSCs viability and SUSD2 surface expression as readouts to identify potent/optimal small molecule(s) which can be used for generation of highly effective eMSCs.

6.2. Materials and Methods

6.2.1. Human endometrial tissue samples

Human endometrial tissue samples were obtained from women who had no endometrial pathology and were not taking any hormonal therapy for at least three months. Human ethics approval was from Monash Health and Monash University Human Research Ethics Committees (Ethics number: CF10/2080, 2010001150) and patients gave written informed consent. The experiments were conducted according to the National Health and Medical Research Council of Australia guidelines for Medical Research.

6.2.2. Isolation and culture of eMSCs

SUSD2 positive eMSCs were isolated following published protocols (Masuda et al., 2012, Gurung et al., 2015) and cryopreserved in 10% DMSO/FBS freezing medium for later use. Cryopreserved eMSCs were quickly thawed in a water bath at 37^{o}C. The cell suspension was slowly transferred drop by drop into bench medium (BM) (5% NCS/DMEM). The cells were centrifuged at 1100 rpm at 4^{o}C for 5 minutes and washed with BM. The cell pellet was suspended in 5 ml SFM (with bFGF and EGF, 10 ng/ml each), and plated in a T25 fibronectin-coated flask. The culture flask was coated with 2 μg/cm^2 of fibronectin in PBS for at least an hour at 37^{o}C beforehand. The cells were cultured in 5%O_2/5%CO_2/90%N_2 (trigas incubator) at 37^{o}C. Media was changed every 2-3 days. When the cells were ~90% confluent, they were passaged and seeded at 5000 cells/cm^2 in fibronectin-coated coated flasks. eMSCs were used for experiments at passage 5 (P5).

6.2.3. Optimisation for cell density in 384-well plates

P5 eMSCs were collected following dissociation with warm TrypLE for 3 minutes at 37^{o}C. Serum free medium with FGF2, EGF and fibronectin (10 ng/ml, 10 ng/ml and 10 μg/ml respectively) was prepared. Fibronectin was added to the culture medium rather than coating the plate for feasibility purposes. Cells were prepared at 100, 200, 500, 700, 1000 cells/well with 0.01% DMSO or 1 μm A83-01 in separate tubes. Cell dispensing was adapted for 384-well plates (Falco 384 well black wall fluorescence plates) using an automated liquid-handling system (Sciclone Workstation), programmed using Maestro software to dispense the required number of cells in each well of the 384-well plate. The cells were incubated at 37^{o}C in a trigas incubator. Medium containing 1 μM A83-01 or 0.01% DMSO was changed every 48 hours for 5 days using the automated system.

Similarly, fixation in 4% paraformaldehyde (PFA), washing and staining with antibodies were performed with the automated liquid handler, which was covered with foil to provide darkness for immunofluorescence staining. At the end of 5 days, the cells were washed twice with PBS and fixed with 4% PFA for 10 minutes at room temperature (RT). Following two with PBS, the cells were blocked with Protein Block (DAKO® Protein Block Serum-Free) for an hour. The cells were then incubated with either PE-conjugated SUSD2 antibody (5 μg/ml, BioLegend) or its isotype-matched control IgG1-PE (Invitrogen) for 2 hr at RT. Then the cells were thoroughly washed with PBS and the nuclei were stained with Hoechst for 5 minutes. Antibodies or stains were diluted in 2% FBS/PBS. Excess Hoechst was washed away with PBS twice and 20 μl PBS was dispensed in each well to prevent the wells drying during image acquisition.

6.2.4. Automated confocal microscopy and image analysis

Confocal image acquisition was carried out with automated ArrayScan BT1 High-Content Systems (ThermoFisher Scientific). Omega XF93 filter at 365 (±50) nm excitation for Hoechst 33258 and 549 (±8) nm excitation for SUSD2-PE using a 10X objective lens were used to detect the nuclei and SUSD2 surface staining, respectively. Once the nuclei were identified in the Hoechst channel, using the algorithm (IN Cell Investigator softwareV1.6) a threshold diameter for the nuclear area was set and areas were marked at the outer region. A secondary 3-micron overlay was encircled beyond the nuclear area to encompass the cytoplasmic area **(Figure 6.1)**. Within this secondary area, the fluorescence intensity for SUSD2 was quantified. A total of nine images were collected per well. Using the in-machine software algorithm, each nuclear signal was converted to identify a cell to quantify total cell number per field. Similarly, a threshold was set to detect SUSD2 signal in the secondary area and calculate average fluorescence intensity and the standard deviation per field. In addition, an arbitrary threshold for high and low SUSD2 expression was set to calculate the percentage of cells expressing high and low SUSD2 intensity Control eMSCs (0.01% DMSO) were used as a negative control while A83-01 treated eMSCs were used as a positive control for the experiments. Mean and standard deviation for the control and treated eMSCs were used to calculated Z-factor value.

6.2.5. Chemical library of A83-01 analogues

A library of 33 analogues of A83-01 was identified in CSIRO chemical database (in collaboration with CSIRO, Clayton, Australia). The primary stocks were calculated at

5 mM in DMSO and the calculation is shown in **(Table 6. 1)**. The secondary stock was to be prepared in SFM at 2 μM which was twice the final concentration. 50 μl each of the secondary stock and cell suspension were to be added to each well of 384 well plate to give a final concentration of 1 μm, with the cells seeded at the optimal cell density.

Table 6. 1 List of Analogues of A83-01

Name of Chemicals	Molecular	Amount mg	Volume (μl) needed
CSIRO-005706BATCH-01	432.50	1	462.4298841
CSIRO-005707BATCH-01	402.52	1.1	546.5621242
CSIRO-005180BATCH-01	418.52	1.3	621.2441609
CSIRO-005714BATCH-01	430.57	1.4	650.3007641
CSIRO-005722BATCH-01	372.43	1.3	698.1215161
CSIRO-005729BATCH-01	406.87	1.1	540.7132499
CSIRO-005730BATCH-01	420.90	0.9	427.6580731
CSIRO-005705BATCH-01	402.45	1.1	546.6463248
CSIRO-005720BATCH-01	416.48	1.2	576.25678
CSIRO-005855BATCH-01	430.57	0.9	418.0504912
CSIRO-005856BATCH-01	456.61	1.2	525.6149695
CSIRO-006212BATCH-01	420.47	1.1	523.2215225
CSIRO-006215BATCH-01	476.58	1.1	461.6223929
CSIRO-006216BATCH-01	414.51	0.9	434.2487135
CSIRO-006217BATCH-01	492.64	1.2	487.1701706
CSIRO-006218BATCH-01	430.57	1	464.5005458
CSIRO-006964BATCH-01	406.48	1.2	590.4358206
CSIRO-006976BATCH-01	436.46	1	458.2306672
CSIRO-006979BATCH-01	406.48	1.2	590.4358206
CSIRO-006980BATCH-01	406.48	1.1	541.2328356
CSIRO-006981BATCH-01	390.37	0.9	461.0958058
CSIRO-006983BATCH-01	418.47	1.1	525.7234074
CSIRO-006985BATCH-01	436.46	1	458.2306672
CSIRO-007168BATCH-01	480.54	1	416.1967112
CSIRO-007197BATCH-01	414.51	1.1	530.7484277
CSIRO-007310BATCH-01	486.59	1	411.0211203
CSIRO-007375BATCH-01	450.56	1.1	488.28125
CSIRO-005556BATCH-01	376.39	1.3	690.7702933
CSIRO-005578BATCH-01	360.39	0.9	499.455589
CSIRO-005600BATCH-01	374.46	1.2	640.9195058
CSIRO-005601BATCH-02	388.49	1	514.8150913
CSIRO-005604BATCH-01	372.43	1.1	590.7182059
CSIRO-005612BATCH-02	436.53	1.5	687.2332676

6.2.6. Statistics

All data are represented as mean ± standard error of mean. Statistical analysis was performed by paired two-tailed non-parametric test (Wilcoxon test). Z factor was calculated with the following formula,

$$\text{Z factor} = 1 - \frac{3\,(\sigma_T + \sigma_U)}{\mu_T - \mu_U}$$

where σ is the standard deviation, μ is the mean in the control/untreated (U) and A83-01 treated eMSCs (T). Assays with Z factor value of less than 0 indicates too much overlap between positive and negative signals, 0 - 0.5 is a marginal assay and 0.5 -1 is considered a positive indication of a successful assay for screening small molecules.

6.3. Results

6.3.1. Identification of eMSCs and defining area for image analysis

After 5 days of culture with or without 1 μM A83-01 in SFM, eMSCs were fixed, stained with SUSD2-PE and counterstained with Hoechst. The principle of our immunofluorescence analysis was based on identifying eMSCs with SUSD2 and demarcating cell nuclei and counting cells with Hoechst. Using algorithm, each cell was identified in the Hoechst channel and the nuclear area was encircled with a blue outline **(Figure 6.1A)**. Similarly, using the PE channel, a cytoplasmic area of 3 μm increment from the nuclear border was outlined in green **(Figure 6.1B)**. Qualitatively, SUSD2 fluorescent intensity was low in the control **(Figure 6.2A)** compared to the treated eMSCs **(Figure 6.2B)**. Using the blue marker, the total number of cells was determined while average SUSD2 fluorescence intensity in the cytoplasmic area was calculated between the blue and green outlines per well. Any cells at the edges of the fields were excluded from quantification.

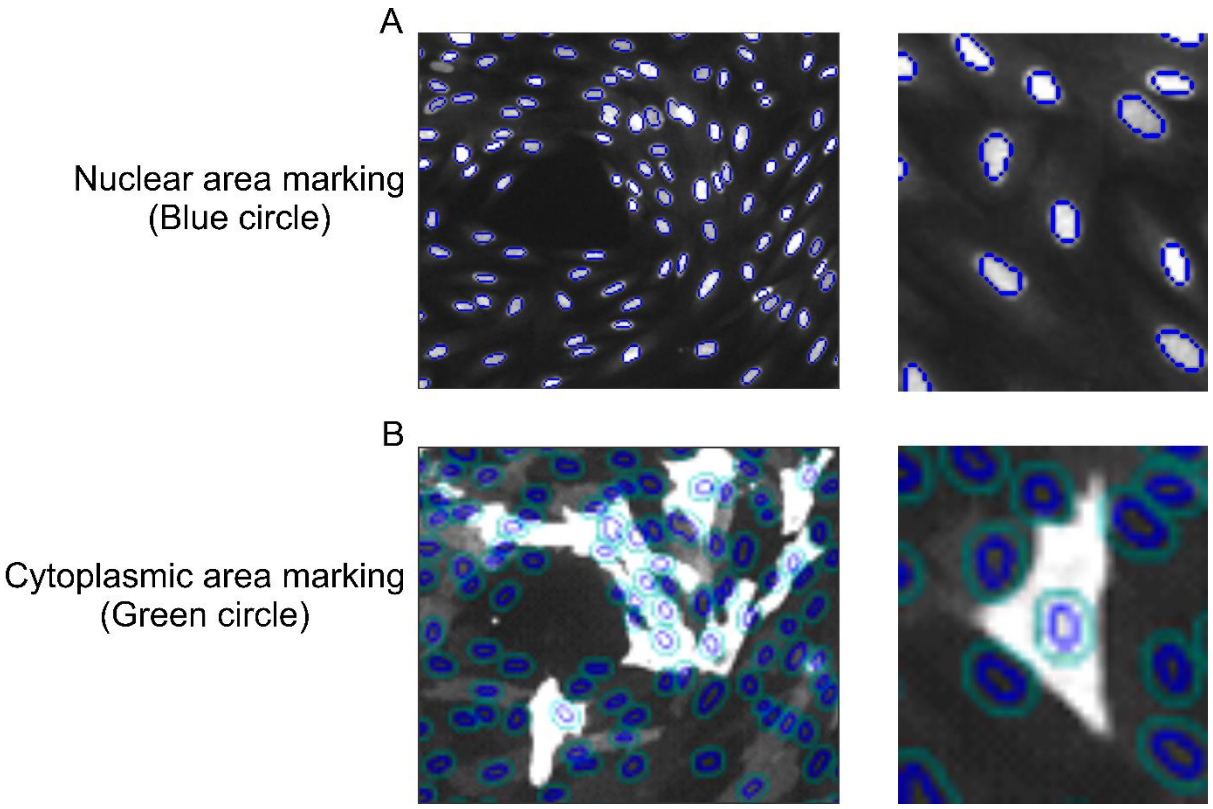

Figure 6.1 Representative images of eMSCs marked for nuclear/Hoechst and cytoplasmic/SUSD2-PE stained areas for cell counting and fluorescence intensity image analysis. Cells labelled with Hoechst 33258 were marked with a blue outline to identify nuclear area **A)**, and a 3-micron increment from the nuclear outline was encircled with a green outline to identify cytoplasmic SUSD2-PE area **B)**.

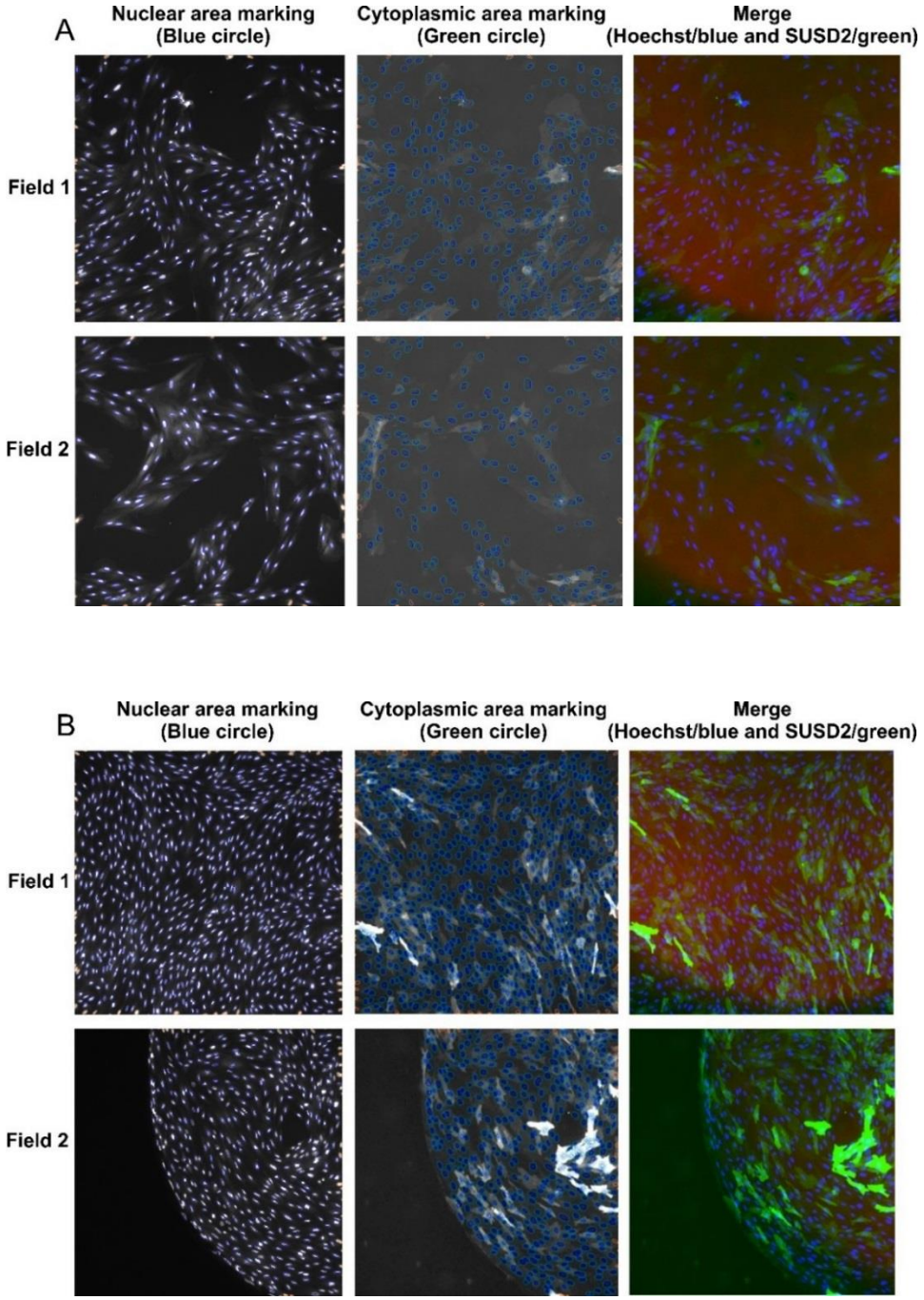

Figure 6.2 Representative fluorescence images of Control and A83-01 treated eMSCs in wells of a 384-well plate. eMSCs were cultured in a 384-well plate with and without 1 μM A83-01 for 5 days. Cells were fixed, immunostained with SUSD2 surface marker (green) and counterstained with Hoechst (blue). Representative images of two fields of view in **A)** untreated and **B)** A83-01 treated eMSCs (10X magnification).

6.3.2. 500 cells per well is ideal for 384-well plate

To determine the optimal cell seeding density eMSCs were plated at a density of 100, 200, 500, 700 or 1000 cells per well in of a 384-black wall clear bottom plate with or without A83-01 for 5 days. It was carried out with a single donor sample but multiple technical replicates. The 100 cells per well density was too low, while 1000 cells per well density had too many cells for proper detection of growth. There was a significant increase in the number of cells with A83-01 treatment compared to the control at 200, 500 and 700 cells per well densities **(Figure 6.3A)**. There was also a significant increase in SUSD2 mean fluorescent intensity (MFI) in well with these cell density **(Figure 6.3B).** In order to identify and segment individual cells in each well for optimal and accurate image analysis measurement, 500 cells per well for the 384-well plate was chosen as ideal.

In our previous studies, we coated culture plates with fibronectin for at least an hour prior to seeding the eMSCs. However, in this study for technical reasons, the 384-well plates were not pre-coated but rather fibronectin was added in the culture medium during the cell seeding process. Although we did not compare this approach with the eMSCs cultured in coated plates, cell attachment was not hampered as the cells grew well during the 5 days as shown in the figures.

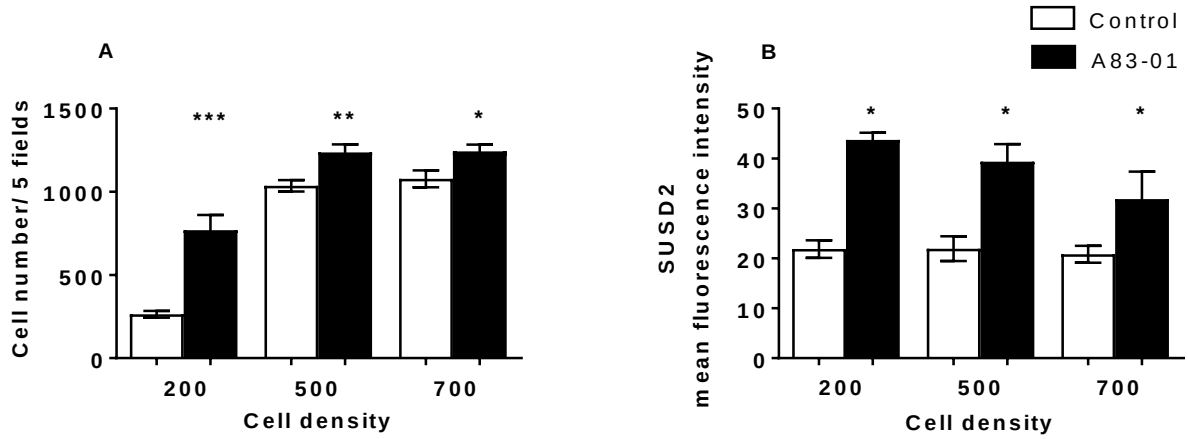

Figure 6.3 Optimisation of cell seeding density per well, duration and SUSD2 MFI assay following A83-01 treatment in eMSCs. Passage 6 eMSCs incubated with 1 μM A83-01 in SFM in trigas at 37 °C for 5 days were assessed by Hoechst and SUSD2 immunofluorescence staining. Total cell number **A)** and SUSD2 MFI **B)** per well of technical replicates were obtained for each cell density in the two groups and plotted as mean ± SEM.

6.3.3. Variation in SUSD2 intensity of A83-01 treated eMSCs

During the analysis cells expressing very high and low SUSD2 levels were clearly evident. To assess if there were any differences in the percentage of high and low SUSD2 expressing cells in the control and treated eMSCs, we put an arbitrary threshold of 125 as high and 15 as low intensity for the PE channel. Using the in-machine algorithm, we calculated the number of cells with MFI <15, 15-125 and >125 **(Figure 6.4 A and B)**.The differences in the number of cells with MFI <15 (black bars) or 15-125 (gray bars) between the two groups were not statistically significant **(Figure**

6.4A). However, there was a significant increase in the total number of cells with high SUSD2 MFI (>125) following A83-01 treatment compared to the control **(Figure 6.4A/**white bars**, B)**. Unfortunately, Z-factor for the average MFI for SUSD2 was low (0.16) while that of MFI>125 was 0.23 (low) for 500 cells/well. In calculating the Z-factor we considered only one standard deviation. Therefore, it is considered a marginal assay.

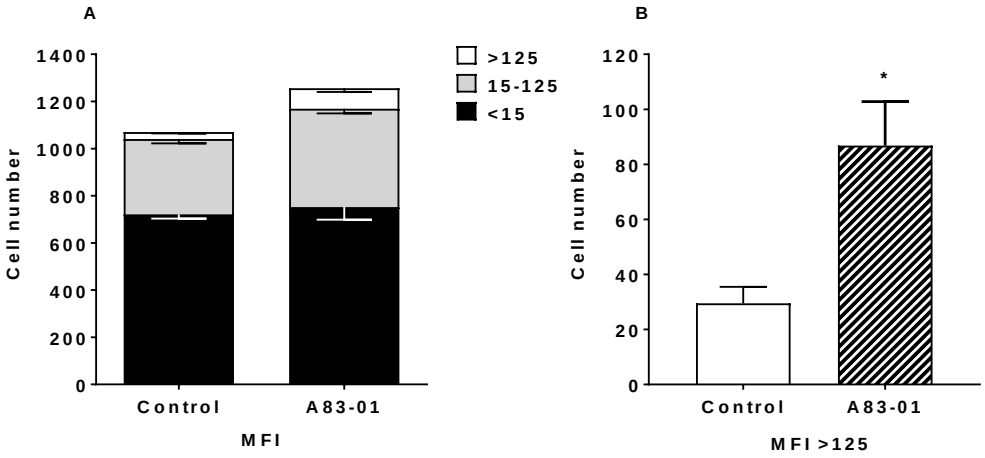

Figure 6.4 Differences SUSD2 MFI following A83-01 treatment. Passage 6 eMSC incubated A83-01 in SFM in 5%O2/5%CO2 at 37 °C for 5 days were assessed by Hoechst and SUSD2 immunofluorescence staining. **A)** A total number of cell with SUSD2 MFI >125 and **B)** A total number of cells in control and treated eMSCs with each bar consisting of MFI >125 (white bars), 15-125 (gray bars) and <15 (black bars) of X technical replicates seeded at 500 cells/well density. Graphs are plotted as mean ± SEM. *P<0.05.

6.3.4. Validating the screening procedure with more samples was unsuccessful

It is critical to validate the procedure and assay for drug screening using multiple biological replicates to test for the reproducibility before testing any chemical library. We, therefore, tested our procedure and assay using two more biological samples with technical replicates at 500 cells per well density in a 384-well plate outlay. Media was changed every 48 hours for 5 days to mimic screening with A83-01 analogues. The experimental plate out lay is shown in **Figure 6.5**. Then the cells were stained with SUSD2 and Hoechst. However, Z Factor for SUSD2 intensity for two biological samples were 0.1 and -0.5 which is below the values ideal for any chemical screening.

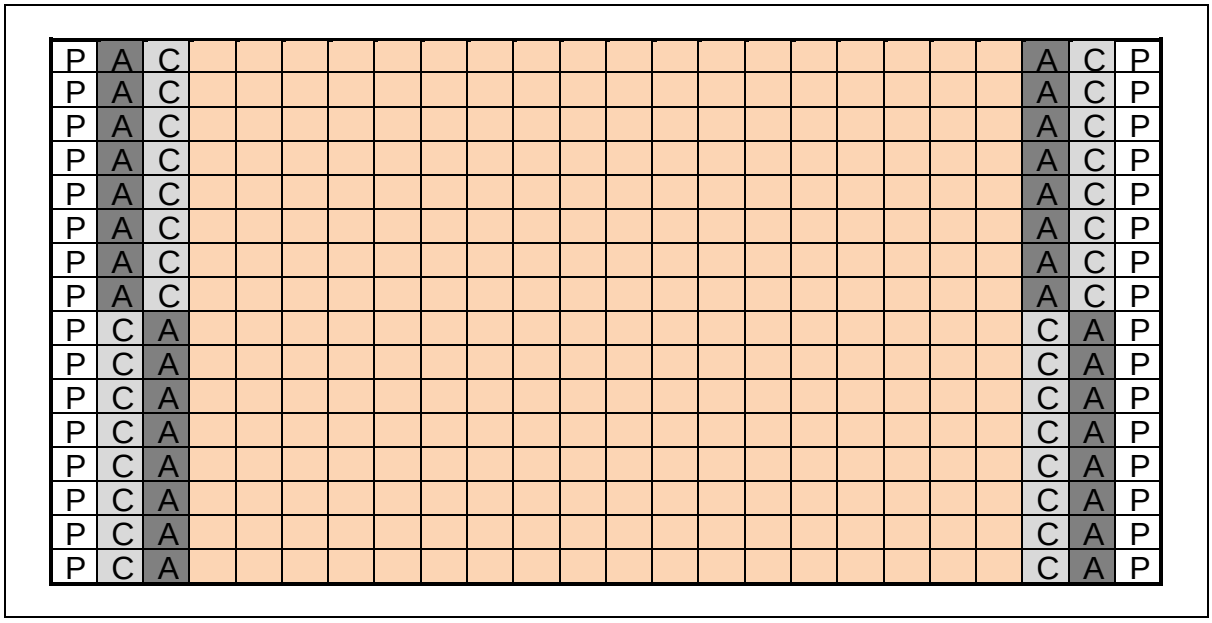

Figure 6.5 Example of a plate layout for A83-01 analogues screening. A 384-well plate showing the layout with the first and last columns for PBS (P), vehicle controls (C) and A83-01 as positive controls (A) arrangement in the outer two columns and all test compounds on the 288 wells of the plates (pink) in triplicates.

6.4. Discussion

In this study, we attempted to establish the parameters for a high content screening assay of eMSCs treated with A83-01, in preparation for testing a analogue library of 33 related compounds. Initial parameters optimised were cell seeding density and SUSD2 expression levels. The optimal cell density was 500 cells per well for 5 days of culture. Consistent with my Chapter 2, SUSD2 MFI increased significantly following A83-01 treatment. However, analysis of our HCS assay showed that it failed to meet the minimum Z factor value of >0.5. Hence the assay was not sufficiently robust for screening the A83-01 analogues. We concluded that this assay in its current form could not clearly separate true positive from false positive readouts for a chemical screening assay.

6.4.1. Overcoming issues with the High content screening assay

SUSD2 is a surface antigen present on MSCs. It is present in highly clonogenic MSCs from bone marrow (Buhring et al., 2007, Sivasubramaniyan et al., 2013), endometrium (Masuda et al., 2012) and spermatogonial progenitors (Harichandan et al., 2013). SUSD2 was our marker for isolating clonogenic cells from the endometrium, however, their culture in SFM resulted in a significant decrease in the proportion of cells expressing SUSD2 with increasing passaging which correlated with a decrease in their clonogenic ability (Gurung et al., 2015). In Chapter 2, we showed that there was a significant increase in the proportion of cells expressing SUSD2 as well as an increase in SUSD2 MFI following treatment of cultured eMSCs with 1μM A83-01. Therefore, we proceeded with optimising a high content screening assay to assess A83-01 analogues. However, the initial optimisation experiments indicated that there was too

much overlap in SUSD2 expression between control and treated cells. Although the assay was marginal when optimising the cell density for culture, it was not the case when assessing the full procedure and assay to mimic screening of A83-01 analogues.

Robustness of HCS assays is dependent on and works best with large differences in measured parameters between control and treatment. One robust assay described in literature used for screening chemicals using HCS assesses cytoplasmic to nuclear translocator proteins (Kau et al., 2003, Trask, 2004, Borchert et al., 2005, Bouck et al., 2011). For example, cytoplasmic NF-κB-p65 protein translocated to the nucleus upon stimulation with IL-1β (Trask, 2004). Microscopic images of such an assay are quite conclusive and define a clear cut separation between the untreated and treated samples (Trask, 2004). From our RNA-seq data, we have identified multiple genes and pathways that are differentially expressed in the A83-01 treated eMSCs compared to control cells such as the Wnt pathway-related genes as well as NF-κB. Therefore, future assays for screening the A83-01 analogues could utilise an assay that measures translocation of differentially expressed proteins from the cytoplasm to the nucleus.

β-catenin in an integral part of the Wnt signalling pathway (Clevers and Nusse, 2012, Yang et al., 2016). The canonical Wnt signalling pathway involves activation of the cysteine-rich domain of a Frizzled family receptor. This leads to the disruption of the β-catenin destruction complex; APC, Axin, and GSK-3β FIX (Clevers and Nusse, 2012). Thus, there is an accumulation of cytoplasmic β-catenin subsequently leading to its translocation to the nucleus and regulation of transcription of Wnt target genes. In the absence of Wnt ligand, cytoplasmic β-catenin is phosphorylated by the

destruction complex and is degraded by ubiquitin-proteasome pathway and cytoplasmic β-catenin level is kept low (Tulac et al., 2003, Clevers and Nusse, 2012). In our RNA-seq data from Chapter 4, Wnt pathway agonists such as *WNT2*, *WNT5A*, *WNT7B*, *WNT11*, *LEF1*, *FZD7*, *FZD8* were downregulated while the Wnt inhibitors such as *DKK1* and *SFRP1* were upregulated. Interestingly, in both groups of cells, *CTNNB1* encoding for β-catenin was equally highly expressed. This suggests that Wnt signalling is active in eMSCs but is significantly inhibited by A83-01. Therefore, analysing the mean nuclear and mean cytoplasmic differences of immunofluorescently stained β-catenin may provide a robust assay for screening A83-01 analogues using eMSCs. A HCS assay screening Wnt activators in primary human pre-osteoblasts set a precedent that this could work for A83-01-treated eMSCs (Borchert et al., 2005).

NF-κB is present in the cytoplasm of almost all cell type (Oeckinghaus and Ghosh, 2009). Its activators include reactive oxygen species, TNF-α and IL-1β (Trask, 2004). In an unstimulated state, NF-κB is tightly secluded in the cytoplasm by a family of inhibitors, the inhibitor of kappa B kinase (IκBs) (Oeckinghaus and Ghosh, 2009). Upon stimulation, the IκBs are phosphorylated leading to proteasomal degradation and release of NF-κB within the cytoplasm, which then translocates into the nucleus. Similar to the Wnt signalling pathway, genes involved in NF-κB pathways were differentially regulated in the A83-01 treated and control eMSCs with an upregulation of NF-κB inhibitors *NFKBIA* and *TNFAIP3* and downregulation of its activator *ROS1* by A83-01. Therefore, this pathway could be exploited to develop another assay for HCS evaluation in the future.

6.4.2. Choosing metric analysis for High content screening assay

Choosing the right statistical method to analyse the HCS data is as vital as optimising the assay. The most common metric used to analyse data for chemical screening is the Z factor. It takes into consideration three standard deviation differences between the negative and positive control samples when the data are normally distributed. A known potential disadvantage of the Z factor for assessing the utility of any assay is that any variable or biologically significant effect resulting from a subtle change could be overlooked by this approach (Yang et al., 2016). “B score” is an alternate statistic to the Z factor. The advantage of using this statistic over Z factor is that it is a non-parametric test and takes into consideration statistical outliers (Malo et al., 2006). This statistical scoring may be more beneficial especially in our situation where the data are not normally distributed.

6.4.3. Limiting Factors

Limitations of this study were that the liquid-handler and the automated microscopy broke down during my candidature and new parts had to be shipped from abroad (taking months) which hindered the progress of my study. There was also a limitation in staff available to look after the machines which were challenging as a new user. The most vital impediment was the limitation of the knowledge of the best assay to test for screening the chemical library. In addition, we also did not have in-depth knowledge in choosing the best statistical analysis for our high content screening assay. I have addressed the limitations and suggested ways to overcome them, therefore, it may be worth attempting screening the chemicals with the above-mentioned assays. In addition, trying early passage eMSCs and selecting for cells from older age women

could be considered as we have observed greater differences in the A83-01 effect on eMSCs isolated from older women.

6.5. References

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Chapter 7

General Discussion

This thesis aimed to develop culture expansion of undifferentiated human mesenchymal stem cells using small molecule/s, especially A83-01, for potential application for their autologous use in women for gynaecological disorders. The identification of MSCs in almost all postnatal tissues (bone marrow, adipose tissue, umbilical blood, menstrual blood, placenta and pre-/post-menopausal endometrium) has made it possible for women to choose to a variety of MSC sources for autologous use. The data presented in this study provide evidence showing generation of homogeneous population of MSCs when cultured in presence of A83-01. In addition, comparative analysis of control and A83-01 treated eMSCs revealed enhanced phenotypic properties following treatment suggesting their utility in the treatment of a wide range of disease.

Chapter 2 assessed the use of a small molecule (A83-01) that inhibits TGF-βR (ALK 4/5/7 inhibitor) signalling during culture expansion of ageing pre-menopausal eMSCs (Gurung et al., 2015). The concentration of A83-01 (1 μM) maintained multiple facets of eMSC function and generated a homogeneous population. Pre-treating eMSCs with A83-01 conserved primary adult stem cell properties, including colony forming efficiency, multilineage differentiation potency and proliferative capacity. Targeting the TGF-βR signalling pathway with a small molecule inhibitor also mitigated apoptosis and senescence in long term eMSC cultures. In summary, inhibition of TGF-βR signalling prevented loss of eMSC potency by preventing apoptosis and senescence. This would be beneficial in generating a homogeneous population of potent MSCs required for a cell-based therapy. This was the reason for further exploring the effects of A83-01 on eMSC culture expansion and determining if its beneficial effects were applicable to other commonly used MSC in the following chapters.

In Chapter 2, I also discovered that TGF-βR signalling negatively regulated the expression of our newly identified MSC surface marker, SUSD2, a type I transmembrane protein with a large extracellular and small transmembrane region (Sivasubramaniyan et al., 2013). A83-01 exerted its effect by blocking phosphorylation of the intracellular SMAD2/3 proteins, key components of the TGF-βR signalling pathway. The most interesting inference I made was that SUSD2 is regulated by TGF-βR signalling pathway, at least in eMSCs. This suggests that SUSD2 promoter has a potential SMAD binding site regulating its expression. Discovery of a regulatory pathway for SUSD2 not only helps in understanding the role of TGF-βR signaling in eMSCs differentiation but also provides new understanding on the role of SUSD2 and its biological functions. This knowledge can be applied in pathology such as breast cancer where tumours with high SUSD2 expression are associated with a doubling of tumor-associated macrophages, mainly of the M2 proangiogenic phenotype (Hultgren et al., 2017). It also suggests that implanting eMSCs with increased SUSD2 adhesion molecules on their surface may have a beneficial effect when used in cell-based therapies when tissue repair is the goal.

Having developed a method for generating undifferentiated homogeneous eMSCs in Chapter 2, the focus of Chapter 3 was to assess whether A83-01 pre-treated eMSCs altered survivability *in vivo*. It was also important to ensure that A83-01 treatment did not harm or affect the survivability of transplanted eMSCs. Three lines of evidence demonstrated that eMSCs survived at least 30 days in immunocompromised NSG mice. Firstly, tracking of genetically labelled human eMSCs using a lentiviral vector to introduce the mCherry gene identified surviving cells for at least 30 days. Secondly, demonstrating human endogenous Alu sequences through qPCR and finally detection

of mCherry^{+} cells by flow cytometric and immunofluorescence image analysis indicated eMSCs survival till day 30. Similar approaches have been employed to track MSC in other studies (Diana et al., 2013). Over time the transplanted SUSD2^{+} eMSCs declined in the number as did mCherry/SUSD2-expressing cells which might suggest cellular differentiation *in vivo* or their removal by the immune system.

In various animal disease models and the majority of clinical trials, transplanted MSCs have reversed the disease process and prolonged survival of the recipients. The beneficial activities of MSCs are likely mediated through their secretion of cytokines that exert an early paracrine effect described as a “touch and go” mechanism (Uccelli et al., 2008). However, MSCs have been detected *in vivo* for varying duration, ranging from a few days to months (Liechty et al., 2000, Mackenzie and Flake, 2001, Togel et al., 2008). This raises the question “Is it more than just a touch and go phenomenon?” Extensively cultured MSCs are a heterogeneous population of cells at various stages of potency, a feature that possibly contributed to observed differences in their survival *in vivo,* as well as suboptimal outcomes (von Bahr et al., 2012, Samsonraj et al., 2015). Similarly, in our small animal skin repair model, eMSCs survived up to 14 days post transplantation, despite exerting anti-inflammatory effects and promoting wound healing with minimal fibrosis well beyond the time of cell detection (Ulrich et al., 2014). Finally, future studies on the interaction of A83-01-treated eMSCs with immune cells *in vitro* and with immunocompetent or disease-model animals will further shed light on how they interact with the host innate and specific immune systems.

In Chapter 4, I found that targeting the pleiotropic TGF-β cytokine by inhibition of receptor kinase signalling with A83-01 elucidated its multifunctional roles in cells

(Massague, 2012). Based on RNA sequencing profiles of A83-01-treated and control eMSCs, this study has provided vast information on the role of numerous signalling pathways and their crosstalk in undifferentiated eMSCs. Many genes involved in TGF-βR, Wnt, PI3K-Akt signalling pathways, apoptosis and fibrosis were downregulated. Similarly, genes involved in the inflammatory response, angiogenesis, cytokine-cytokine receptor interaction and those directly related to potency were upregulated following A83-01 treatment. I found increased in fibroblast-related genes in cultured eMSCs in agreement with Barragan et al confirming their spontaneous differentiation (Barragan et al., 2016). The downregulation of these genes with A83-01 treatment confirmed that blocking TGF-βR signalling rescued them. My comparative study highlights that A83-01 treatment of eMSCs may positively influence their subsequent immuno-modulatory behaviour, increase survivability and enhance tissue repair properties. Areas for further investigation emanating from the sequencing data include investigations on immunoregulatory, angiogenic and anti-fibrotic functional roles of A83-01 treated versus control eMSCs. Given the noteworthy upregulation of antifibrotic and stemness genes, another important future study is to examine and compare the decidualisation capacity of A83-01-treated eMSCs *in vitro* in embryo implantation studies*,* as well as their reparative properties in animal models of pelvic organ prolapse, Asherman's syndrome and thin endometrium. Overall, this study suggests the possibility of using A83-01-treated eMSCs for the treatment of a wide range of diseases.

In extending my studies of A83-01 on eMSCs to other MSC types frequently used in clinical trials, in Chapter 5, I found that A83-01 has a similar effect on postmenopausal MSCs and other related endometrial sources such as placenta and menstrual blood.

Indeed, A83-01 had a similar effect on generating homogeneous SUSD2-expressing populations on late passage MSCs from female reproductive tissues, having similar potential to promote proliferation and prevent senescence and apoptosis. However, this was not the case for bmMSCs and adMSCs. Some differences in the cell cycle and Annexin-V/PI analysis, especially in the bone marrow MSCs, also suggest the necessity for validating findings with multiple assays. It was surprising to observe much greater proportions of apoptotic and senescence cells in untreated bmMSCs and adMSCs cultures compared to eMSCs. Differences in MSC properties from these tissue sources have been reported previously, not only in different age groups but also between male and female (Siegel et al., 2013, Sundelacruz et al., 2015). bmMSCs from young female donors are more clonogenic, express and/or secrete more anti-inflammatory cytokines and have greater ability to suppress allogeneic T-cells proliferation than male bmMSCs (Siegel et al., 2013). Similarly, female bmMSCs had greater capacity at rescuing pulmonary hypertension induced severe Bronchopulmonary dysplasia than male bmMSCs in a rat model (Sammour et al., 2016). These findings suggest the possibility of not only donor age and passaging but also gender, which could account for the disparity between bmMSCs and adMSCs and female reproductive tissue MSCs in this study. Evidence suggests that eMSCs have a role in maintaining an allogenic fetus, as defects in their epigenetic status and function lead to recurrent pregnancy loss (Teklenburg et al., 2010, Lucas and Frenette, 2014). This physiological function of eMSCs may confer their superior transplantability than MSCs from other sources due to their programming to manage the acceptance of an allogeneic embryo.

In Chapter 6, I established the parameters for a high content screening assay (HCS) of eMSCs treated with and without A83-01 in preparation to test an analogue library of 33 A83-01 related compounds and a broader range of small molecules targeting other signalling pathways. Other pathways besides TGF-βR signalling are involved in MSC differentiation. Understanding the signalling pathways regulating their fate *in vitro* is paramount to overcome the limitation of generating an undifferentiated homogeneous population of MSCs with sufficient potency for effective and prolonged therapy (Ding and Schultz, 2004). Although I was successful in optimising the cell density, duration of culture and SUSD2 staining protocol, unfortunately, I was not able to validate the process using HCS assay with a Z-factor value of >0.05. Nonetheless, from the RNA seq data analysis, we identified valuable genes that could be pursued through protein analysis to provide a different readout in the HCS. This could be further optimised and applied in HCS assay of small molecules to generate undifferentiated MSCs for clinical application. Most small molecule screenings are performed on embryonic and induced pluripotent stem cells, aiming to find small molecules maintaining their self-renewal, reprogramming and in lineage-specific reprogramming (Zhang et al., 2012). Little has been done to investigate the use of small molecules or other factors in maintaining the self-renewal, stemness and potency of mesenchymal stem cells, an underestimated impediment in cell therapy and regenerative medicine.

Future directions

Current approaches for treating common gynaecological problems such as pelvic organ prolapse, Asherman's syndrome and thin dysfunctional endometrium are limited. An alternative approach is required and using autologous cell-based therapy is promising. The studies in this thesis highlight the detrimental effects the expansion process has on the functional properties of MSCs. Further, they demonstrated that if the signalling pathways activated during culture expansion are targeted with a small molecule such as A83-01, a homogeneous undifferentiated MSC population can be generated. Given that a short treatment was effective in late passage cells, further study on longevity of the effects and how reversible they are could be considered. Culture in A83-01 containing medium from the first initiation of MSC cultures could also be investigated. This will help in determining the optimal timing for treating MSC prior to their use in patients, and in addition for obtaining TGA approval in the future.

The utility of A83-01-treated MSCs in scaffolds as a tissue-engineering construct could be studied especially in the treatment of POP. Similarly, *in vivo* studies using these cells to re-cellularise a decellularised uterus/endometrium may provide a proof of concept of their potential use in women with Asherman's syndrome and thin dysfunctional endometrium. From literatures and the findings from my thesis, targeting TGF-β pathway with A83-01 or its derivatives in vivo may be used to promote healing by stimulating endogenous MSC proliferation and promoting angiogenesis, decreasing fibrosis and regulating immune cells to promote healing, hence eliminate the need of an exogenous cell therapy. Nonetheless, delivery method, concentration and duration of treatment need to be optimised for different disease conditions.

Summary

The human endometrium is a highly regenerative tissue from where highly clonogenic MSCs can easily be isolated from biopsy tissues obtained without anaesthesia. MSCs from other reproductive tissues can also be easily isolated. From this study, I identified a small molecule, A83-01, which maintains culture expanded MSCs in the undifferentiated state more so from reproductive tissues than from bone marrow and adipose tissues. RNA sequencing data from this thesis also supported beneficial effects of A83-01 on reversing the negative impact of culture expansion of eMSCs necessary to generate enough cells for clinical use. This thesis also highlights the value of screening and identifying small molecule/s that can be used in generating undifferentiated potent MSCs with improved efficacy *in vivo*.

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APPENDICES

Appendix 1

Gurung, S., Werkmeister, J.A., and Gargett, C.E. 2015. Inhibition of Transforming Growth Factor-beta Receptor signaling promotes culture expansion of undifferentiated human Endometrial Mesenchymal Stem/stromal Cells. Scientific reports **5**:15042. doi:10.1038/srep15042.

Appendix 2

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Appendix 3

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Appendix 4

Gurung, S., Werkmeister, J. A. & Gargett, C. E. 2016. Book chapter “Endometrial MSC and Tissue Engineering for Pelvic Organ Prolapse Repair” Editor Kerry Atkinson in “The Biology and Therapeutic Application of Mesenchymal Cells” John Wiley & Sons.

Appendix 5

Letouzey, V., Tan, K.S., Deane, J.A., Ulrich, D., **Gurung, S.**, Ong, Y.R., and Gargett, C.E. 2015. Isolation and characterisation of mesenchymal stem/stromal cells in the ovine endometrium. PloS one 10:e0127531. doi:10.1371/journal.pone.0127531.

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Inhibition of Transforming Growth Factor-β Receptor signaling promotes culture expansion of undifferentiated human Endometrial Mesenchymal Stem/stromal Cells

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Human endometrial MSC (eMSC) are a novel source of MSC easily harvested from the highly regenerative uterine lining. We have developed protocols for eMSC isolation from single cell suspensions using magnetic bead-sorting using a perivascular marker antibody to SUSD2 and culture expansion in serum free medium (SFM). Similar to other MSC, eMSC spontaneously differentiate into fibroblasts during culture expansion decreasing their purity and efficacy. The aim of this study was to determine if A83-01, a TGF-β receptor inhibitor prevents eMSC differentiation in culture. SUSD2$^+$ eMSC were cultured in SFM with bFGF/EGF in 5% O_2/5% CO_2. At passage 6, eMSC were incubated with or without A83-01 for 7 days, then analysed for MSC properties. A83-01 dose dependently promoted SUSD2$^+$ eMSC proliferation and blocked apoptosis via the SMAD 2/3 pathway. Fewer A83-01 treated cells were autofluorescent or stained with β-galactosidase, indicating reduced senescence. A83-01-treated cells had higher cloning efficiency, differentiated into mesodermal lineages and expressed MSC phenotypic markers. These data suggest that A83-01 maintains SUSD2$^+$ eMSC stemness, promoting proliferation by blocking senescence and apoptosis in late passage cultures through binding to TGF-β receptors. Small molecules such as A83-01 may enable the expansion of undifferentiated MSC for use in tissue engineering and cell-based therapies.

Mesenchymal stem/stromal cells (MSC) have been identified in almost all adult human tissues[1] since Friedenstein and colleagues discovered colony-forming fibroblasts in bone marrow in the 1970s[2]. MSC are typically characterised by their clonogenicity, multipotency[3] and surface phenotype[4]. In addition, MSC home to damaged tissues[5], and have anti-inflammatory and immunomodulatory properties[6]. Increasingly, MSC are recognized for their biological effects in repairing damaged tissues through secretion of soluble bioactive molecules, including growth factors such as vascular endothelial growth factor[7], anti-fibrotic factors such as hepatocyte growth factor and prostaglandin E2[8], angiogenic factors[9] and molecules that inhibit apoptosis and activate tissue specific progenitor cells. MSC-conditioned medium recapitulates the activity of MSC *in vitro* indicating a paracrine effect that initiates cellular signalling that ultimately enhance tissue repair[10,11]. These MSC properties have led to their use in numerous clinical

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trials for a variety of diseases, including graft versus host disease[12], cardio-vascular disease as a cell-based therapy[13] or in tissue-engineered constructs for bone (www.clinicaltrials.gov).

MSC have recently been identified in the highly regenerative uterine lining (endometrium). Human endometrial mesenchymal stem/stromal cells (eMSC), like other mesenchymal stem/stromal cells are a rare group of quiescence cells (~1–4%) found in a perivascular location[14,15]. In the endometrium, eMSC are found in the functionalis layer that is shed during menstruation and in the remaining basalis layer from which the new functionalis grows each month[16,17]. eMSC can be prospectively isolated from endometrial biopsy tissues using co-expression of the MSC markers, CD140b and CD146 by flow cytometry sorting or with a single marker SUSD2 using magnetic beads[14,15]. eMSC isolated using the W5C5 antibody that recognises the SUSD2 antigen have typical *in vitro* MSC properties, in addition to reconstituting stromal tissue *in vivo* and significantly reducing inflammation and promoting neovascularisation when delivered as a tissue-engineering construct in an animal model of wound repair[14,18]. SUSD2 is a novel marker, recently identified, as an alternate to CD271 for purifying human bone marrow MSC (bmMSC)[19]. SUSD2 is a type I transmembrane protein that has a large extracellular region with domains known to have roles in cell adhesion, homodimerisation, signal transduction and migration[20] through interaction with LGALS1 (galactosidase-binding, soluble, 1) and UGGT1 (UDP-glucose ceramide glucosyltransferase-like 1) proteins[21]. SUSD2 is also highly expressed in brain especially in the hippocampus where it plays a role in neuritic growth and excitatory synapses which involve its cell adhesive properties[21].

eMSC require expansion for use in clinical applications similar to bmMSC[14,22,23]. However like other MSC, eMSC undergo spontaneous differentiation to fibroblasts during the culture expansion process, decreasing their purity[24]. Heterogeneity and decreased efficacy of culture-expanded MSC result in reduced clinical effect. In addition, the regenerative potential of MSC declines with age[25]. Freshly isolated, culture expanded SUSD2$^+$ eMSC underwent spontaneous differentiation indicated by decreasing proportions of SUSD2$^+$ cells and increasing SUSD2$^-$ cells with increasing passage[18]. The MSC markers designated by the International Society of Cellular Therapy (ISCT) do not indicate the "stemness" of culture expanded MSC. During culture expansion, MSC age losing CFU activity, tri-lineage multipotency, telomere length and ability to generate neotissue *in vivo*, despite expressing the standard ISCT MSC markers[24,26,27]. For example, bmMSC lose differentiation and proliferative capacity even though expressing high levels of CD44, CD271, CD90 and CD105 during extended culture[28]. Thus, these typical ISCT MSC markers cannot be used to monitor the differentiation state of MSC during culture. The novel marker SUSD2 and CD146 may be superior markers to monitor the status of MSC during the culture expansion process[22].

The loss of clonogenicity, multipotency and onset of senescence upon extensive culture of bmMSC results in increased senescence-associated beta-galactosidase and p16 gene expression, as well as changes in DNA methylation, limiting the utility of MSC as a cell-based therapy[29]. The maintenance of a stem/progenitor cell population during culture expansion requires activity of signalling pathways involved in self-renewal and proliferation while preventing differentiation[30]. Several small molecules targeting signaling pathways involved in maintaining pluripotency or blocking differentiation have been used for pluripotent cell cultures. Inhibition of the GSK3β, MEK and TGF-β signalling pathways have been used in rat and human induced pluripotent stem cells (iPSCs) to prevent spontaneous differentiation and maintain their stemness during prolonged culture[31]. The ROCK inhibitor, Y27632, has been used to prevent dissociation-induced cell death of human embryonic stem cells[32]. The PDGFR-IV tyrosine kinase inhibitor (#521233, Calbiochem) increased expression of pluripotency genes *OCT4* and *NANOG*, and increased MSC potency from multipotent to a pluripotent state[33]. Transforming-growth factor beta receptor (TGF-βP), platelet-derived growth factor receptor (PDGF-R) and basic-fibroblast growth factor receptor (bFGFR) pathways have crucial roles in specifying MSC differentiation into osteogenic, myogenic, adipogenic and chondrogenic lineages[34]. Controlling MSC self-renewal and differentiation with small molecule inhibitors or activators of one or more of these key signalling pathways, should generate a homogeneous MSC population during culture expansion.

The use of eMSC for cell-based therapy requires their expansion in culture conditions that supports homogenous growth and maintains self-renewal and multipotency. A83-01 is a potent selective inhibitor of the TGF-βRs ALK4, 5, and 7. A83-01 inhibits SMAD2 phosphorylation[35,36], maintains self-renewal and proliferation of rat and human induced pluripotent stem cells in cultures without feeder layers[35]. The aim of this study was to determine whether A83-01 maintained growth and prevented spontaneous differentiation of eMSC during culture expansion. In this study, we showed that A83-01 promotes proliferation of late passage SUSD2$^+$ cells in serum free medium (SFM), an effect mediated by SMAD2/3 signaling. A83-01 also prevented senescence and apoptosis of cultured eMSC, suggesting that TGF-β has a role in regulating SUSD2 expression and eMSC growth, apoptosis and senescence and therefore may have a role in spontaneous MSC differentiation during culture expansion. Small molecules such as A83-01 may provide an approach for the expansion of undifferentiated MSC for use in tissue engineering and cell-based therapy.

Results

A83-01 dose dependently promotes eMSC proliferation. In our earlier studies, we observed that SUSD2$^+$ cells diminished in number with increasing passage[18], despite their high purity on initial seeding following SUSD2 magnetic bead sorting[14]. To examine the effect of the TGF-βR inhibitor, A83-01 on

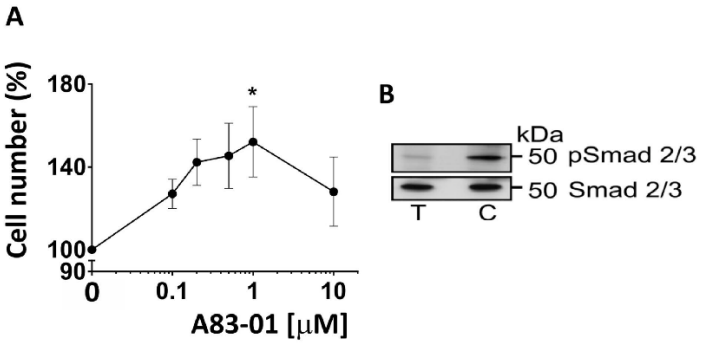

Figure 1. Dose Response curve of A83-01 promotion of eMSC proliferation. (A) Passage 3 eMSC incubated A83-01 in SFM in $5\%O_2/5\%CO_2$ at 37 °C for 7 days was assessed by MTS cell viability assay. Means for triplicates were obtained for each sample at each concentration, then normalised to vehicle control DMSO (100%) and plotted as mean ± SEM of n = 6 patient samples. **(B)** Passage 6 eMSC lysates with or without 1 μM A83-01 were immunoblotted with anti-SMAD 2/3 or anti-pSMAD 2/3 antibodies. A83-01 inhibited TGF-βR-induced phosphorylation of SMAD 2/3. (C = control, T = treated).

eMSC proliferation, passage 3 eMSC were cultured in SFM in 5% O_2 with A83-01 concentrations ranging from 0–10 μM for 7 days. Control medium was supplemented with vehicle. The MTS cell viability end-point assay was used to assess the effect of A83-01 on eMSC growth. As shown in Fig. 1A, A83-01 dose dependently increased the number of viable cells with maximal effect at 1 μM concentration ($p < 0.05$) by day 7. This result suggests that TGF-βR signaling regulates cell growth in negative manner. All further experiments were carried out with P6 eMSC using A83-01 at 1 μM concentration. A83-01 blocks the phosphorylation of SMAD2/3 (Fig. 1B) thus indicating its activity via SMAD pathway.

Surface Phenotype Expression of A83-01 treated eMSC. We next examined the phenotype of A83-01 treated eMSC. Single-color flow cytometry analysis of 5 MSC markers showed that untreated P6 eMSC cultures comprised 69%-SUSD2$^+$, 53%-CD140b$^+$, 1%-CD146$^+$, 95%-CD90$^+$ and 0%-CD271$^+$ positive cells (Fig. 2A), suggesting loss of the MSC phenotype and spontaneous differentiation. It is noteworthy that CD90, the representative ISCT MSC marker[4] did not change over the period of culture, and that P6 eMSC did not express CD271 (bmMSC marker) whether incubated with or without A83-01. There was a significant increase in the percentage of SUSD2$^+$ (94%, $p < 0.05$) and CD140b$^+$ (83%, $p < 0.05$) cells when the P6 cells were treated with 1 μM A83-01 for at least 7 days. There was also an increase in the mean fluorescence intensity for the SUSD2 marker on the SUSD2$^+$ cells (Fig. 2B,C), indicating an increase in the number of SUSD2 molecules per cell ($p < 0.05$). This was also evident by immunofluorescence (Fig. 2D). However, A83-01 had no effect on the CD146 expression, which was downregulated during culture expansion (Fig. 2A).

A83-01 maintained functional properties of late passage eMSC. We next investigated whether inhibition of the TGF-βR signalling pathway in late passage eMSC cultures altered their MSC functional properties. The cloning efficiency of P6 A83-01 pre-treated eMSC was significantly greater ($p < 0.05$) than control cells (Fig. 3A,B). The A83-01 pre-treated eMSC also generated larger colonies than untreated eMSC.

Next, we tested whether A83-01 pre-treated eMSC retained MSC multilineage differentiation capacity. P6 eMSC pre-treated with or without 1 μM A83-01 were cultured in differentiation induction media or 1% FCS growth medium (control) to assess differentiation into adipocytes, osteocytes and chondrocytes (Fig. 3C). A83-01 pre-treated and untreated cells showed similar phenotype changes in adiopogenic medium with similar numbers of cells containing Oil Red O stained lipid droplets. Similarly for osteogenic differentiation, the amount of Alizarin Red stained calcium deposits was comparable. In contrast, chondrogenic differentiation of the cell pellets was greater for the A83-01 pre-treated cells, as a strong Alcian Blue stained matrix in a cartilage-like organoid was observed, while the untreated eMSC pellet disintegrated easily with little evidence of chondroitin sulphate matrix deposition (Fig. 3C). There was no differentiation in non-induction medium.

A83-01 effect on pluripotency and stem cell gene expression. We next examined the expression of pluripotency and MSC genes suggested to have a role in maintaining MSC self-renewal. Quantitative RT-PCR of A83-01 treated and untreated cells failed to detect pluripotency genes *OCT4*, *SOX2* and *NANOG* in either group (results not shown) although they were demonstrated in the human iPS cells positive control. Consistent with the flow cytometry data, *SUSD2* was downregulated in the control

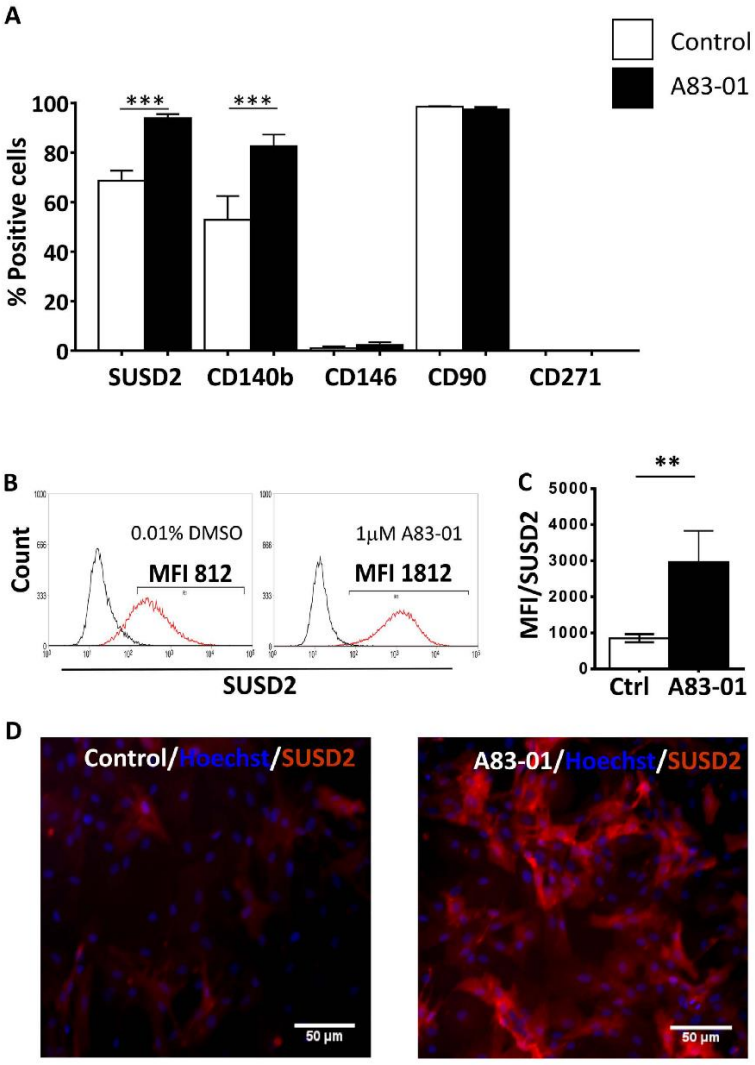

Figure 2. Phenotype of P6 eMSC cultured with or without A83-01 in serum free medium in 5%O_2. (A) % Positive cells for MSC surface markers on passage 6 eMSC (n = 8) cultured in 1μM A83-01(black bars) or in 0.01% DMSO (white bars) for 7 days and assessed by single-colour flow cytometry. **(B)** Representative flow cytometric histograms of $SUSD2^+$ eMSC treated with (black bar) and without (white bar) 1μM A83-01 and MFI summarised in **(C)**. **(D)** SUSD2 expression on control (left panel) and A83-01 treated (right panel) eMSC by immunofluorescence (red). Data are mean ± SEM of n = 8 different patient samples. $^{**}p < 0.01$, $^{***}p < 0.001$.

group and highly expressed in the A83-01 treated cells ($p = 0.0078$) (Fig. 4). The expression of *CD146* and *MMP3* genes was reduced in A83-01 treated cells ($p = 0.04$ and $p = 0.0078$, respectively) (Fig. 4). There was also an increase in the expression of *AOC3* ($p = 0.031$), a marker of $SUSD2^+$ cells[37] and *FRZB* ($p = 0.015$) (Fig. 4), a gene encoding for a Wnt ligand binding protein, in A83-01 treated group while no difference was observed in the expression of *NOTCH2, NOTCH3* and *DKK* genes (Fig. 4).

A83-01 blocks apoptosis and senescence in P6 eMSC. To identify the mechanism of action of A83-01 in increasing eMSC proliferation (Fig. 1), we undertook cell cycle analysis (Fig. 5A) with propidium iodide to label DNA. Figure 5A,B shows that A83-01 treatment increased the proportion of cells in G2/M phase ($p < 0.05$) indicative of an increased rate of cell division. There were also significantly fewer A83-01 treated cells in the sub G1/G0 phase of the cycle compared with control cells indicating fewer apoptotic cells with fragmented DNA content in the A83-01 treated cells (Fig. 5A,B). We then quantified

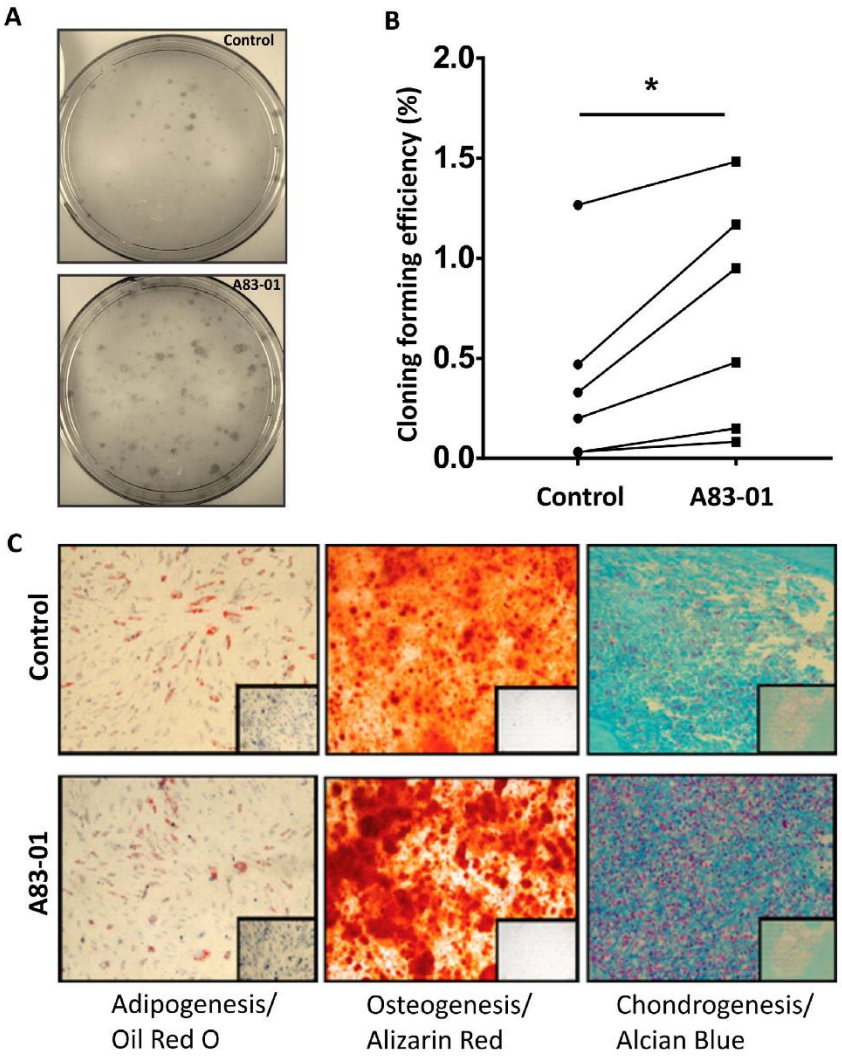

Figure 3. Functional MSC properties of P6 eMSC cultured with or without A83-01 in serum free medium. **(A)** Representative culture plates seeded at clonal density (50 cell/cm^2). **(B)** Graph shows Colony Forming Efficiency of P6 eMSC pre-treated with 1μM A83-01 or 0.01% DMSO vehicle for 7 days in 5% O_2 in SFM followed by clonal culture at 50 cells/cm^2 in SFM for 4 weeks. **(C)** Multilineage mesodermal differentiation of 0.01% DMSO treated control and 1μM A83-01 treated P6 eMSC showing adipogenic, osteogenic, and chondrogenic differentiation (controls were cultured in 1% serum media) for four weeks in 5%O_2. Oil Red O was used to visualise cellular lipid vesicles for adipogenic differentiation, Alizarin Red to detect calcium mineralisation for osteogenic differentiation and Alcian Blue to detect acidic polysaccharides in the extracellular matrix for cartilage differentiation. Representative images from n = 3 samples. Data are mean ± SEM of n = 6 different samples *$p < 0.05$.

the apoptotic cells using Annexin V flow cytometry to assess early phase apoptosis. The inclusion of PI was to detect late apoptotic and necrotic cells. Culture expanded P6 eMSC significantly reduced the percentage of live cells and increased the proportion of apoptotic cells as shown by the increased binding of Annexin V to exposed phosphatidylserine (PS) on the outer leaflet of the plasma membrane (Fig. 5C,D). The increased PI staining in the untreated eMSC also indicated increased necrotic cells. These changes were mitigated by pre-treatment with 1μM A83-01 ($p < 0.05$) (Fig. 5C,D).

To further understand the action of A83-01, we examined unstained P6 eMSC from treated and control groups by UV light to quantify autofluorescence, as a measure of senescence. As shown in Fig. 5E, control P6 eMSC were significantly more autofluorescent than the A83-01 treated P6 eMSC ($p = 0.001$).

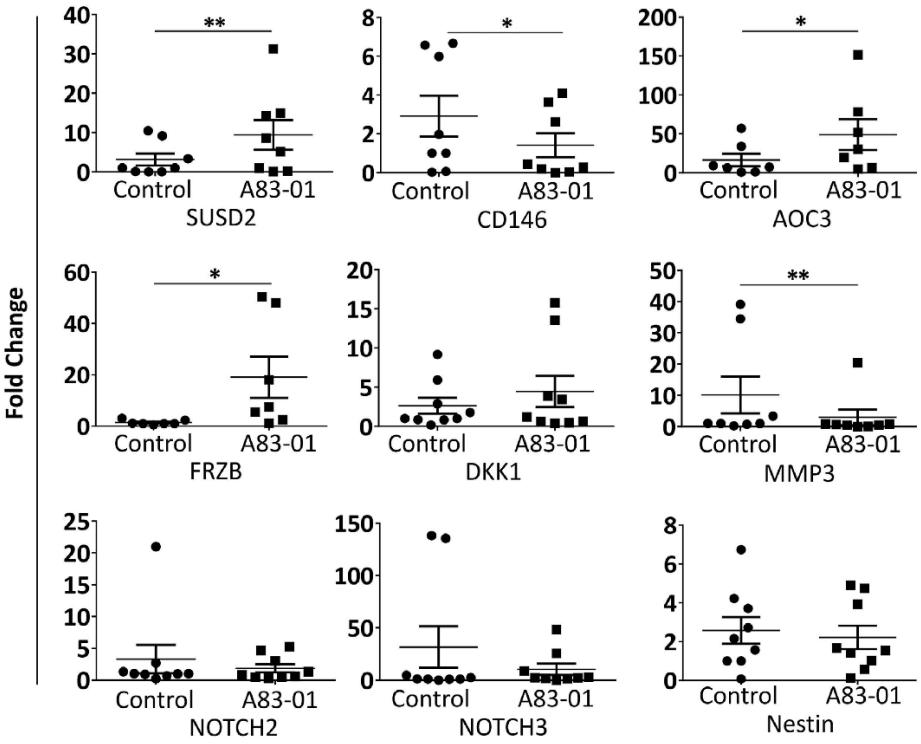

Figure 4. Quantitative RT-PCR analysis of MSC genes. P6 eMSC cultures treated (black squares) or untreated control (black circles) with 1 μM A83-01 in 5%O_2/5%CO_2/90%N at 37 °C for 7 days. qRT-PCR analysis of *SUSD2*, *CD146*, *AOC3*, *FRZB*, *MMP3*, *DKK1*, *NOTCH3*, *NOTCH2* and *NESTIN*. β-Actin or GAPDH were used to normalise the mRNA level, and fold change was calculated using $2^{-\Delta\Delta CT}$. Data are mean ± SEM on n = 7 different tissue samples. *$p < 0.05$; **$p < 0.01$.

Therefore, we measured senile associated β-Gal (SAβ-Gal) activity by incubating cells with X-Gal. As shown in Fig. 5F, A83-01 treated P6 eMSC showed little β-Gal staining whereas the untreated control eMSC displayed blue staining indicative of senescent cells. Furthermore the A83-01-treated cells were smaller and more numerous, in agreement with our findings above.

Discussion

The main findings from this study are that A83-01, a small molecule TGF-βR inhibitor, prevented the typical loss of undifferentiated MSC during culture expansion. Specifically, we showed that A83-01 treatment prevented loss of $SUSD2^+$ eMSC in late passage cultures by promoting the mitosis and proliferation of P6 $SUSD2^+$eMSC and by preventing their apoptosis and senescence. A83-01 treated $SUSD2^+$ cells in late passage culture retained their MSC properties, showing greater clonogenicity then untreated cells. In particular there were greater numbers of large colonies which undergo serial cloning and are more proliferative than those initiating small colonies[38]. Their multilineage differentiation capacity was maintained as well as expression of key MSC genes; *SUSD2*, *AOC3*[37] and *FRZB*[39]. We further identified that the signalling pathway blocked by A83-01 was TGF-βR mediated apoptosis via SMAD2/3 phosphorylation. Since multiple pathways work together in regulating MSC fate and TGF-βR pathway signalling is pleiotropic, targeting this pathway may provide an ideal method for maintaining undifferentiated MSC in cell production protocols for clinical use.

We showed culture expansion of eMSC lead to a loss of clonogenicity and expression of SUSD2, CD140b and CD146 surface markers while CD90, a standard ISCT MSC marker does not change. This was also shown by loss of $SUSD2^+$-expressing eMSC when induced by TGF-β1 to differentiate into smooth muscle cells[40]. These properties support the concept that eMSC spontaneously differentiate into fibroblasts lacking the expression of perivascular markers, clonogenicity, and osteogenic and chondrogenic differentiation capacity. Further molecular characterisation of differentiation at the transcript and protein levels is feasible with qRT-PCR and western blotting respectively. TGF-βR signalling is necessary for chondrogenic differentiation[34]. However, while the experimental medium for eMSC culture expansion contained A83-01, the chondrogenic differentiation medium contained TGF-β1 without A83-01 to

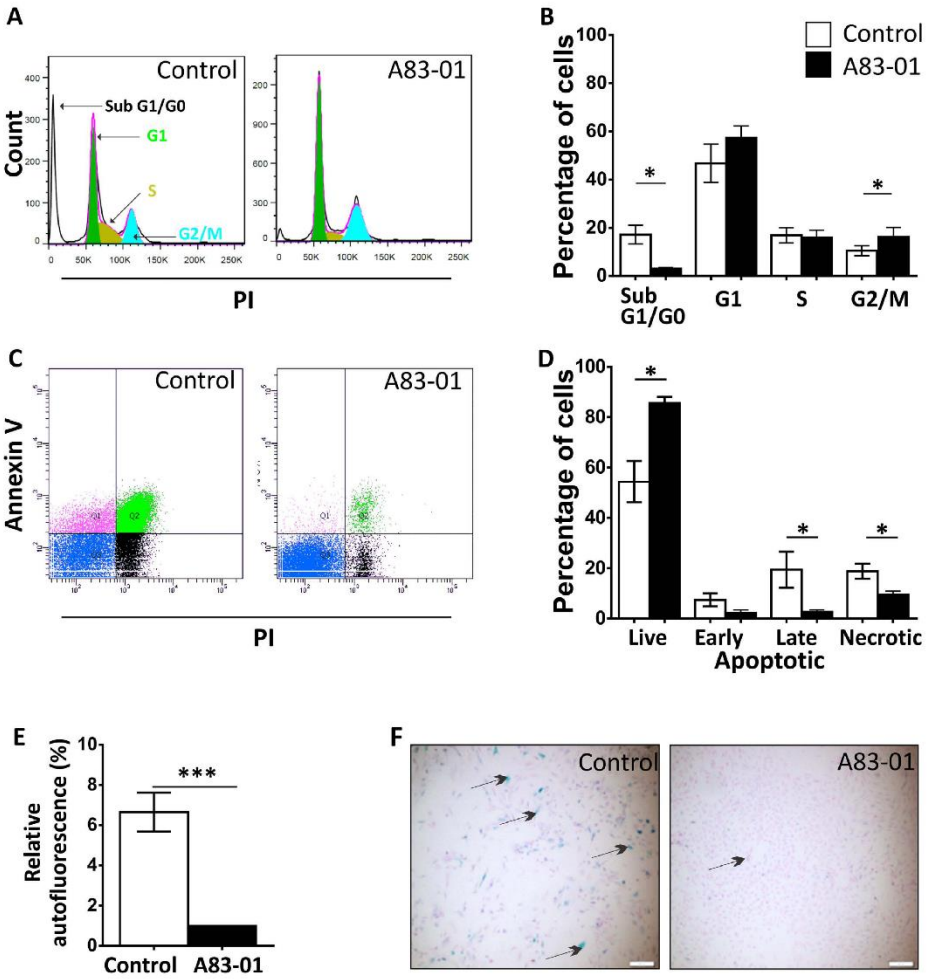

Figure 5. A83-01 blocks apoptosis and promotes eMSC proliferation. P6 eMSC treated with 1 μM A83-01 or 0.01% DMSO cultured for 7 days in SFM in 5%O_2/5%CO_2/90%N were assessed by **(A)** Cell cycle analysis of PI stained cells. Shows representative PI staining on a linear axis of a flow cytometry plot **(B)** the percentage of cells in SubG1/G0, G1, S and G2/M stages of the cell cycle (black bar A83-01 treated, White bar control). Data are mean ± SEM, n = 7 patient samples; *$p < 0.05$. **(C)** Annexin-V and PI staining. Representative flow cytometric plots. The lower left quadrant of each panel shows the viable cells, upper left early apoptotic; upper right late apoptotic and lower right necrotic cells and **(D)** graph showing the percentage of live, apoptotic and necrotic cells. Data are mean ± SEM, n = 6 patient samples, *$p < 0.05$. **(E)** Relative autofluorescence by flow cytometry of unstained P6 cells treated (black bar) and untreated (white bar) with 1 μM A83-01. Data are mean ± SEM, n = 10, ***$p < 0.005$. **(F)** Representative images showing the staining of senescence-associated β-galactosidase (SA-β-gal) in cultured P6 eMSC treated with or without 1 μM A83-01.

assess chondrogenic differentiation potential of A83-01 treated and untreated cells. One advantage of using small molecules rather than siRNA to modulate receptor activity is that their inhibitory effect is reversed as soon as the small molecules are removed. We were therefore able to show that chondrogenic differentiation was enhanced in A83-01 pre-treated cells. A83-01 not only increased the expression of SUSD2 proteins but also CD140b. In contrast, CD146 gene expression was greater in the untreated group but did not appear to be translated into protein as it was not detected by flow cytometry. Furthermore, expression of CD146 on cultured MSC is regulated by factors such as hypoxia, growth factors, and metalloproteases[41,42]. Culture expanded MSC are more autofluorescent than the primary cells indicating replicative senescence and loss of proliferative ability[43,44] an effect observed in P6 control eMSC which was mitigated by A83-01 treatment.

Endometrial MSC are an attractive source of cells for tissue engineering and cell-based therapies because they can be harvested with minimal discomfort to patients, have standard MSC properties *in vitro* and *in vivo*, and they can be cultured in serum free conditions, offering a readily available cell source for allogeneic as well as autologous use. The necessity to expand MSC for clinical use due to their rarity and their subsequent spontaneous differentiation limits the full potential of eMSC and MSC in general.

TGF-β belongs to a superfamily of TGF cytokines which has multiple functions. TGF-β plays a vital role in MSC differentiation along with PDGF and FGF-2 pathways[34,45]. TGF-β is synthesized by endometrial stromal cells under the influence of physiological female hormones, fluctuating during different phases of menstrual cycle. TGF-β production increased in the secretory and menstrual phases but was diminished in the proliferative phase, suggesting that TGF-β promotes differentiation[46]. Similarly, TGF-β promoted differentiation of SUSD2$^+$ eMSC when cultured on polyamide/gelatin meshes[40]. TGF-β can independently, as well as in association with Wnt and NOTCH signaling pathways, regulates proliferation and differentiation of MSC[47,48]. Gene profiling of purified eMSC shows that there is an increased fold change in FRZB receptor indicating an interaction with the Wnt signalling pathway. Pluripotency genes are not present in freshly isolated eMSC[39] and we found that P6 eMSC did not express pluripotency markers nor did A83-01 treatment upregulate their expression.

Apoptosis or programmed cell death mainly results from activation of cellular caspases. The TGF-βR pathway also participates in apoptosis via SMAD activation, in association with Death associated protein 6 and TGF-βR inducible transcription factor[49,50]. In epithelial cells and hepatocytes, TGF-βR induces apoptosis and inhibits proliferation[49–51] through the activation of TGFβ-inducible early gene-1 (TIEG1) via phosphorylation of SMAD2/3 and formation of reactive oxygen species. TIEG1 inhibits cell growth and leads to apoptosis[49]. P6 eMSC treated with 1μM A83-01 showed significantly increased growth by preventing apoptosis compared to the untreated cells, however it is not known if TIEG1 is involved.

Human Lgr5$^+$ liver stem cell cultures in mouse culture medium was not supported for more than three weeks and revealed highly active TGF-β signalling. This was also mitigated by blocking with A83-01 which improved cloning efficiency with extended time in culture of the liver stem cells[52]. A83-01 is a selective inhibitor of TGF-βR type I ALK4/5/7. It has a thiourea group in its structure, conferring copper ion chelation properties. Thioureas prevent copper mediated oxidative cellular damage by chelating copper in the medium[53]. One of the potential mechanisms by which A83-01 acts in SUSD2$^+$ eMSC may be the prevention of spontaneous differentiation and apoptosis mediated via the copper chelating thiourea moiety. Our study also demonstrated for the first time that TGF-βR signalling is an essential negative regulator of SUSD2 expression through SMAD2/3 signalling in eMSC. In addition, the TGF-βR pathway is involved in regulating eMSC proliferation, senescence and apoptosis.

Conclusions

In summary we have shown that TGF-βR signaling is involved in eMSC cell fate *in vitro*. A83-01, a small molecule TGF-βR inhibitor, enhanced the expression of SUSD2 and CD140b, maintaining eMSC clonogenic phenotype during prolonged culturing, promoting cell proliferation and preventing apoptosis and senescence. Small molecules such as A83-01 that promote eMSC proliferation in the undifferentiated state may provide an approach for the expansion of undifferentiated MSC for use in tissue engineering and cell-based therapies.

Materials and Methods

Human endometrial tissue samples. The experimental protocols were conducted under the ethical guidelines according to the National Health and Medical Research Council (NHMRC) of Australia's National Statement on Ethical Conduct in Human Research. Human ethics approval was obtained from the Monash Health and Monash University Human Research Ethics committees. Human endometrial tissues samples were collected from pre-menopausal women who were undergoing endometrial curette or hysterectomy for non-endometrial pathologies and who were not taking any exogenous hormones for three months prior to the surgery, following informed patient consent.

Isolation and magnetic bead sorting of SUSD2$^+$ eMSC. eMSC were isolated according to our previously published protocol[14]. Briefly, endometrial tissues from hysterectomy sample were carefully scraped off the underlying myometrium. Both hysterectomy and curette tissues were mechanically minced and digested with 0.5% collagenase type I and 40μg/ml deoxyribonuclease type I (both Worthington Biochemical Corporation) in Dulbecco's modified Eagle's medium (DMEM/F12) for 90 and 60 minutes, respectively in a humidified incubator at 37 °C on a rotating MACSmix (Miltenyi Biotech). The tissue digest was filtered through 40μm cell strainer (BD Biosciences) to separate the epithelial gland fragments and undigested tissues. The red blood cells in the filtrate were separated from the single stromal cells by density gradient centrifugation using Ficoll-Paque (GE healthcare Bio-science). eMSC were obtained by incubating the stromal cells in Phycoerythrin (PE)-conjugated anti-human SUSD2 (10μg/ml, BioLegend)) in 0.5% FCS/PBS (bead medium) and anti-PE magnetic-activated cell sorting microbeads (Miltenyi Biotec) for 30 minutes each in the dark on ice. The conjugated pellet was resuspended in bead medium and applied to a Miltenyi column (Miltenyi Biotec, #130-042-201) in a magnetic

field. The separated cells, containing the SUSD2^{+} eMSCs in the column were eluted in bead medium and the cells counted.

Cell culture and assessment of cell proliferation. The SUSD2^{+} eMSC were initially maintained in DMEM/F12 medium containing 10% Fetal calf serum (FCS) (Invitrogen), 1% antibiotic-antimycotic (Life Technologies) and 2 mM glutamine and slowly changed to an in-house DMEM/F12 serum free medium supplemented with basic fibroblast growth factor (FGF2, 10ng/ml) and epidermal growth factor (EGF, 10 ng/ml) (SFM) at 37 °C in 5%O_2/5%CO_2/90%N, as described previously[22]. The cells were seeded at 5000 cells/cm^2 density at subsequent passages in fibronectin (10 ug/ml) pre-coated culture flasks. Cell proliferation assays were performed at passage 3 by seeding 1000 cells in 100 μl SFM per well in fibronectin pre-coated 96-well plates with or without A83-01, concentrations varying from 0–10 μM. Media was changed every second day and contained A83-01 at the same concentration. Following 7 days of culture, 20 μl of MTS tetrazolium reagent (Promega) was added to each well and incubated for 2 hours and the soluble formazan product was quantified using a micro plate reader (SpectraMax Plus384; Molecular Devices) at 490 nm. Further experiments were done at passage 6 where the cells were separated into two groups, one group was treated with 1μM A83-01 and the control with (0.01% DMSO) vehicle. The data was normalised to the control and reported as a percentage.

Immunophenotyping. eMSC were trypsinised with TrypLE™ (Life technologies, #12604-021)) and resuspended at 10^5 cells/tube. Cells were washed with 5% heat-inactived newborn calf serum in DMEM (bench medium) and incubated with PE-, APC- or FITC-conjugated primary antibodies or matched-isotype controls in bench medium for 30 minutes in the dark on ice. Primary antibody used was CD146 (1:1 supernatant, clone CC9, kind gift from Prof David Haylock CSIRO). PE-conjugated antibodies were SUSD2 (1:20, Biolegend, #327406), CD140b (1:20, R&D systems FAB1263P) and CD271 (1:20, Miltenyi Biotec). APC-conjugated antibody was CD90 (1:20, BD Pharmingen). Isotype control antibodies at the same concentration as the primary antibody were included for each run and were used to set the electronic negative control gate on the flow cytometer. Finally, cells were washed with bench medium and fixed with 4% paraformaldehyde (PFA) in 2%FBS/PBS. Samples were analysed using a MoFlo Flow Cytometry (Beckman Coulter) and Summit software (version 5.2., Beckman Coulter).

Immunofluorescence microscopy. Passage 6 (P6) eMSC were cultured on coverslips with or without 1 μM A83-01 for 7days and then fixed in 4% PFA followed by protein block (Dako, X0909) for 10 minutes each at room temperature with washing in between with PBS. PE-conjugated SUSD2 antibody (1:200, BioLegend, #327406) in 2% FCS/PBS was incubated for 2 hours at room temperature in dark. Isotype control IgG1 antibody was used as a negative control. Hoechst 33258 (1:2000, Molecular Probes) was used to visualise nuclei. Images were visualised and photographed using a Delta Vision microscope, and analysed using ImageJ software (ImageJ-win32.Ink).

Immunoblotting. Cell lysates were prepared using lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% triton X-100) with mini protease inhibitor cocktail tablet (Roche) and phosphatase inhibitor sodium vanadate (2 mM). The following antibodies were used: anti-SMAD 2/3 antibody (#3102S), antiphospho-SMAD 2/3 (#8828S), horseradish peroxidise conjugated secondary antibody (#7074S) from Cell Signalling Technology. The specific protein was detected by treating the membrane for two minutes with enhanced chemiluminescence (# 133406, Abcam) which provides the HRP substrate, and capturing the signal in X-ray films.

Quantitative RT-PCR. RNA was isolated using PureLink® RNA mini Kit (Life technologies, #12183018A) and further treated with DNase (PureLink™ DNase, Invitrogen) to obtain DNA-free total RNA. First-strand cDNA was synthesized using SuperScript III first-strand synthesis system (Invitrogen). 50 ng of cDNA was amplified and detected using TaqMan probes for *OCT4, NANOG* and *SOX2*, and SYBR Green Super Mix for *SUSD2, CD146, AOC3, MMP3, FRZB, DKK1, NOTCH3, NOTCH2* and *NESTIN*. The PCR conditions consisted of initial denaturation at 95 °C for 10 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds and annealing/polymerisation at 60 °C for 60 seconds. Primer sets are detained in Table 1. GAPDH or β-Actin was used as an endogenous control to normalise the target gene expression and fold change was calculated using the $2^{-\Delta\Delta CT}$ method.

Mesenchymal stem/stromal cell properties. To assess colony-forming ability, P6eMSC pre-treated and untreated with 1 μM A83-01 for 7 days were seeded at 50 and 100 cells/cm^2 on fibronectin-coated 100 mm culture dishes (BD Falcon) in SFM in a tri-gas incubator 5%O_2/5%CO_2/90%N for four weeks. The cells were then formalin-fixed for 10 minutes and stained with haematoxylin (AMBER SCIENTIFIC). The colonies were washed twice with distilled water and counterstained with Scott's tap water to develop the blue colour. Colony efficiency was calculated by counting the number of colonies divided by the number of cells seeded and the percentage determined.

To assess multipotency, the remaining cells were cultured in adiopogenic and osteogenic, and control medium (1% fetal calf serum) on 13-mm coverslips, and for chondrogenic differentiation the cells were cultured as 3D pellets in chondrogenic induction media for 4 weeks at 37 °C in 5%CO_2/5%O2 as

GENE	PRIMER SEQUENCE
OCT4	F:CAGTGCCCGAAACCCACAC
	R: GGAGACCCAGCAGCCTCAAA
NANOG	F: TAATAACCTTGGCTGCCGTCTCTG
	R: GCCTCCCAATCCCAAACAATACGA
SOX2	F: ACACCAATCCCATCCACACT
	R: GCAAACTTCCTGCAAAGCTC
SUSD2	F: AGAGCTGGATGGACCTGAAA
	R: ATGCCAGCATGATGGAGAC
CD146	F:GAAGCATGGGGCTTCCCAG
	R:CCTCCGGAGCTTTGTAGACG
AOC3	F:TCAGCTGGGAGAGGATTTGG
	R:CGGAAGTAGATGGAGTCGGC
MMP3	F:AGCAAGGACCTCGTT TTCATT
	R:GTCAATCCCTGGAAAGTCTTCA
FRZB	F:CCTGCCCTGGAACATGACTAA
	R:CAGACCTTCGAACTGCTCGAT
NESTIN	F:GAAACAGCCATAGAGGGCAAA
	R:TGGTTTTCCAGAGTCTTCAGTGA
DKK1	F:GATCATAGCACCTTGGATGGG
	R:GGCACAGTCTGATGACCGG
NOTCH2	F: GTTTGTGTGGATGGGGTCAA
	R: TCCACATCCTCTGTGCAGAA
NOTCH3	F:GGACCTGCCGTGGCTATA
	R:ACGTCGTCCTCACAGTTATCA
GAPDH	F:TGTGGGCATCAATGGATTTGG
	R:ACACCATGTATTCCGGGTCAAT
β-ACTIN	F:GGGCATGGGTCAGAAGGATT
	R:AGTTGGTGACGATGCCGTG

Table 1. RT-PCR Primers Used.

described previously[22]. To detect the differentiation, the cells were fixed with 4% PFA and incubated with 1% Oil Red O for adipogenesis, 4% Alizarin Red (pH 4.1) for osteogenesis and 1% Alcian blue (pH 2.5) on paraffin embedded sections (5 μm) of the micromass pellet for chondrogenesis. Stained cells were examined under an Olympus BX41 microscope (Olympus) and images were taken with 10X objective lens using the DP25 digital camera (Olympus).

Cell cycle analysis and apoptosis by flow cytometry. To assess the cell cycle status, P6 A83-01 treated and untreated cells were detached, pelleted and fixed in ice-cold 70% ethanol at 4 °C overnight. They were washed with 2%FBS/PBS and incubated with 50 μl RNAse (100 μg/ml, Sigma) at room temperature for 15 minutes. 200 μl of propidium iodide (PI) (50 μg/ml, Sigma P4170) was added and the cells were analysed immediately by flow cytometry using BD FACS Canto™ II on PI-linear scales. The data were analysed using Flow Jo 7.6.3.

To assess apoptosis, P6 A83-01 treated and untreated cell-pellets were stained with Annexin V-APC/PI kit following the manufacturer's protocol (#88–8007, eBioscience). Briefly, cells were trypsinised and resuspended in 100 μl binding buffer, 5 μl of Annexin V-APC solution was added to the cell suspension and incubated for 15 minutes at room temperature protected from light. Following washing with the binding buffer, 5 μl of PI was added to the cells suspended in 200 μl binding buffer and events immediately acquired by flow cytometry using BD FACS Canto™ II and analysed with Flow Jo 7.6.3.

Cell senescence by β-galactosidase and auto-fluorescence. Senescent cells were assessed by staining for beta-galactosidase activity. P6 A83-01 treated and untreated eMSC were cultured on coverslips for 7 days as described above, then fixed in 4% PFA for 10 minutes and stained in freshly prepared X-Gal (1 mg/ml in DMSO) staining reagent (5 mM $K_3Fe(CN)$, 5 mM $K_4Fe(CN)$, 2 mM $MgCl_2$, 150 mM NaCl) in citrate buffer at pH6 for 24 hours at 37 °C. The cells were washed twice with PBS and

counter stained with nuclear fast red (Sigma-Aldrich, 0.1% w/v) for 10 minutes, then examined under an Olympus BX41 microscope (Olympus). Images were taken with 10X objective lens using the DP25 digital camera (Olympus).

Statistical Analysis. Non parametric Friedman's test with Dunn's multiple comparison post hoc tests were used to test for multiple groups and Wilcoxon matched-pairs signed rank tests were used to test for statistical significance between treated and control groups. Data are presented as mean ± standard error of mean. Differences were considered statistically significant at $p < 0.05$.

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Author Contributions
S.G. performed the experiments, collected and assembly data, analysed the data, and wrote manuscript. C.E.G. conceived and designed the experiments, provided the financial support, helped with data analysis and interpretation, editing and final approval of manuscript. J.A.W. collaborated by designing, data analysis and interpretation and final approval of manuscript.

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Stem Cells in Endometrial Physiology

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Abstract

Human endometrial mucosa is a dynamically remodeling tissue, undergoing cyclical morphologic and functional changes in response to fluctuating sex steroid hormones each menstrual cycle during a woman's reproductive life. Postmenopausal endometrium responds similarly to exogenous estrogen. Cyclical endometrial regeneration also occurs in nonmenstruating rodents, although to a lesser extent. The recent identification of rare populations of endogenous epithelial progenitor cells, mesenchymal stem/stromal cells (MSCs), the side population (SP) cells, and label-retaining cells (LRCs) suggests these stem/progenitor cell populations may play a key role in endometrial regeneration during menstrual and estrus cycles. This review summarizes the identification of epithelial progenitors, MSC, SP, and LRC, and discusses their contribution to endometrial tissue regeneration, maintaining tissue homeostasis, decidualization, and placentation. Markers for human endometrial MSC have been identified, revealing their perivascular location in both the functionalis and basalis layers. These markers also allow their purification from biopsy tissue and menstrual blood. These findings have advanced our understanding of normal endometrial physiology and will provide new insight into endometrial proliferative disorders (endometriosis, endometrial cancer). The ability to prospectively isolate endometrial MSC will enable their utilization in cell-based therapies for reproductive tract pathologies.

Keywords
- endometrium
- progenitor cells
- mesenchymal stem cells
- endometrial regeneration

Regenerative Potential of Human Endometrium

The human endometrium is a dynamically remodeling mucosa, undergoing 400 to 500 monthly cycles of morphologic and functional changes during reproductive life. Cyclical changes in sex steroid hormones regulates these processes to prepare a receptive endometrium for embryo implantation.[1] In the absence of an embryo, the functional layer sheds during menstruation, whereas the basalis layer is retained. A new functional layer regrows from the basalis, generating 4 to 10 mm of mucosal tissue in the first half of the subsequent cycle in response to rising circulating estrogen levels.[2]

The first step in human endometrial regeneration is the rapid repair of the surface epithelium, which occurs concomitantly with endometrial breakdown.[3] It occurs in a low estrogen environment when endometrial epithelial cells lack estrogen receptor-α (ER-α).[4] There is evidence for three mechanisms involved in epithelial repair. Epithelial cells from basal gland stumps migrate over the denuded surface,[5] small stromal cells interacting with a fibrinous matrix undergo mesenchymal-to-epithelial transition,[6] and a gene profiling study suggests that fragments of shedding endometrium are trapped during reepithelialization,[3] contributing to the biosynthesis of a new matrix through interactions between $\alpha_5\beta_1$ and $\alpha_3\beta_1$ integrin receptors on migrating epithelial cells and matrix fibronectin.[7]

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Once the luminal epithelium has resurfaced and circulating estrogen levels rise, rapid regeneration of endometrial glands, stroma, and vasculature occurs as the proliferative phase progresses. Endometrial stem/progenitor cells were postulated to generate a pool of rapidly proliferating transit amplifying epithelial, stromal, and vascular cells to regenerate new glands, stroma, and vasculature of the endometrial functionalis.[8]

Endometrial regeneration is also observed postpartum and in postmenopausal women treated with estrogen therapy.[9] Pregnancies have been reported following endometrial ablation or resection. These observations suggest that stem/progenitor cell populations reside deep in the basalis layer of the endometrium.[10] This level of cell loss and tissue regeneration without scarring also occurs in other regenerative tissues where stem/progenitor cell populations continuously replenish lost cells to maintain tissue homeostasis.[11,12]

Role of Stem/Progenitor Cells in Human Endometrial Regeneration

Adult stem cells are rare populations identified in almost all adult tissues,[13,14] and are clonogenic, self-renew, have high proliferative and differentiation potential, properties that can be functionally assessed in vitro.[10] Human endometrium contains small populations of epithelial progenitor cells, MSC and SP cells likely responsible for its monthly regeneration and maintaining tissue homeostasis.[10]

Human Endometrial Epithelial Progenitor Cells

Epithelial progenitor cells have been identified as clonogenic cells in endometrial single cell suspensions derived from hysterectomy tissue to ensure basalis glands were examined (►**Fig. 1a**).[15] Large epithelial colony-forming units (CFU) comprised 0.8% of epithelial cells and small CFU 0.14%.[15] Individual large epithelial CFU serially cloned three to four times when seeded at very low densities (10–20 cell/cm^2), indicating substantial self-renewal in vitro, which was not a feature of small epithelial CFU. Single epithelial cells initiating large clones underwent 34 population doublings at standard culture seeding densities (2,000 cells/ cm^2) producing 10^{11} epithelial cells, indicative of high proliferative potential. Epithelial cells from large CFU differentiated into large cytokeratin-expressing gland-like structures in three-dimensional (3D) culture provided an endometrial stromal cell feeder layer was present.

Markers of Endometrial Epithelial Progenitor Cells

No specific surface markers enabling the purification of human endometrial epithelial progenitor cells demonstrating classic adult stem cell properties have yet been reported. However, SSEA-1 (CD15), a Lewis X epitope of mouse embryonic stem cells and human neutrophils, distinguishes basalis from functionalis epithelial cells (►**Fig. 1a**).[16] SSEA-1 is also expressed in glands of postmenopausal endometrium.[16] Gene profiles comparing pre- and postmenopausal EpCAM-purified endometrial epithelial cells showed that premenopausal basalis epithelium was similar to that from postmenopausal women.[17,18] In 3D Matrigel cultures, SSEA-1$^+$ epithelial cells formed polarized spheroids indicating differentiation. SSEA-1$^+$ epithelial cells have some properties of adult stem/progenitor cells, such as longer telomeres and greater telomerase activity.[16] However, it is not known whether clonogenic, self-renewing epithelial cells are found within the SSEA-1$^+$ population. The future identification of epithelial progenitor markers will enable the examination of their role in endometrial regeneration in normal cycling endometrium and also in gynecologic disorders associated with abnormal epithelial proliferation: endometrial cancer, endometriosis, and adenomyosis.

Human Endometrial Mesenchymal Stem/stromal Cells

Mesenchymal stem/stromal cells (MSCs) are defined as plastic adherent clonogenic cells with a characteristic surface phenotype and capable of differentiating into multiple mesodermal lineages generating adipocytes, osteoblasts, and chondrocytes in vitro.[19] A rare population (1.25%) of putative endometrial stromal stem/progenitor cells were first identified as stromal CFU in cycling endometrium.[15] Stromal CFU were of two types: large (0.02%) and small (1.23%). Cells initiating large CFU underwent serial cloning at very low seeding densities (10–20 cell/cm^2) at least three to four times but small CFU did not.[20] Stromal CFU were found at all stages of the menstrual cycle and in postmenopausal endometrium, suggesting a role in regenerating the stroma.[21] Single cells initiating large stromal CFU underwent 30 population doublings when cultured at higher density, producing more than 10^{11} cells, six to seven orders of magnitude more than small CFU.[20] Large CFU differentiated into adipocytes, osteoblasts, myocytes, and chondrocytes, and expressed typical MSC markers, indicating MSC properties.

Freshly isolated endometrial stromal cells also generated CFU in serum-free medium containing FGF2, EGF, PDGF-BB, or TGF-α, indicating their corresponding receptors were expressed and functional on stromal CFU. These growth factors likely contribute to the many factors defining the endometrial niche in vivo that maintains cellular homeostasis. Stromal CFU activity was significantly lower in serum-free-compared with serum-containing medium, highlighting the unknown factors present in the serum that regulate endometrial cell proliferation in vitro.[21] A fibronectin matrix–enhanced endometrial MSC (eMSC) (CD140b$^+$CD146$^+$) attachment and FGF2 and EGF supplementation of serum-free medium augmented eMSC proliferation in 5% O_2 (physiologic normoxia).[22]

Markers of Human Endometrial MSC

The identification of specific surface markers of clonogenic stromal cells is necessary to demonstrate their location in tissues, purify them for characterizing their properties and genomic signature, and to harness their therapeutic potential. Coexpression of CD140b (PDGFRβ) and CD146 and a single perivascular marker, SUSD2 (W5C5 antibody), have been used to purify human endometrial eMSC.[23–25] These markers identified a perivascular location closely associated with endothelial cells in endometrium, pointing to a likely pericyte

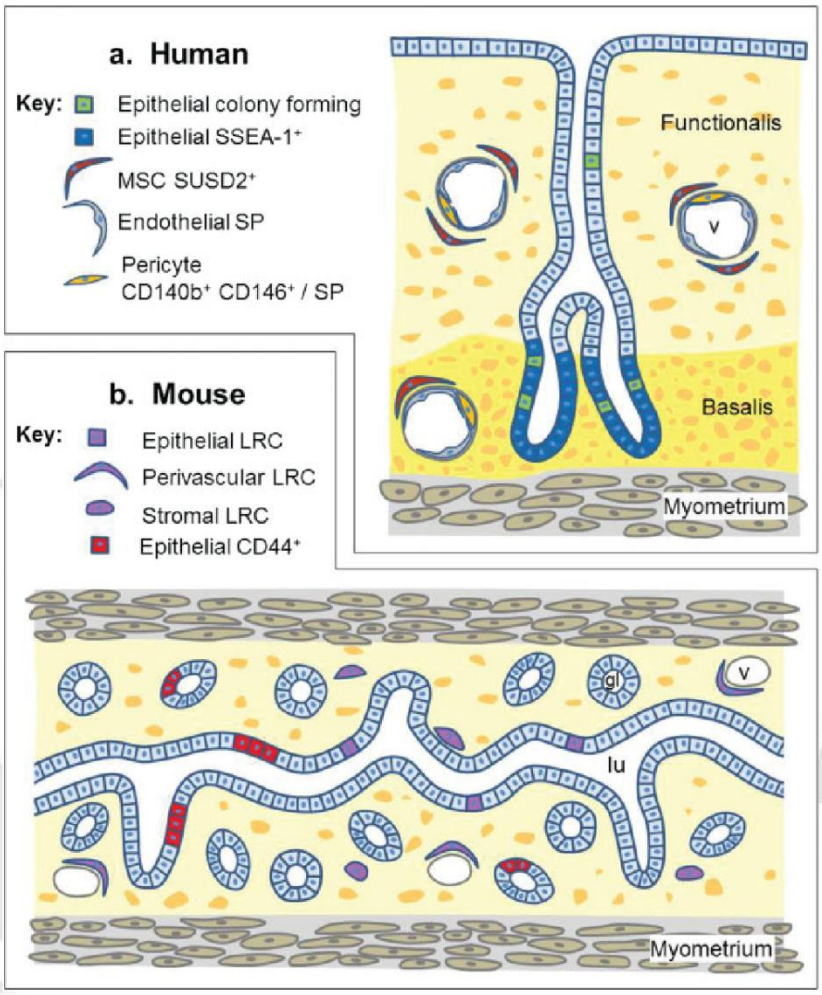

Fig. 1 Schematic showing location of the different stem/progenitor cells types identified in human and mouse endometrium. (**a**) In human endometrium, colony-forming epithelial progenitors are postulated to be present mainly in the basalis layer. SSEA-1 (stage-specific embryonic antigen 1) distinguishes basalis from the functionalis epithelial cells. SUSD2$^+$ mesenchymal stem/stromal cells with in vitro and in vivo mesenchymal stem/stromal cell (MSC) properties are located in the perivascular region. PDGFRβ/CD140b$^+$CD146$^+$ endometrial MSCs (eMSCs) are pericytes. Side population (SP) cells are a heterogeneous population comprising CD31$^+$ endothelial cells and CD140b$^+$CD146$^+$ pericytes. (**b**) In mouse endometrium, epithelial label-retaining cells (LRCs) are located in the luminal epithelium whereas stromal LRC are present in the perivascular region and in endometrial stroma. CD44 identifies the luminal and glandular epithelial/progenitor cells with the ability to generate glandular structures in vivo. Abbreviations: gl, gland; lu, lumen; v, vessels.

identity of human eMSC (►**Fig. 1a**).[23,25] Perivascular SUSD2$^+$ cells are found in the functionalis and basalis layers, indicating they are shed in menstrual blood and can be harvested from endometrial biopsies.[8] SUSD2$^+$ cells differentiate into smooth muscle cells (SMCs) and fibroblasts when cultured in tissue engineering constructs.[26] SMC and fibroblasts generate extracellular matrix that provides tissue elasticity and creates a physiologic environment, demonstrating the therapeutic potential of eMSC.[26]

The ultimate proof of adult stem cell activity is to show the cell's ability to reconstitute tissue in vivo using xenograft models. Transplantation of unfractionated human endometrial epithelial and stromal single-cell suspensions (5×10^5 cells) beneath the kidney capsule of ovariectomized and estrogen-supplemented immunocompromised NOG mice reconstituted functional endometrium comprising cytokeratin$^+$CD9$^+$ glandular structures, CD10$^+$CD13$^+$ stroma, and α-SMA$^+$ myometrial layers.[27] The endometrial xenografts responded to cyclical exogenous sex hormones producing tortuous glands and differentiated decidual cells followed by large blood-filled cysts suggestive of menstruation. Human SUSD2$^+$ eMSC transplanted under the kidney capsule of NSG (equivalent to NOG) mice generated human vimentin$^+$ endometrial stroma-like tissue and migratory endothelial

cells in the kidney parenchyma[25] similar to xenografted endometrial SP cells.[28] These studies demonstrate that SUSD2$^+$ eMSC contribute to the regeneration of functionalis stromal fibroblasts and blood vessels each menstrual cycle.

Gene expression profiling of freshly isolated CD140b$^+$CD146$^+$ eMSC showed their similarity to endometrial fibroblasts (CD140b$^+$CD146$^-$), but also 762 differentially expressed genes.[29] Principal components analysis confirmed their relatively close relationship compared with endothelial cells (CD140b$^-$CD146$^+$), indicating that fibroblasts are a lineage cell of eMSC.[29] The gene profile of human eMSC highlights their pericyte identity as genes involved in angiogenesis, inflammation, and immunomodulation were identified as signature eMSC genes, together with steroid hormone/hypoxia response, cell communication, proteolysis, self-renewal, and multipotency genes.[29] A secretome and RNA seq analysis of passage 2 decidualized SUSD2$^+$ eMSC revealed increased immunomodulatory and trophic factor genes, chemokine ligand-7, leukemia inhibitory factor (LIF), and CXCR4 receptor, but lower MHC class I and no class II genes compared with SUSD2$^-$ endometrial fibroblasts.[30] Collectively these data suggest that eMSC are clonogenic, multipotent pericytes, capable of responding to hormonal changes during the menstrual cycle and having roles in regenerating the endometrial stroma and vasculature on a monthly basis. eMSC are also responsible for optimal endometrial stromal decidualization to support embryo implantation and early placentation.[30] The immunomodulatory factors secreted by eMSC confirm their likely immunosuppressive properties similar to the bone marrow (BM) and adipose tissue MSC[31] and indicate a role for eMSC in allowing the survival of the implanted allogenic embryo.[30]

Human Endometrial Side Population Cells

The side population (SP) is considered a universal marker for adult stem cells in a variety of species, organs, and tissues.[32] The SP are identified by a low fluorescent phenotype due to efflux of Hoechst 33342 dye via cell membrane transporter, adenosine triphosphate (ATP)–binding cassette subfamily G member 2 (ABCG2) highly expressed in SP cells.[33]

SP cells have been reported in freshly isolated and cultured human endometrial cells.[28,34–36] The percentage of endometrial SP shows menstrual cycle-associated changes, being highest in the early proliferative phase, decreasing in the early secretory phase, and lowest in the late secretory phase,[28,34,36] suggesting that transit-amplifying cells and differentiated cells increase from the proliferative to the secretory phase.

There are differences in CFU activity between cultured and freshly isolated endometrial SP cells.[28,34,36] Cultured endometrial SP cells have higher CFU activity than non-SP or main and replicative population (MRP) cells,[34,36] while fresh SP cells had lower CFU activity.[28] Differences in cell cycle status between freshly isolated and cultured SP cells (G0 and G1/S/G2/M, respectively) account for this differential CFU activity.[36] The quiescence of freshly isolated SP cells accords with adult stem cell properties.[37] It is also possible that in vitro CFU and differentiation assays do not provide a microenvironment or stem cell niche for maintaining adult stem cell properties, resulting in differences between laboratories.

Cultured SP cells negative for both epithelial (CD9) and stromal (CD13) markers differentiated into CD9$^+$E-cadherin$^+$ gland-like organoids and CD13$^+$ stromal clusters in long-term Matrigel cultures.[34] Estradiol and progesterone also induced cultured SP cells to decidualize in vitro.[36] Freshly isolated SP cells also gave rise to epithelial, stromal, and endothelial cells in vitro.[28] Although differentiation is an important stem cell property, the SP is heterogeneous, presumably containing stem/progenitor cells of each endometrial cell lineage. Indeed, the endometrial SP contained CD326 (EpCAM$^+$) cells (epithelial, 27%), CD10$^+$ cells (stromal, 14%), CD31$^+$ cells (endothelial, 51%), CD34$^+$ cells (endothelial, hematopoietic stem cell, 46%), and CD146$^+$ cells (endothelial, MSC, 25%).[38] Of these lineages, CD31$^+$, CD34$^+$, CD146$^+$, and CD140b$^+$CD146$^+$ cells (eMSCs) were significantly more abundant in endometrial SP cells than in MRP cells,[38] substantiating the endothelial and MSC-like potential of endometrial SP cells.

In vivo xenotransplantation of freshly isolated endometrial SP cells under the kidney capsule of immunodeficient NOG mice generated endometrium-like tissues with a well-delineated gland structure, although the reconstitution rate was low (8%).[28] No reconstitution was observed with xenotransplanted MRP cells.[28] In a competitive reconstitution assay between fluorescent and luminescent protein–labeled endometrial SP or MRP cells and nonlabeled unfractionated endometrial cells, the labeled SP-derived cells were more abundantly distributed in the glands, stroma, and endothelium of the reconstituted endometrium compared with MRP-derived cells.[38] These data suggest multiple differentiation potential of endometrial SP cells in vivo, although multiple stem/progenitor populations are likely present. The inclusion of unlabeled, unfractionated endometrial cells in this reconstitution assay provides an appropriate microenvironment or stem cell niche, which substantially improved the low endometrial reconstitution rate by SP cells alone. Surprisingly, a cell line derived from freshly isolated SP cells reconstituted endometrial-like tissue at 100% success rate.[35]

ABCG2 is highly expressed in SP cells.[32] Endometrial ABCG2$^+$ cells therefore largely correspond to SP cells and are located in and near the vascular wall of small endometrial vessels in both functional and basal layers.[28] The similar perivascular location of eMSCs and some endometrial SP cells (►**Fig. 1a**) indicates that endometrial SP cells contain a small but significant population of eMSCs.[38] It also suggests that multiple stem/progenitor cell types are present in the endometrial SP.

Investigation of the endometrial SP is hampered by its heterogeneity and low clonogenicity making single cell culture unrealistic. However, an increasing body of evidence indicates that endometrial stem/progenitor cells are enriched in the SP fraction. Further studies are needed to elucidate the identity and detailed role of SP cells in endometrial physiology and pathology.

Menstrual Blood Mesenchymal Stem/Stromal Cell–Like Cells

The perivascular location of eMSC in the functionalis suggests that MSC would be detected in the menstrual blood.[8] Indeed,

many groups have cultured MSC as the plastic adherent cells from menstrual blood in a similar manner to the bone marrow MSC.[39,40] These cultures comprise eMSC and stromal fibroblasts and rarely contain epithelial cells,[41] emphasizing the basalis location of endometrial epithelial progenitors. Cultured menstrual blood cells have been redifferentiated into the decidual cells.[42] Most research on menstrual blood cells has focused on their broad differentiation capacity into ectodermal, mesodermal, and endodermal lineages and their potential use in cell-based therapies.[43]

Role of Bone Marrow–Derived Cells in Endometrial Regeneration

Bone marrow (BM)–derived cells appear to migrate to many organs and differentiate into tissue-specific cells.[44] However, the concept of BM stem cell transdifferentiation is contentious.[45] Evidence of a BM-derived contribution to endometrial tissue comes from studies tracing donor-specific markers following BM transplantation in patients and experimental animals. The Y-chromosome is used as a donor marker in sex-mismatched transplants, or HLA-type mismatches in patients[46] and transgenic reporters in mice.[47,48] Endometrial BM-derived cells are identified by coexpression of donor and endometrial markers, in the absence of BM-derived immune cell markers. Rates of BM-derived cell engraftment into the endometrium vary from less than 1% to 48% for epithelial cells and 52% for stromal cells, depending on the context and method of detection.[46,49–53] In animal models, uterine injury, but not hormonally driven regeneration, reportedly increases, whereas granulocyte colony–stimulating factor and cigarette smoke exposure reduce endometrial engraftment.[50] BM-derived endometrial endothelial cells have also been described.[54,55]

BM harbors rare populations of the hematopoietic stem cells (HSCs), MSC, and endothelial progenitors (EPC), all of which could incorporate into endometrial tissue.[10] A transgenic mouse model tracing $CD45^+$ cells of HSC lineage identified BM-derived endometrial epithelium in a small number of cells in a small number of animals,[47] supporting an HSC origin for some endometrial epithelial cells. However, HSC-derived endometrial stroma was not observed in patients and baboons transplanted with mobilized and purified $CD34^+$ HSCs. Also arguing against a role for HSC, granulocyte-stimulating factor-mobilized bone marrow HSCs reduced the engraftment of BM-derived stromal cells in mouse endometrium.[50] Intravenous transplantation of irradiated mice with either freshly isolated bone marrow MSC or EPC showed contributions to the epithelium, stroma, and endothelium, whereas an HSC progenitor population contributed to the stroma and endothelium.[48] Freshly isolated MSC or EPC and cultured BM-derived hypoblast-like stem cells also enhanced endometrial regeneration. Coincident irradiation injury to the ovary will, however, affect estrogen-driven endometrial regeneration. Though small numbers of labeled BM cells were observed in the endometrium of the transplanted mice, their incorporation into the ovary and their subsequent effect on ovarian function remain unknown. The variability in detected rates of endometrial engraftment and apparently conflicting results in these studies reflect the technically challenging nature of definitively identifying BM-derived epithelial and stromal cells, which has also proven problematic in the lung, liver, and kidney.[56] Further careful investigation is required to clarify the extent of nonhematopoietic BM-derived cell engraftment in the endometrium and its biological significance in endometrial regeneration and repair.

Role of Endometrial Stem/Progenitor Cells in Mouse Endometrial Regeneration

Label-retention assays have identified quiescent or slow cycling stem/progenitor cells in mouse endometrium. A label (e.g., nucleoside analogue BrdU) is incorporated into proliferating endometrial cells during development or periods of remodeling or repair. A chase period of days to weeks dilutes the label to undetectable levels in actively dividing cells while label is retained in populations of quiescent or slow cycling cells that may be stem/progenitor cells. Label-retaining cells (LRCs) are dynamic, and the timing and context of labeling and length of chase determine which cells incorporate and retain label.[57]

Both stromal and epithelial LRC have been identified in mouse endometrium. Stromal LRC typically persist longer than epithelial LRC reflecting higher proliferation rates of the epithelium.[58,59] Stromal LRC are a heterogeneous population some of which express ER-α[60] or the stem cell marker Sca-1.[61] Some stromal LRC are perivascular, expressing smooth muscle actin-α suggesting they are pericytes or mural cells (►**Fig. 1b**).[58,62] Epithelial LRCs are found in the luminal epithelium (►**Fig. 1b**) and, unlike most endometrial epithelial cells, do not express ER-α.[58] However, labeling of adult regenerating endometrium with a short chase (4.5–8.5 days) also yields glandular LRC expressing ER-α.[63] Stromal and luminal epithelial LRCs have been implicated in estrogen-mediated endometrial regeneration,[58,60] glandular LRC in estrogen-independent epithelial repair,[62,63] and stromal LRC in postpartum endometrial repair.[61]

Cell surface markers have been sought for stem/progenitor cells in mouse endometrium. CD44 is a widely expressed multifunctional cell surface protein expressed by endometrial epithelial cells with enhanced capacity to generate glandular structures in an in vivo assay.[64] $CD44^+$ epithelial cells do not express ER-α or progesterone receptor, and survive in hormonally deprived endometrium where they display Wnt pathway activation that may be related to their persistence. A combination of estrogen and progesterone drives the expansion of $CD44^+$ epithelial progenitors, presumably through an indirect mechanism as they lack steroid hormone receptors. The relationship between epithelial LRC and $CD44^+$ epithelial progenitors is not clear.

Summary

Endometrial stem/progenitor cells are present in the endometrium and likely play a vital role in regulating dynamic changes in tissue homeostasis during normal endometrial

function. Investigation of the contribution of BM-derived endometrial cells has yielded conflicting results and needs further stringent investigation. Furthermore, the role of BM-derived endometrial cells in endometrial physiology and function is not known. The identification of markers for human eMSC has revealed their perivascular location in both functionalis and basalis. These markers enable their purification and investigations of their role in endometrial regeneration and decidualization. Identification of human endometrial epithelial progenitor cell markers is eagerly awaited and will increase our knowledge on their role in endometrial physiology. Markers of endometrial epithelial progenitors and eMSCs will enable their therapeutic use in disorders affecting women's health.

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Commentary

Endometrial Mesenchymal Stem/Stromal Cells, Their Fibroblast Progeny in Endometriosis, and More[1]

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The human endometrium is a highly regenerative tissue, undergoing monthly cycles of growth, differentiation, and shedding during a woman's reproductive life. Resident mesenchymal stem/stromal cells (MSC) have recently been identified and characterized in human endometrium by numerous investigators (reviewed in [1]) and likely have a key role in its regenerative capacity. In many of these investigations, some or all of the classic properties of bone marrow MSC [2] have been demonstrated: clonogenicity, multipotency (differentiation into mesodermal lineages, adipocytes, osteocytes, and chondrocytes), and a surface phenotype that distinguishes them from leukocytes, hematopoietic, and endothelial cells. Others have shown their self-renewal in vitro [3] and ability to reconstitute endometrial stromal vascular tissue in vivo [4–6].

Specific markers enriching for the most highly clonogenic endometrial MSC (eMSC), MCAM (CD146) PDGFRB , have revealed their in vivo identity as pericytes [7], and a single marker, SUSD2, as perivascular cells [6] in both the functionalis and basalis layers of the endometrium. Likewise, these and several other markers have identified the in vivo perivascular niche of MSC in many other tissues [8], including bone marrow, placenta, and umbilical cord, and have been used to purify MSC from stromal fibroblast populations also present in these tissues [9]. Similar to bone marrow fibroblasts [10], endometrial stromal fibroblasts (eSF) also exhibit MSC properties [11], except they are weakly clonogenic, generating small, non-self-renewing cell clones, and lack proliferative capacity or the ability to generate neotissue in vivo [10]. Their presence contributes to the heterogeneity of MSC cultures. In response to progesterone, eSF differentiate into decidual cells, which have essential roles in the establishment and maintenance of pregnancy [12]. In contrast to bone marrow MSC, SUSD2 and CD146 PDGFRB eMSC differentiate into decidual cells in vitro [13–15], indicating a unique property of eMSC.

The perivascular location of eMSC in the endometrial functionalis indicates that they are shed in menstrual blood and may have a role in initiating endometriosis, which is thought to result from retrograde shedding of menstrual debris into the peritoneal cavity [1]. In women with endometriosis, eSF are progesterone resistant, likely contributing to infertility associated with this condition.

MSC are an attractive cell type for regenerative medicine due to their anti-inflammatory, immunosuppressive, and tissue reparative properties, with ~600 clinical trials registered on clinical trials Web sites for a wide range of immune and degenerative disorders [16]. Despite this, the fate and mechanism of action of MSC are poorly understood, confounded by the spontaneous differentiation of MSC to fibroblasts during culture expansion and frequent use of heterogeneous cell preparations of nonpurified fibroblast preparations for clinical application [17]. Allogeneic menstrual blood-derived stromal fibroblasts are being trialed for congestive cardiac failure [18], and autologous eMSC are being developed as a cell-based therapy for urogynecological applications [19].

In a recent issue of *Biology of Reproduction*, an elegant gene profiling study combined with hierarchical clustering and principal component analysis [20] identifies a unique set of lineage genes for eMSC and eSF. Comparison of these in vivo (freshly isolated) phenotypes with their short- and long-term clonal culture counterparts by Barragan et al. confirms the lineage commitment of eMSC to eSF, demonstrating that endometrial pericytes are the lineage precursors of eSF in vitro [14]. The authors also demonstrate that eMSC from women with endometriosis exhibit progesterone resistance that is inherited by their eSF progeny. In contrast, the prominent pro-inflammatory phenotype of endometriosis eSF is not inherited from eMSC but rather induced by unidentified in vivo niche factors. Finally Barragan et al. [20] demonstrate that eMSC rapidly and spontaneously differentiate into eSF in culture, adopting the lineage genes of eSF, and that this process occurs with less fidelity in eMSC derived from endometrium of women with endometriosis. Just as the identification of specific markers for purifying eMSC and eSF has opened the door to investigating their respective roles in endometrial physiology and pathophysiology [1], the discovery of sets of lineage genes for these cells by Barragan et al. opens new avenues of research designed to gain deeper insights into their function at the transcriptional level in both health and disease.

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Barragan et al. [20] provided an in depth study designed to define the transcriptional phenotype of eMSC and eSF in vivo and after short- and long-term culture. The goal was to identify the cellular ontogeny of progesterone resistance characteristic of endometriosis to enable further characterization in in vitro studies. Principle component analysis clearly distinguished the freshly isolated from cultured cells, and hierarchical clustering segregated the gene profiles on cell type followed by disease, which reversed in long-term cultures, suggesting convergence of cell phenotypes and/or magnification of disease phenotype.

The study first determined a set of lineage signature genes for freshly isolated eMSC versus eSF from control samples, identifying 550 upregulated genes in eMSC and 1370 in eSF [20]. The majority of these lineage genes were conserved in the eMSC and eSF of endometriosis samples. These conserved eMSC lineage genes included pericyte markers, hypoxia-related genes, and genes involved in activated Notch and TGFβ signaling pathways, SLIT ligands, WNT inhibition, and various growth factors, chemokines, and cytokines. Conserved eSF lineage genes included phospholipase A2, membrane metallo-endopeptidase (CD10), hormone receptor and metabolism, growth factor, cytokine and receptor genes, ROBO receptors for SLIT ligands, and WNT and Hedgehog signaling pathway genes.

The Barragan et al. study [20] has made a major contribution to the MSC discipline, and the eMSC field in particular, by addressing the issue of spontaneous MSC differentiation into stromal fibroblasts and generation of heterogeneous cultures during expansion. In a comprehensive experimental design, the authors compared gene profiles of pure populations of freshly isolated eMSC (CD146 PDGFRB) and eSF (CD146 PDGFRB) with early and long-term primary clonal cultures of both cell types. After accounting for the altered expression of culture adaptation genes, 44% of the in vivo eSF lineage genes were still expressed in late passage cultures, providing a robust definition of the eSF molecular phenotype. Importantly, very few eMSC lineage genes were upregulated in eSF cultures, including markers (SUSD2, CD146) used to select eMSC populations. In contrast, highly purified eMSC spontaneously differentiated in culture, downregulating the expression of 81% of their in vivo lineage genes and upregulating 55% of eSF lineage genes until only 19 genes were differentially expressed between late passage eMSC and eSF cultures. This extensive phenotypic analysis confirms previous studies showing loss of SUSD2 expression in purified eMSC cultures [15, 21]. It also recapitulates findings for freshly isolated bone marrow MSC (bmMSC) purified using nerve growth factor receptor (NGFR, CD271) in which 35% of lineage genes were downregulated during subsequent culture in a much smaller study examining only 94 genes [22]. As for eMSC, a WNT receptor (*FRZB*) was downregulated during culture expansion of CD271 bmMSC. Several genes from the same family were also downregulated in both eMSC (*ANGPT2, BMP8A, CDH2, CDH6, TGFBR2*) and bmMSC (*ANGPTL4, BMP5, CDH5, TGFBR3*). Likewise, several eSF lineage genes were upregulated in cultured CD271 bmMSC, including *OMD, PDGF-C* and *-D, IGF2, SFRP4*,and*FABP4*. Recently, it was shown that spontaneous differentiation of freshly isolated SUSD2 eMSC into SUSD2 eSF during culture expansion was blocked by a small molecule TGFβ receptor inhibitor A83-01 [23] in serum-free medium lacking TGFβ, implying a role for autocrine TGFβsignaling. Under these conditions, inhibition of TGFβsignaling prevented apoptosis and senescence of eMSC. Concurring with these observations, Barragan et al. [20] showed that fresh CD146 PDGFRB eMSC expressed higher levels of the drivers of differentiation *TGFB-1* and *-2* ligands and the *TGFBR2*receptor than fresh CD146 PDGFRB eSF and that these genes were downregulated in eMSC in early and late cultures, implying that the TGFβ signal acts as a switch to initiate eMSC differentiation and subsequent apoptosis. These complementary studies highlight the importance of TGFβ signaling in maintenance of the eMSC phenotype.

Specific markers used for purifying perivascular MSC are frequently downregulated on culture expansion either due to spontaneous differentiation to stromal fibroblasts or culture adaptation. The surface marker *CD146*, *SUSD2*, amino oxidase, copper containing 3 (*AOC3*), and regulator of G-protein signaling 5 (*RGS5*) lineage genes of freshly isolated eMSC are lost during culture expansion [20, 23]. The bmMSC marker CD271 is also expressed in eMSC [6] and downregulated during culture expansion [23]. All are potential markers for monitoring eMSC cultures for the presence of undifferentiated MSC using flow cytometry. However, the percentage of CD271 , AOC3 , or CD146 cells is drastically and rapidly reduced and does not predict the percentage of clonogenic cells remaining in serum-free eMSC cultures [23] (Gurung and Gargett, unpublished results). In adipose MSC cultures, CD146 is cleaved by matrix metallo-proteinase 3 (MMP3) [24]. This mechanism may also operate during culture of eMSC because *MMP3*expression was markedly upregulated in eMSC cultures in the Barragan et al. study [20] and in A83-01-containing medium [23]. In contrast, the percentage of SUSD2 cells in eMSC cultures reflects their functionality (clonogenicity) and currently appears to be the most reliable marker for monitoring eMSC cultures [23], an important consideration for cell production for clinical applications. Barragan et al. [20] also found that SUSD2 expression remained low in control eSF cultures but was upregulated in eSF cultures derived from eutopic endometrium of women with endometriosis. The possible inclusion of endometriosis samples in another study may explain the appearance of SUSD2 cells in SUSD2 cultures [15].

The main purpose of the Barragan et al. study [20] was to determine whether eSF are the progeny of eMSC precursors and if they inherit progesterone resistance from eMSC or acquire this pathological change in situ as eSF. To answer these questions, the authors compared the molecular phenotypes of freshly isolated and cultured eMSC and eSF obtained from controls and endometriosis samples. In freshly isolated samples, there was considerable similarity in molecular phenotypes, with more than 95% of the top 200 in vivo lineage genes conserved in endometriosis samples, although there were fewer differentially expressed genes in endometriosis eMSC and eSF compared with controls. Of the 320 differentially expressed genes between freshly isolated endometriosis and control eMSC, 22 eMSC lineage genes were downregulated in endometriosis, including thrombospondin-4 (*THBS4*) and *FLT1*, and 61 eSF lineage genes were upregulated, including*PDGFRA, PDGFC, LOX*,and*WNT5A*. Pathway analysis revealed that the upregulated genes were associated with proteolysis and downregulated with colony formation, cell death, and survival, suggesting a role for eMSC in initiating endometriosis lesions when refluxed into the peritoneal cavity during menstruation. Of the 413 differentially expressed genes in freshly isolated eSF from controls and endometriosis samples, 22 eMSC lineage genes were upregulated, including*ANGPT2, FRZB*,and*TGFB2*as well as genes associated with inflammation (*CXCL2, IL8, NFKB1*). There were 62 eSF lineage genes downregulated, including*PLA2G7, IL17RB, DKK1*, and *FGF9*. Pathway analysis showed upregulated genes were associated with numerous pathways,

including inflammation, leukocyte trafficking, angiogenesis and epithelial cell function, indicating a striking pro-inflammatory phenotype of eutopic eSF. This key feature of endometriosis indicates the important contribution of eSF to the pathogenesis of endometriosis and associated infertility [25].

Barragan et al. also demonstrated a progressive change in phenotype of cultured eSF from endometriosis samples with a widening in differential gene expression compared with controls [20]. Only 33% of eSF lineage genes were expressed in eutopic eSF compared with 44% in controls, indicating diminished phenotypic stability in endometriosis eSF cultures. Also more eMSC lineage genes (36%) were retained in endometriosis eMSC cultures compared with control eMSC (23%), resulting in a total of 78 differentially expressed genes in long-term cultures from endometriosis samples compared with 19 in controls. This suggests that eMSC from endometriosis samples have reduced differentiation capacity, which may confer greater ability to initiate lesions in the peritoneal cavity. Despite the increased number of differentially expressed eMSC genes in endometriosis samples in late passage cultures, pathway analysis showed a narrow range of activated genes mainly in lipid metabolism and proteolysis, which may confer invasiveness.

For the first time, Barragan et al. demonstrated that progesterone resistance is a feature of cultured endometriosis eMSC, and that eSF inherit this inability to decidualize in vitro from its precursor, eMSC [20]. Both eMSC and eSF from long-term cultures of endometriosis samples were unable to secrete IGFBP1 in response to progesterone plus estrogen. This suggests that endometriosis is indeed a disorder of the endogenous endometrial stem/progenitor cell population [1], in particular eMSC. There does not appear a direct link between the susceptibility genes identified in genomewide associations studies [26] and the decidualization pathway, suggesting an acquired defect. Epigenetic changes in eutopic and ectopic endometrium are associated with key pathways in steroid hormone production and metabolism [27], but whether altered methylation of eMSC genes have a role is currently unknown. In contrast to endometriosis eSF inheriting a decidualization defect from their eMSC precursors, Barragan et al. showed that the pro-inflammatory phenotype of eSF was not acquired from eMSC in vitro, suggesting that eSF obtained their pro-inflammatory properties in the endometrial in vivo niche, possibly involving systemic mediators [20]. Finally the authors provide a model of the disease phenotype of eMSC and eSF in endometriosis that elegantly summarizes the numerous findings of their study.

In summary, the study by Barragan et al. makes excellent use of gene profiling, bioinformatics, and in vitro studies to generate and interpret important new information on the molecular phenotype and relationship between the pericyte eMSC and eSF, their respective roles in endometriosis and associated infertility, and much more. It shows that the decidualization defect of endometriosis eSF is inherited from eMSC, but not pro-inflammatory characteristics, which are acquired by eSF in vivo. The identification of lineage genes for eMSC and eSF and their comparisons between freshly isolated purified cell populations and their cultured counterparts also informs the MSC field, clearly revealing spontaneous differentiation of MSC to fibroblasts, at a time when the definition of MSC is being questioned. As with all well-designed discovery studies, it also opens new questions for further study. Questions such as what mechanisms lead to the decidualization defect in eMSC of women with endometriosis? Is this due to epigenetic changes known to occur in eutopic endometrium and endometriotic lesions [27], and if so, are the hypo- or hypermethylated genes specific to this stem/progenitor cell type? Or is there a role for micro-RNAs? Are there pharmacological approaches using small molecules or biologicals targeting eutopic eMSC that might promote appropriate differentiation to eSF without the decidualization defect? How is the pro-inflammatory phenotype conferred on eSF in the endometrial niche? Are the lineage gene signatures maintained in eMSC and eSF of ectopic lesions? And could gene profiling ectopic eMSC produce data supporting the concept of retrograde menstruation of endometrial stem/progenitor cells in the pathogenesis of endometriosis?

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SECTION IV

The role of bioengineering in the therapeutic applications of mesenchymal stromal cells

CHAPTER 41

Endometrial mesenchymal stromal cell and tissue engineering for pelvic organ prolapse repair

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Chapter menu

41.1 Introduction

Pelvic organ prolapse (POP) is a very common condition affecting millions of women worldwide. Recent notifications by the US Food and Drug Administration (FDA) warning of the high rate of complications with transvaginal placement of polypropylene (PP) mesh have led to withdrawal of many vaginal meshes from the market. This now severely limits the available treatment options for large numbers of women with POP. There is an urgent need for an effective therapy for this major hidden disease burden of women. This chapter will review recent studies in an emerging field in regenerative medicine that suggest a tissue engineering (TE) approach for the treatment of POP. It will detail new mesh designs that are more likely to succeed than the present PP meshes. It will describe the potential of cell-based therapy for POP, highlighting a newly identified source of mesenchymal stem/stromal cells (MSCs) from human endometrium. Characterization of endometrial MSCs (eMSCs) and the use of specific markers for their enrichment will be discussed. Approaches undertaken to produce eMSCs under good manufacturing practice conditions will be emphasized. Finally, the need for a large animal model of vaginal repair that can assess new TE constructs for a longer duration will be highlighted as a necessary step before the first clinical trials of a cell-based therapy can be commenced in women with POP. Figure 41.1 shows the anatomy of the human uterus, fallopian tubes, ovaries and vagina.

The uterus is made up of an external layer of smooth muscle called the myometrium and an internal layer called the endometrium. The endometrium has three layers: the stratum compactum, the stratum spongiosum (which make up the stratum functionalis), and the stratum basalis. The stratum compactum and stratum spongialis develop into the stratum functionalis during the first half of the menstrual cycle (proliferative phase).

41.2 Pelvic floor disorders

Pelvic floor disorders (PFDs) encompass stress urinary incontinence, fecal incontinence, and POP. The overall

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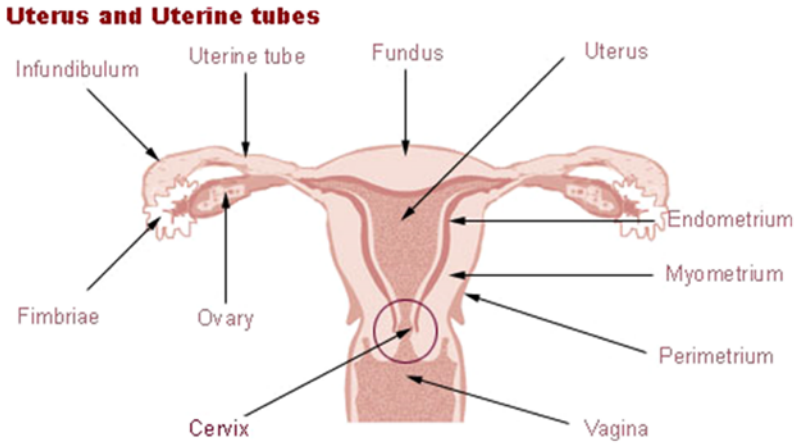

Figure 41.1 Anatomy of the human uterus, fallopian tubes, and vagina. This work is in the public domain in the USA because it is a work prepared by an officer or employee of the United States Government as part of that person's official duties under the terms of Title 17, Chapter 1, Section 105 of the US Code. http://training.seer.cancer.gov/ss_module09_cervix_corpus/cervix_unit01_sec01_intro.html.

prevalence of PFD in women is 35%, which increases to 52% for women above 70 years of age [1–3]. Women as young as 18 years of age are affected by this condition, and the prevalence increases significantly with age [4,5]. Major risk factors include pregnancy, vaginal delivery, and obesity. Although many pregnant women seek elective cesarean section to prevent PFDs, compelling evidence suggests that the incidence of PFD in women undergoing this elective procedure does not decrease compared with nulliparous women or those undergoing vaginal birth delivery [1,6].

41.3 Pelvic organ prolapse

POP is a major hidden burden affecting millions of women worldwide. POP is the downward descent or herniation of pelvic organs, including the bladder, bowel, and/or uterus, into the vagina due to a weakened or damaged pelvic floor support structures [7]. POP accounts for over two-thirds of PFDs [7]. The support structures of female pelvic organs comprise the endopelvic fascia of the vaginal wall, the suspensory ligaments, and the levator ani muscles (pelvic floor muscles) [3,7]. Pregnancy and vaginal delivery can injure these support structures, through damage or denervation to muscles [8], overstretching or tearing of the ligaments, and weakening of the endopelvic fascia. In addition to the immense stretching of the pelvic floor during vaginal delivery, 10–20% of women giving birth to their first child have prolonged labor, instrument delivery, a large infant weight, or an episiotomy, all of which contribute to pelvic floor injury leading to POP [9]. Subsequent pregnancies and child birth and aging further weaken these tissues. Symptoms of POP include urinary incontinence, bowel incontinence, and/or sexual dysfunction [9].

One in four women suffer from POP, for which surgical reconstruction is frequently required. Women have an 11–20% probability of requiring an operation for POP repair in their lifetime [4,10–12]. POP is the leading indication for hysterectomy in postmenopausal women [13], and in Australia approximately 22 000 POP repair operations are conducted every year. In the USA the figure is 300 000 per year [7]. Although the lifetime risk for POP is higher than breast or lung cancer [14], most women suffer in silence because of the embarrassing symptoms [15]. A survey showed that only one in three US women had reluctantly discussed bladder issues with their doctor because of a perceived stigma and associated embarrassment. With increasing rates of obesity, delayed first childbirth, and rising life expectancy, the disease burden related to POP in terms of decreased quality of life, social isolation, lost productivity, and the effects on families and health-care systems will be substantial [3]. A comparative study in the USA estimated that 17 million women were affected by urinary incontinence, and the cost was estimated to be $19.5 billion [16]. Long-term, safe, and more effective therapies and earlier interventions are needed for affected women in order to improve their quality of life.

41.3.1 Surgical treatment for pelvic organ prolapse

Depending on which organs and support structures are affected and the severity of POP, the ultimate treatment is reconstructive surgical repair of the vaginal wall transvaginally (colporrhaphy). Native tissue surgical treatment has a high failure rate, with 30% of cases requiring further surgery for recurrent POP and with the time frame between surgeries decreasing with increasing age [4]. This high failure rate of native tissue repair prompted clinicians to adapt commercial synthetic abdominal hernia meshes for POP repair. These meshes aimed to provide additional support of the damaged pelvic tissues and to improve long-term outcomes. Synthetic PP meshes were introduced in 1997 and gained FDA approval in 2004. Since then, surgical methods for repair of POP have increased in number and complexity. In the USA, mesh had

been used in a third of POP surgeries and inserted transvaginally in ~75% of cases [17]. These mesh-augmented repair operations delivered seemingly higher "cure" rates, particularly for anterior vaginal wall damage, compared with colporrhaphy using native tissue surgery [18–20]. More recently, however, serious complications, such as mesh exposure into the vagina, bladder, or bowel, mesh shrinkage, vaginal shortening, pain, dyspareunia, and infections, have been reported in as many as 10–15% of cases [21–23]. This alarming complication rate led to two notifications by the FDA in 2008 and 2011 warning of the complications with transvaginal placement of surgical mesh [17,24]. These notifications have led to the withdrawal of some vaginal meshes from the market by several leading device companies. There is now a real risk that successful treatment options for women will be reduced and ineffective for a large number of women with POP.

41.3.2 New meshes for treatment of pelvic organ prolapse

Meshes used for clinical purposes may either be biological or synthetic. Biological scaffolds are typically natural tissues such as autologous rectal fascia and fascia lata (the deep fascia of the thigh that encloses the thigh muscles), allogeneic, such as cadaveric tissue, or decellularized xenografts, such as porcine small intestinal submucosa (SIS). Synthetic meshes, predominantly fabricated from PP, are the most commonly used for POP repair. Biological materials can be used as an untreated, decellularized matrix or can be further stabilized by chemical cross-linking. In either case, they are largely biodegradable and have limited efficacy due to their variable degradation rate, lack of longevity, variable tissue integration and host response, and concerns over transmission of diseases. Synthetic meshes are often preferred over biological meshes for applications requiring long-term support of tissues because they have more consistent properties and there is a more predictable host response [25]. While currently used PP meshes provide good mechanical tensile support for the damaged vaginal wall, they are generally stiffer than the natural tissue, resulting in compromised distensibility, poor compliance, and less integration into vaginal fascial tissues [26]. In addition, synthetic materials, while normally inert and nonimmunogenic, can induce a relatively vigorous foreign-body reaction leading to fibrotic tissue deposition [27].

The ideal mesh for POP has not yet been designed [28]. Adverse effects associated with current meshes can lead to erosion where the implanted material protrudes into the vaginal, bladder, or bowel lumen. POP meshes should be biocompatible and provide sufficient mechanical strength to support damaged tissue, but should also be elastic and flexible enough to allow compliance, movement, and integration with the natural tissue [25,26].

The physical properties of mesh depend upon polymer composition of the material, filament type, weaving or knit pattern, pore size, weight, and coating [26,27]. The current range of meshes will vary in one or more of these properties. Evolution of mesh design has largely focused on reduced weight with accompanying large pore diameter, which is conducive to cell infiltration, new fibro-collagenous deposition, and tissue remodeling [26,29].

Our recent work has used these current design strategies to further improve the biomechanical and biological performance of new fabricated polymer mesh types. The *in vitro* structural and mechanical properties of three commercially available PP meshes were compared with these new fabricated meshes from alternative polymers [26]. One of these meshes fabricated from polyamide (PA) monofilament yarn was optimized with a pore size of 1000 μm and dip-coated with 12% porcine gelatin (PA/G) to provide a platform for delivering cells to the surgical site (Figure 41.2D). The new PA/G meshes were less stiff on uniaxial biomechanical testing, more flexible and elastic on multiaxial testing, and with lower bending rigidity than commercial PP meshes. These favorable properties allow the mesh to conform to the highly mobile human vaginal tissue, with a less predictable likelihood to erode into neighboring organs. *In vivo* evaluation of the fibronectin-coated PA/G mesh designs in a rat abdominal hernia model showed significantly improved tissue integration by promoting angiogenesis, exerting a moderate foreign body reaction and generating good collagen deposition for up to 90 days compared with commercial PP mesh [27]. This new mesh with superior biomechanical and biocompatibility properties may be a more suitable starting point for developing a TE construct for POP therapy.

41.4 Tissue engineering

TE is an emerging field in regenerative medicine that uses a combination of cells and biomaterials of synthetic or natural origin (Figure 41.2D and E) The goal is to replace or regenerate damaged or diseased human tissues and restore or improve the long-term outcomes of surgical therapy [26]. In the urogynecological field, preclinical studies using animal models and clinical trials are scarce and have predominantly focused on the use of cell-based therapies for stress urinary incontinence. There are few studies that have explored the potential use of cell-based or TE approaches as potential therapies for POP. We are proposing to use a novel cell source with newly designed meshes to generate a TE approach for treating women with POP (Figure 41.2). The following summarizes the *in vitro* studies and studies in preclinical animal models (Figure 41.2F).

41.4.1 Candidate cells for tissue engineering applications for pelvic organ disorders

41.4.1.1 Skeletal muscle-derived cells and muscle fibers

Murine skeletal muscle-derived stem cells (MDSCs) can differentiate into smooth muscle cells (SMCs) *in vitro* and express the pluripotency genes *OCT4* and *NANOG* [30]. MDSCs were grown on porcine SIS (Cook, Biotech®) and on a polymeric Vicryl mesh, and were found to stimulate vaginal tissue repair with reduced fibrosis in a rat model. It was postulated that MDSCs had

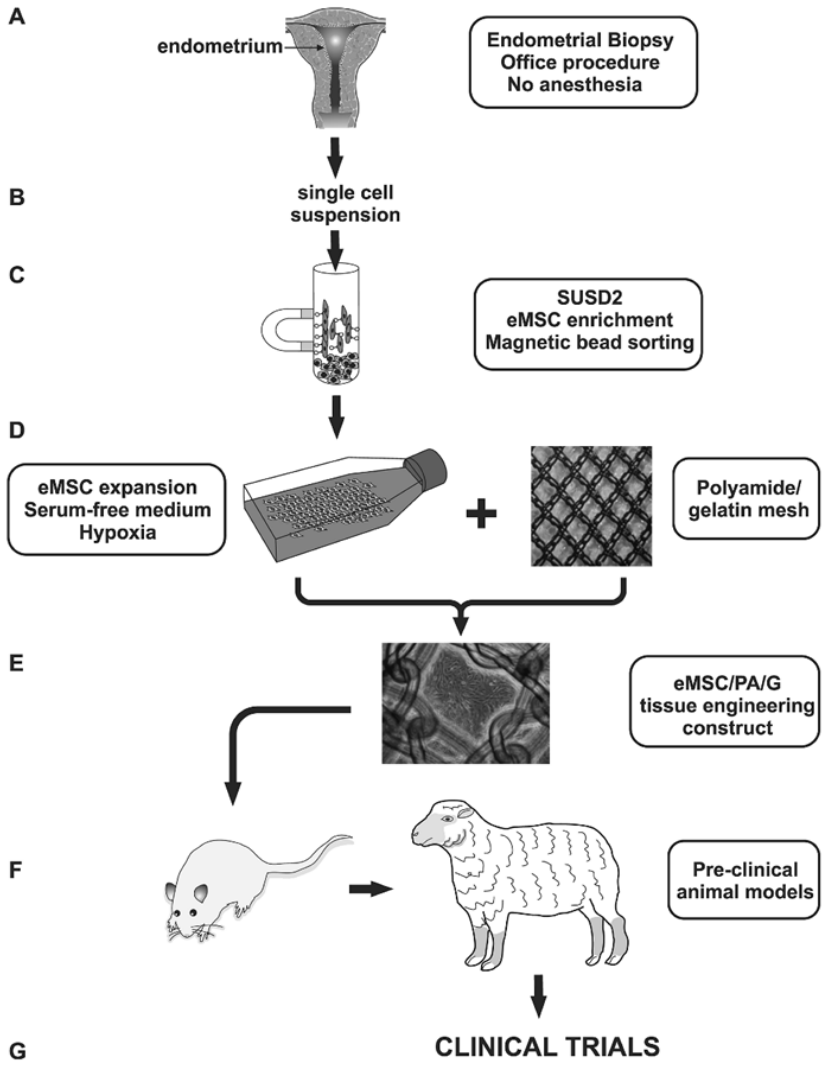

Figure 41.2 Schematic showing a TE approach for the treatment of POP with eMSCs. Endometrium is the mucosal lining of the uterus. It can be collected (A) by biopsy in an office-based procedure without the need for anesthesia. (B) Endometrial tissue is mechanically and enzymatically dissociated into a single-cell suspension and (C) eMSCs immunoselected using SUSD2 (W5C5) antibody by magnetic bead sorting. (D) eMSCs are culture expanded in serum-free medium in physiological (5% O_2) hypoxia and (E) seeded on fibronectin-coated PA/G composite mesh, which is cultured for a further 48 h before transplanting into (F) small animal models (rats) and a preclinical large animal model (sheep) for proof of concept and then (G) human clinical trials.

transdifferentiated into SMCs and stratified keratin 5^+ squamous epithelium when implanted into the injured vaginal tissue as a TE construct. α-smooth muscle actin (α-SMA) was used to evaluate the resulting tissue generated, but it could not discriminate between SMCs and myofibroblast differentiation. This is important, because the latter mediates the fibrotic process. In addition, it is unclear whether the differentiated cells had arisen from a stem cell precursor or a mixed population of heterogeneous cells. Further studies on the functional properties of the implants and their effects *in vivo* are required.

Fresh skeletal muscle fibers or cultured autologous skeletal-muscle-derived cells have also been seeded on synthetic biodegradable methoxy(polyethylene glycol)–poly(lactic–co-glycolic acid) meshes and implanted subcutaneously into the rat abdomen [31]. The TE constructs were well tolerated, and in contrast to the previous study the cultured skeletal muscle cells had disappeared by 8 weeks. However, there was generation of fragmented striated muscle tissue in the skeletal muscle fiber tissue construct [31], indicating superiority of fresh skeletal muscle fibers to culture-expanded skeletal muscle cells for surgical reconstruction in an animal abdominal wall model of POP repair surgery [31].

41.4.1.2 Fibroblasts

Human vaginal fibroblasts have also been suggested as an alternative cell source for a TE approach to treat POP. Vaginal

fibroblasts were expanded, added to a collagen solution, labeled with the fluorescent dye DiL and applied to biodegradable poly (D,L-lactic–co-glycolic acid) meshes [32]. The TE construct was implanted subcutaneously into mice, where a well-organized multilayered neofascia of collagen was generated. The DiL-labeled cells survived and were identified within the construct after 12 weeks, indicating the contribution from the human vaginal fibroblasts.

The feasibility of using autologous vaginal fibroblasts for the treatment of POP is attractive; however, POP vaginal fibroblasts are less than ideal. Vaginal fibroblasts from the prolapsed site in women with POP showed delayed collagen contractility and lower matrix metalloproteinase-2 production compared with fibroblasts from normal premenopausal women [33]. The immunoreactive collagen and elastin scores of the vaginal connective tissue from women with POP were higher than with normal vaginal tissue but contained fewer fibroblasts [34]. This increased collagen staining does not accurately reflect total collagen content, nor does it take into account alterations in the ratio of type III/type I collagen. A higher ratio, as found in women with POP, is characterized by mechanically inferior tissue, suggesting that pathological changes in vaginal wall connective tissue composition changes the quality and the functional ability of the vaginal fibroblasts, and this should be considered when opting to use them [33].

Buccal mucosal fibroblasts are a readily available cell source for TE. They express the typical MSC/fibroblast surface markers, but do not differentiate into adiopogenic and chondrocyte lineages and have limited osteogenic differentiation ability. Despite this, buccal mucosal fibroblasts produce collagen I *in vitro*, an important component of extracellular matrix [35]. Cultured buccal fibroblasts attach well in heat-annealed poly(L-lactic acid) (PLA) scaffold, produce collagen and elastin under intermediate stress forces *in vitro*, and have been suggested as a cell type for use in the treatment of POP [36]. However, *in vivo* work in an appropriate animal model is required to assess the utility and long term effects of buccal fibroblasts for tissue repair.

41.4.1.3 Mesenchymal stem/stromal cells

MSCs were first discovered in the bone marrow as an adherent, clonogenic, nonhematopoietic cell population that undergoes extensive proliferation in culture and differentiates into multiple mesodermal lineages, including fat, bone, cartilage, stroma, tendon, and muscle [37]. MSCs have since been identified in multiple organs, including adipose tissue, dental pulp, umbilical cord tissue, placenta, and endometrium [38,39]. MSCs are an attractive source of cells for regenerative medicine, as evidenced by the ~400 clinical trials now listed on clinical trial websites (such as ClinicalTrials.gov) and the 2000 or more patients who have received varying forms of MSC as experimental therapy without any serious adverse events [40,41]. A systematic review and meta-analysis of clinical trials involving MSCs given intravenously or intra-arterially showed systemically delivered MSCs to be safe [41]. Thus far there have been no clinical trials of MSC or cell-based therapy for POP. MSCs home to sites of tissue damage and act in a paracrine manner, secreting bioactive signaling molecules that promote angiogenesis, limit fibrosis, enhance anti-inflammatory and immune suppressive function, inhibit apoptosis, and induce tissue-specific progenitor cells to proliferate – processes that collectively repair damaged tissue [40,42]. In preclinical animal models, MSCs have also been delivered directly to damaged tissue in scaffolds as TE constructs for repairing bone, cartilage, fat, tendon, muscle, and connective tissues. Scaffolds provide structural support and sites for cell adhesion, allowing delivery and retention of large numbers of cells at the site of tissue injury [43]. In this mode the MSCs integrate and proliferate in the neotissue and/or have paracrine effects, modulating tissue repair without long-term engraftment [44]. MSCs seeded on to a scaffold deliver larger doses of secreted bioactive molecules locally to an injury site compared with systemic delivery of MSCs [42]. Preclinical studies are required to determine the efficacy of MSCs, their mechanism of action, and whether MSC-seeded materials are superior in regenerating tissues than materials alone [40].

In order to provide consistency among investigators in the definition of MSCs, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed minimal criteria in 2007 to minimize experimental variability in MSC research [45]. MSCs should be more than 95% positive for the surface markers CD90, CD105, and CD73 and negative for the hematopoietic lineage markers CD45, CD14, and CD11 as well as for the co-stimulatory molecules CD80 and CD40. However, MSCs defined by such methods still include heterogeneous populations with different levels of "stemness." Thus, the markers defined above are not suitable for the prospective isolation of MSCs from tissues. Furthermore, MSCs undergo spontaneous replicative differentiation and senescence during culture expansion, losing their stemness and regenerative ability despite expression of the listed phenotypic markers [46,47]. These markers fail to discriminate between undifferentiated MSCs and differentiated mature fibroblasts (see Chapter 30). Culture conditions, including seeding density, confluency, and growth factors, also have the potential to influence MSC phenotype marker expression.

Clinical translation of MSCs for cell-based therapies includes autologous and allogeneic sources. For autologous use, the easy accessibility of a cell source is vital. Common sources, such as bone marrow and adipose tissue biopsy, involve painful procedures for procurement and require some form of anesthesia. These sources often produce low yields. A new source of MSCs from human endometrium (i.e., eMSCs) has been proposed as an alternative autologous (or allogeneic) source because of their easy accessibility (Figure 41.2E). eMSCs can be obtained via endometrial biopsy in an office-based procedure, incurring minimal pain, scarring, or morbidity, and generally do not require an anesthetic [48–50].

41.5 Endometrium is highly regenerative and contains stem/stromal cells

The endometrium or mucosal lining of the uterus is a dynamic tissue. Following menstruation as much as 10 mm of endometrium is regenerated in the first half of the subsequent menstrual cycle. The endometrium then differentiates in preparation to receive an embryo in the second half of the cycle, and sheds if there is no pregnancy [51,52]. This repeats for 400–500 cycles of growth, differentiation, and shedding during a woman's reproductive life. Endometrial regeneration also occurs in the postpartum period and in postmenopausal women taking estrogen therapy [53,54]. This level of cellular turnover and scarless mucosal regeneration in the endometrium is similar to that observed in other highly regenerative tissues, such as the skin, gastrointestinal tract, and bone marrow [52], where resident stem/progenitor cells have known roles in maintaining tissue homeostasis [50].

41.5.1 Human endometrial mesenchymal stem/stromal cells

Key properties of any stem cell include the ability to form colonies from single cells, to self-renew into daughter stem cells, and to differentiate into one or more lineages and reconstitute tissue *in vivo*. Colony-forming unit (CFU) cells were first identified in stromal cell suspensions from human endometrium in 2004 [55]. These CFUs–fibroblast (CFU-Fs) comprised 1.25% of stromal cells. There was a range of clone sizes, but only the rarer large CFUs demonstrated classic MSC properties: self-renewal *in vitro* assessed in serial cloning assays, mesodermal lineage differentiation to adipocytes, chondrocytes, osteoblasts, and myocytes, and a characteristic surface phenotype [56]. There were variations in the clonogenic activity at different stages of the menstrual cycle and in noncycling endometrium, but these differences were not statistically significant [57]. Nonetheless, 0.02% of freshly isolated endometrial stromal cells generated large colonies. Cells initiating a large colony could be serially cloned four or five times at low seeding densities (10 cells/cm^2) before CFU activity was exhausted, indicating extensive self-renewal capacity *in vitro* [56]. Single cells initiating the large colonies also demonstrated high proliferative ability, undergoing 30 population doublings and generating several billion cells. The culture-expanded cells from the large colonies expressed the characteristic but not unique MSC markers CD29, CD44, CD73, CD105, and CD146 and were negative for CD45, CD34, CD31, and stromal precursor antigen-1 (Stro-1) [56]. Freshly isolated endometrial stromal cells also generated colonies in serum-free medium supplemented with fibroblast-derived growth factor (FGF)-2, epidermal growth factor, platelet-derived growth factor (PDGF)-BB, or transforming growth factor (TGF)-α, although the cloning efficiency was lower in the absence of serum [55]. These findings indicate that multiple factors are likely to act as niche factors *in vivo*.

41.5.1.1 Prospective isolation and functional properties of human endometrial mesenchymal stem/stromal cells

Efforts are in progress to find markers to identify MSCs in many tissues and in different species. Identification of markers for the prospective isolation of MSCs is important in order to demonstrate their location in tissues, characterize their properties and genomic signature, and to harness them for their therapeutic properties. To identify markers of human endometrial stromal CFUs, stromal cells were flow-sorted using four commonly used MSC markers (CD90, CD146, CD133, and Stro-1) and examined for CFU activity [58]. CD90^{high} and CD146^{+} individually enriched for stromal CFUs and immunostaining identified their perivascular location in human endometrium. Stro-1, a bone marrow MSC marker, did not enrich for endometrial stromal CFUs despite its perivascular location. Importantly, endometrial stromal cells co-expressing two markers, CD140b and CD146, contained almost all stromal CFUs and accounted for 1.5% of the total stromal cells [38]. Single CD140b^{+} CD146^{+} cells underwent multilineage differentiation into the typical MSC mesodermal lineages of adipocytes, chondrocytes, osteocytes, and myocytes [38]. They had the characteristic MSC surface marker phenotype and were negative for the hematopoietic markers CD34, CD31, and CD45. Together, these data showed that almost all eMSCs are found in the CD140b^{+} CD146^{+} subpopulation. Examining the co-expression of these two markers by immunofluorescence revealed the perivascular location of eMSCs in the layer that is shed at menstruation and in the remaining basal layer of human endometrium. Their close association with endothelial cells pointed to the likely pericyte identity of eMSCs. It was also noted that the CFU ability of the cells following flow cytometry was threefold less compared with unsorted cells, indicating that flow cytometry sorting adversely affects cell viability [58]. Therefore, it was important to find a single marker of eMSCs to enable a simpler method of enrichment using magnetic bead sorting (Figure 41.2C) [59]. Screening endometrial stromal cell suspensions with several novel antibodies with perivascular specificity [60] by flow cytometry and immunohistochemistry identified a single marker detected by the W5C5 antibody [59].

The W5C5 antibody targets the extracellular domain of Sushi domain containing 2 (SUSD2), a type I transmembrane adhesion molecule. SUSD2 has a large extracellular region containing a somatomedin B, adhesion-associated domain in MUC4 and other proteins (AMOP), a von Willebrand factor (vWF), and a Sushi domain. Although the biological function of SUSD2 is not fully known, somatomedin B, AMOP, and von Willebrand factor domains are components of proteins that participate in cell adhesion, homodimerization, migration, homing, and signal transduction [60]. Large-scale mapping of human protein–protein interactions by mass spectrometry indicates that SUSD2 may be involved in cell-to-cell binding [61]. Clonogenic bone marrow MSCs reside in the CD271^{bright} but not in the CD271^{dim} population. Furthermore, the W5C5 antibody

exclusively identified the CD271^{bright} bone marrow MSCs but not the dim population [62]. Apart from functional and phenotypic differences, the CD271^{bright}/W5C5^{+} bone marrow MSCs have bright nuclear staining compared with the dark immature lymphoblastic appearance of the CD271^{dim} cells. This supports W5C5 as a promising marker candidate for isolating MSCs.

As with bone marrow MSCs, the majority of endometrial CFU-Fs are found in the W5C5^{+} fraction. W5C5^{+} magnetic-bead-sorted endometrial stromal cells generated more colonies than flow-cytometry-sorted W5C5^{+} cells and flow-cytometry-sorted CD140b^{+} CD146^{+} cells, indicating the utility of magnetic bead sorting with a single marker. Exploring the relationship between W5C5 and existing eMSCs markers showed that 85% of fresh W5C5^{+} cells expressed CD140b and 60% expressed Stro-1, while all the W5C5^{+} CD146^{+} cells were positive for CD140b. There was no difference in the colony-forming ability between the W5C5^{hi} and W5C5^{+} CD146^{+} cells [59]. W5C5^{+} stromal cells demonstrated typical MSC properties: mesodermal lineage differentiation into Oil Red O-stained adipocytes that expressed peroxisome proliferator-activated receptor gamma 2, osteogenic cells which stained for alkaline phosphatase and expressed core binding factor-α1, also known as RUNX, and chondrocytes surrounded by Alcian blue-positive cartilage matrix expressing collagen X. In addition, W5C5^{+} endometrial stromal cells differentiated into early stage SMCs staining for α-SMA as well as later differentiation stages by staining for smooth muscle myosin heavy chain and to CD31^{+} endothelial cells [59]. The surface phenotype of the W5C5^{+} beaded population showed that they predominantly expressed CD146 and CD90, endometrial perivascular and stromal markers respectively [59]. The identification of CD140b^{+} CD146^{+} and W5C5^{+} as potential markers of eMSCs and their presence in the endometrial layer that is shed during menses indicates that eMSCs can be harvested from endometrial biopsy samples as well as from menstrual blood [50]. The ultimate proof of the regenerative capability of stem/progenitor cells is their ability to reconstitute the parent tissue from which they were derived. Human vimentin-positive endometrial stromal-like tissue was generated when W5C5^{+} eMSCs were transplanted under the kidney capsule of severely immunodeficient NOD SCID GAMMA mice [59]. In addition to the stromal-like tissue, migratory cells were also observed in the mouse kidney parenchyma, which were morphologically similar to CD31^{+} endothelial cells demonstrated in xenografts from endometrial side population cells [63]. Therefore, both *in vitro* and *in vivo* studies have demonstrated that W5C5^{+} eMSCs differentiate into endometrial fibroblasts and vascular cells [59,64].

Other surface markers have also been identified for isolating eMSC-like cells. Mesenchymal stem cell antigen-1 (MSCA-1) is a novel molecule on the surface of MSCs that is suitable for their prospective isolation [65]. The molecular identity of MSCA-1 is tissue nonspecific alkaline phosphatase (TNAP) [65]. TNAP/MSCA-1 co-localized with a subpopulation of CD146^{+} endometrial cells at a similar percentage as for CD146^{+} CD140b^{+} eMSCs [58], but not in differentiated stromal fibroblasts from bone marrow or endometrium. TNAP/MSCA-1 is not a suitable single marker for isolating eMSCs as immuno-localization showed not only endometrial perivascular cell staining but also the luminal surface of the glandular epithelium [65]. However, co-expression of TNAP/MSCA-1 with CD146 can be used to prospectively sort and isolate eMSCs.

All specific markers identified for eMSCs (CD140b^{+}, CD146^{+}, SUSD2, and TNAP/CD146) show that eMSCs are present in both stratum functionalis and stratum basalis layers of the endometrium and are located perivascularly. Thus, it was anticipated that menstrual blood would also contain a subpopulation of eMSCs. Indeed, adherent menstrual blood cells cultured for five passages have been immunoselected with CD117 (c-kit) beads but yielded only 9% [66]. Further culturing for three more passages increased this yield to 77% of CD117^{+} cells. However, the existence of fresh c-kit-expressing stromal cells in menstrual blood still remains to be demonstrated. Immunostaining of endometrial tissue over the lifespan from the female fetus to the atrophied tissue of postmenopausal women demonstrated that the CD117^{+} stromal cells were present as scattered single cells in the endometrial stroma and myometrial stroma, but mostly in the endometrial glands in adult life [67]. As with TNAP, CD117 is also expressed on endometrial glandular epithelial cells. Thus, a simple isolation procedure of eMSCs without epithelial contamination is not possible with TNAP or CD117.

41.5.1.2 Endometrial mesenchymal stem/stromal cell phenotype and gene expression profile

Gene expression profiling on freshly isolated endometrial stromal cells sorted using flow cytometry on the basis of CD146 and CD140b expression has provided insight into the global state of eMSCs' gene expression and their inferred functions [64]. The three cell types compared were CD146^{+} CD140b^{+} (eMSCs), CD146^{-} CD140b^{+} (fibroblasts), and CD146^{+} CD140b^{-} (endothelial cells) [64]. Hierarchical clustering and principle component analysis showed greater similarity of gene profiles between eMSCs and stromal fibroblasts, which were distinct from endothelial cells. Despite the relatively similar profiles of eMSCs and endometrial fibroblasts, 762 genes were differentially expressed between these cell types, approximately half the number of differences observed between eMSCs and endometrial endothelial cells. This study indicated a relatively close relationship between eMSCs and endometrial stromal fibroblasts, and that endometrial fibroblasts are a lineage cell of eMSCs. Importantly, the CD146^{+} CD140b^{+} population expressed significantly greater levels of the single marker SUSD2 now used to enrich for eMSCs [68].

The pericyte phenotype of eMSCs was confirmed by the expression of pericyte markers such as the regulator of G-protein signaling (*RGS5*), α-smooth muscle actin-2 (*ACTA2*), angiopoietin 2 (*ANGPT2*), insulin-like growth factors (*IGFs*), nerve growth factor (*NGF*), platelet-derived growth factor (*PDGF*), transforming growth factor-β (*TGF-β*), parathyroid hormone-like hormone (*PTHLH*), and syndecan. Gene families responsible for

self-renewal and differentiation, such as Notch, TGFB, Wnt, IGF, FGF, Hedgehog members, and cyclic adenosine-monophosphate-mediated and G-protein-coupled receptor-mediated signaling were also upregulated in eMSCs [64]. Furthermore, this study confirmed that endometrial CD140b$^+$ CD146$^+$ cells are clonogenic and can differentiate into mesodermal lineages.

Gene profiling of passage 2 culture-expanded SUSD2$^+$ eMSCs identified 12 perivascular genes upregulated in SUSD2$^+$ eMSCs. Perivascular markers, including melanoma cell adhesion molecule (CD146), vascular endothelial protein 1 (also known as amine oxidase, copper containing 3 (*AOC3*)), *NOTCH3*, a smooth muscle myosin (*MYTH11*), and elastin (*ELN*) were enriched in cultured SUSD2$^+$ eMSCs. These genes translated as the proteins were also identified around endometrial arterioles by immunostaining [69]. Desmin (DES), α-SMA (ACTA2), and chondroitin sulfate proteoglycan 4 (CSPG4), which are essential for regulating pericyte recruitment and sprouting during angiogenesis, were also highly expressed, indicative that the pericyte signature genes of the primary cells were retained in cultured eMSCs [68,69].

The Notch target genes *HEY2* and *HEYL*, but not *HEY1*, were found to be highly expressed in SUSD2$^+$ eMSCs compared with SUSD2$^-$ fibroblasts [69]. These *HEY2* and *HEYL* transcripts were significantly downregulated in human SUSD2$^+$ eMSCs treated with γ-secretase Notch inhibitor, DAPT. This was reflected by decreased SUSD2 mRNA expression and SUSD2$^+$ eMSC number to 53% and 64% respectively. This study showed for the first time that *SUSD2* is a downstream target gene in the Notch pathway in human eMSCs. Further knockdown and overexpression assays are required to confirm the involvement of Notch signaling in eMSC function. However, other signaling pathways may also be involved in eMSC regulation.

Gene profiling of freshly isolated CD140b$^+$ CD146$^+$ eMSCs did not detect the pluripotency genes *OCT4* (*POU5F1*), *SOX2*, and *NANOG* when compared with the National Institutes of Health-approved human embryonic stem cell line H7 using quantitative real time reverse transcriptase polymerase chain reaction [64]. In contrast, others have demonstrated the expression of OCT4 (POU5F1) but not NANOG [70] and the embryonic stem cell markers SSEA-4 and c-KIT (CD117) [66] using flow cytometry in culture-expanded endometrial regenerative cells cultured from menstrual blood. Some studies suggest that the *OCT4* gene is expressed by adult stem/progenitor cells and primarily regulates self-renewal, but others have shown that only pluripotent stem cells such as human embryonic stem cells and induced pluripotent stem cells express these pluripotency genes. There are at least three *OCT4* transcripts (*OCT4A*, *OCT4B*, and *OCT4B1*) and four protein isoforms (OCT4A, OCT4B-190, OCT4B-265, and OCT4B-164). OCT4A is the transcription factor responsible for the pluripotency properties in human embryonic stem cells, while *OCT4B* has no such role [71]. Many studies showing the expression of pluripotency genes, especially *OCT4* in adult stem cells, including endometrium, have failed to distinguish the *OCT4* isoforms or did not exclude the possibility of pseudogene artefacts [71].

Recently, much interest has focused on the soluble factors secreted by MSCs, the homing capacity of bone marrow MSCs to sites of injury, and their immune modulatory and paracrine properties. These include secretion of vascular endothelial growth factor, hepatocyte growth factor, TGF, and prostaglandin E_2.

A recent *in vitro* study highlighted the role of eMSCs in endometrial stromal cell decidualization (differentiation) and potentially in placentation [68]. A secretome analysis on passage 2 of SUSD2$^+$ eMSCs and SUSD2$^-$ endometrial fibroblasts following induction of decidualization (differentiation) *in vitro* with a cyclic adenosine monophosphate analogue and a stable progesterone (medroxy progesterone acetate) showed that decidualized eMSCs differentially secreted 12 immunomodulatory and trophic factors compared with SUSD2$^-$ fibroblasts. There was a 43-fold increase in secretion of chemokine ligand-7 (capable of attracting monocytes, lymphocytes, granulocytes, natural killer cells, and dendritic cells) and an 18-fold increase in secretion of leukemia inhibitory factor (which is a multifactorial cytokine indispensable for embryo implantation and stromal cell decidualization, as well as having roles in trophoblast attraction and differentiation). In contrast, the SUSD2$^-$ fibroblasts had only a fivefold and fourfold increase of these cytokines respectively. Therefore, eMSCs may not only have a role in the decidual response but may also function as a key immune modulator, particularly for the establishment of pregnancy given that the fetus is an allograft.

It is likely that eMSCs also have a homing capacity as they show high levels of CXCR4, a chemokine receptor for stromal-cell-derived factor 1, which is a potent chemoattractant that enables migratory activity through tissues and is upregulated in eMSCs. Furthermore, eMSCs display low levels of major histocompatibility complex class I antigens and lack major histocompatibility complex class II molecules, indicating their likely low immunogenicity. Because of these characteristics, eMSCs have been suggested as a possible source of cells for cell-based therapies and TE.

41.6 Culture expansion of endometrial mesenchymal stem/stromal cells toward current good manufacturing practice conditions

Adult stem/progenitor cells in the tissues are rare and account for around 1% of the total tissue. In bone marrow, MSCs account for less than 0.01% of marrow cells, which decreases even further with age [72,73]. In the endometrium, clonogenic MSCs account for around 1–5% of the stromal cells [38,59]. Owing to their limited number in tissues, MSCs need to be enriched using specific markers (see earlier) and then expanded in tissue culture to obtain sufficient numbers of cells for clinical trials. To date, there are no standard culture conditions that satisfy the growth of

MSCs from all tissue types. For clinical translation, culture of MSCs ideally requires serum and xeno-free conditions compliant with current good manufacturing practice. Using Xcelligence and cell viability assays, fibronectin was identified as a superior attachment factor for eMSCs compared with collagen, gelatin, or Cell Start XF [74]. In comparison with various serum-free media (designed for bone marrow MSC culture), an in-house serum-free medium supplemented with FGF-2 and epidermal growth factor and cultured in physiological oxygen concentration (5% O_2) was found optimal for eMSC growth. Under these conditions the cultured eMSCs retained their surface phenotype, cloning efficiency, and multilineage differentiation ability [74]. Substitutes for fetal calf serum include human serum, human platelet lysate, or chemically defined serum-free media [75–77]. However, serum and platelet lysates are complex products with many undefined components and significant lot-to-lot variation, hindering the development of a uniform approach to MSC expansion. Serum-free medium supplemented with chemically defined growth factors and other small molecules is the likely approach for future eMSC expansion.

Not all serum-free media support MSC growth equally [74,78,79]. For example, eMSCs grew well in Lonza-TP-SF medium, but underwent early differentiation, as demonstrated by loss of the MSC markers CD140b, CD146, and SUSD2 but not the characteristic MSC markers CD73, CD90, and CD105, highlighting the inability of classic MSC markers to distinguish MSCs from fibroblasts [74] (and see Chapter 30). Similarly, MSCs derived from umbilical cord and from adipose tissue were supported by StemPro XF medium containing 2% human serum supplementation and by xeno-free medium (RegES) respectively [75,80]. This suggests that optimal culture conditions may need to be defined for each MSC source because MSCs from different tissues have subtly different microenvironments *in situ* by which they maintain tissue homeostasis [74].

41.7 Tissue engineering for pelvic organ prolapse repair

Having developed a new mesh material more closely simulating vaginal tissue biomechanics and having characterized a new source of easily obtainable eMSCs, a TE construct comprising eMSCs and PA/G was developed (Figure 41.2E). *In vivo* evaluation of this TE construct comprising passage 6 eMSCs labeled with Vybrant DiO dye showed that eMSCs attached well on the PA/G mesh (Figure 41.2E). The labeled cells could be identified *in vitro* for up to 35 days and *in vivo* for at least 14 days [49]. In a wound-healing fascial defect model the PA/G–eMSCs TE construct (Figure 41.2E) was implanted subcutaneously in the dorsal region of a nude rat and studied for 3 months (Figure 41.2F). PA/G–eMSCs mesh promoted early neovascularization at 7 days, as assessed by α-SMA immunostaining and image analysis. A greater initial influx of $CD45^+$ leucocytes, predominantly proinflammatory type M1 $CCR7^+$ macrophages, was observed at 7 days in the PA/G–eMSCs mesh, but by 30 days a prohealing M2 $CD263^+$ macrophage phenotype predominated. (CD263, otherwise known as TRAIL-R3, is present on neutrophils and macrophages and is a glycophosphatidylinositol-anchored protein that lacks the cytoplasmic death domain. Thus, it is unable to induce apoptosis and serves as a negative regulator of apoptotic signaling by competing for binding of TRAIL with death receptor 5.) In comparison, the PA/G mesh alone elicited a smaller M1 response at 7 days, which was still predominant at 30 days [49]. By 90 days, significantly fewer $CD68^+$ macrophages surrounded the PA/G–eMSCs mesh filaments compared with PA/G mesh alone. The collagen deposited around the mesh was of rat origin identified by a specific anti-rat-collagen antibody. There was no difference between the total collagen production in the mesh with or without cells or in the ratio of collagen III/collagen I + III over 90 days of implantation (Figure 41.3A). Picrosirius red (Figure 41.3B) also showed similar total collagen content, but birefringence analysis showed that the quality of collagen fibrils laid down differed between the mesh alone and the eMSC-seeded mesh, which comprised well-organized physiological thin fine fibrils (Figure 41.3D) with crimping (Figure 41.3F) [81]. In comparison, the PA/G alone resulted in a greater proportion of dense bands of scar-like fibrils (Figure 41.3C) and without crimping (Figure 41.3E) [49,81]. These differences may have contributed to the biomechanically less stiff and more extensible mesh/tissue complex generated from the eMSC-seeded mesh, suggesting greater compliance and matching to the soft tissue of the vaginal wall than those without cells (Figure 41.4). Such new tissue deposition and biomechanical properties in the eMSC-seeded mesh implants are likely to be more favorable for vaginal tissue repair by allowing greater mobility [49].

The PA mesh with gelatin (10% w/v in H_2O) cross-linked with glutaraldehyde (0.025% w/v) was optimized for eMSCs growth and gelatin degradation *in vitro* [82]. Endometrial eMSCs differentiated into SM22-α (a marker of adult smooth muscle) and smooth muscle myosin-heavy-chain-expressing SMCs on the PA/G mesh in serum-free induction medium containing TGF-β1 and PDGF. eMSCs also differentiated into fibroblast-like cells on the PA/G mesh in 10% serum-supplemented induction media containing connective tissue growth factor (CTGF) and ascorbic acid. CTGF, also known as CCN2, is a matricellular protein of the CCN family of extracellular-matrix-associated heparin-binding proteins. The CTGF-differentiated cells produced collagen I and Tenascin-C, with total collagen production and Masson's trichrome staining indicative of new tissue formation. Tenascin-C is important in regulating cell proliferation and migration, especially during developmental differentiation and wound healing.

SMCs and fibroblasts are vital cellular components required for regenerating the fibromuscular wall of the human vagina, to provide elasticity and strength [82]. However, in the *in vivo* rat model of fascial repair the SMCs and collagen produced were of rat origin, suggesting that the human eMSCs mediated their effects by an induced paracrine mechanism [49]; this is possible given the secretome of cultured $W5C5/SUSD2^+$ eMSCs [68]. To

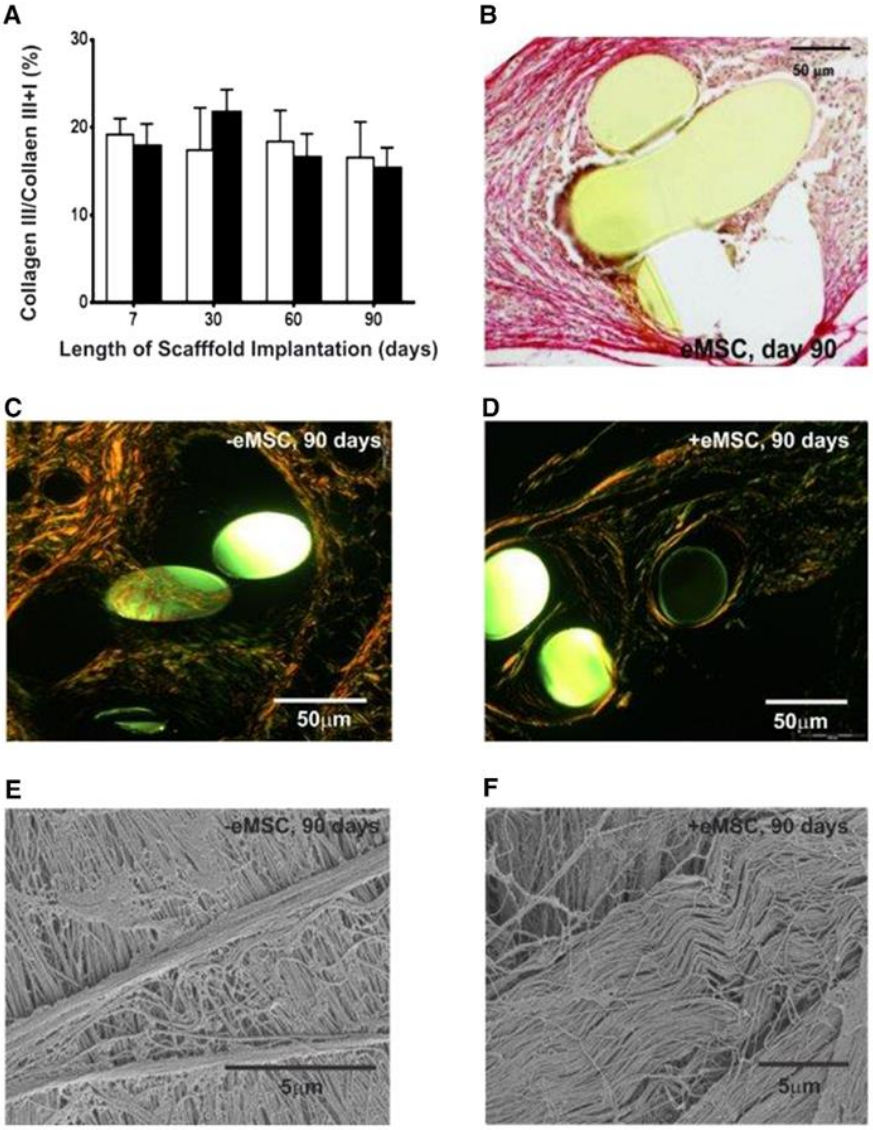

Figure 41.3 Collagen assessment in explanted PA/G meshes with or without eMSCs. (A) PA/G meshes with (black bars) or without (white bars) eMSCs were subcutaneously implanted in the dorsal region of immunodeficient nude rats. The tissue constructs were explanted and the percentage of total collagen III within and around the meshes was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The percentage of collagen III changed little over the period of time in the two groups. Data are mean plus/minus standard error of the mean of $n = 8$ animals/group. (B) Conventional light microscopy image of Sirius-red-stained section to show collagen in explanted PA/G–eMSC mesh construct after 90 days. (C,D) Birefringence microscopy images of Sirius-red-stained sections from explanted PA/G mesh (C) and PA/G–eMSC mesh (D) after 90 days assessed using and scored according to the presence of predominantly thick (orange, red) or thin (green) birefringent collagen fibers. Rats implanted with PA/G–eMSC mesh had a greater number of thin collagen fibers, while the mesh alone had more dense and thick collagen fibers. (E,F) The explanted PA/G meshes with (F) or without (E) eMSCs were carefully extracted from the surrounding muscle and processed for scanning electron microscopy. The micrographs were taken at 1.2 kV using a Schottky field-emission variable pressure scanning electron microscope. Unseeded mesh had relatively narrow collagen bundles (up to 1.3 mm in width) with straight collagen fibrils. Seeded mesh consisted of wider collagen fiber bundles (up to 8 mm in width) with crimped configuration. (See color plate section.) *Source*: reproduced with permission from Mary Ann Liebert Inc. publishers from Ulrich *et al.* [49] *Tissue Engineering A* 2014 and Elsevier from Edwards *et al.* [81] *Acta Biomateriala* 2015.

confirm whether eMSCs are capable of producing collagen and modulating biocompatibility of the PA/G mesh *in vivo* in the vaginal environment, a preclinical large animal model of vaginal repair was required (Figure 41.2F). Currently, this new PA/G–eMSCs TE construct appears promising for POP corrective surgery.

In another study, seven different candidate materials, both biological and synthetic (AlloDerm, porcine dermis, PP, sheep

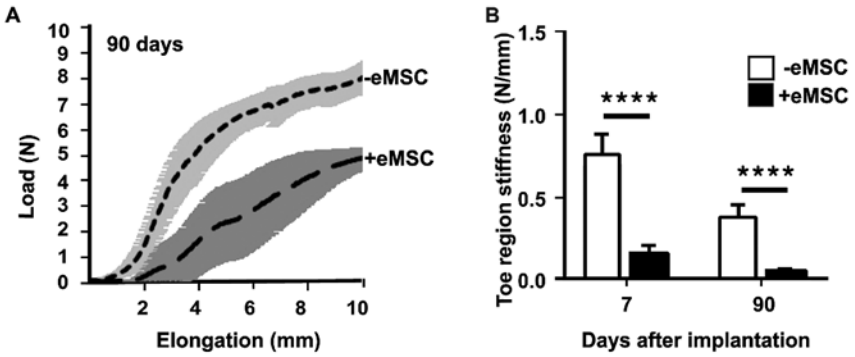

Figure 41.4 (A) Load–elongation curves for unseeded and eMSC-seeded PA/G meshes 90 days postimplantation. Error bars are 95% confidence intervals of $n = 6$ animals/group. (B) Toe region stiffness of PA/G (white bars) and eMSC–PA/G mesh from (A). *Source*: Reproduced with permission from Mary Ann Liebert Inc. publishers from Ulrich *et al.* [49] *Tissue Engineering A* 2014.

forestomach, small intestine submucosa, acellular cadaveric dermis, and PLA scaffold), were assessed *in vitro* for cell attachment, contracture, and extracellular matrix production [83]. Alloderm is a commercially produced acellular dermal matrix. Among these materials, biodegradable PLA and SIS meshes seeded with human buccal fibroblasts showed favorable *in vitro* properties for potential clinical use. For example, these TE constructs increased new extracellular matrix production, particularly collagens I and III and elastin. Both collagen types are a vital component of the extracellular matrix, but the ratio of collagens I and III was not addressed. The biomechanical properties of the PLA mesh with or without buccal fibroblast seeding showed that strength, strain, and Young's modulus for the PLA tissue construct were similar to the biomechanical properties of native tissue from women without pelvic dysfunction [84]. Similar findings were observed when adipose-derived MSCs were used with the PLA mesh [36]. Young's modulus, also known as the tensile modulus or elastic modulus, is a measure of the stiffness of an elastic material. It is defined as the ratio of the stress (force per unit area) along an axis to the strain (ratio of deformation over initial length) along that axis. A rubber band, for example, has an extremely low Young's modulus.

In a rat model with PLA mesh seeded with or without cultured human or rat adipose-derived MSCs and implanted for 7 days, angiogenesis was only promoted in the cell-seeded mesh, as demonstrated by CD31 (platelet endothelium adhesion molecule-1, an endothelial marker) immunostaining [85]. Deposition of collagens III and I was demonstrated qualitatively by immunohistochemistry, and increased macrophage infiltration and foreign body reaction was shown by CD68 immunohistochemistry. CD68 is a glycoprotein that binds to low-density lipoprotein and is only expressed on monocytes and macrophages. There was no difference in the macrophage infiltration in the seeded and unseeded meshes, and macrophages enclosed the PLA fibers, indicating an innate immune response to the synthetic material. This 7-day study with PLA examined the short-term acute effects of fibroblasts on implanted meshes; the long-term effect of the scaffold plus cells still needs to be demonstrated. In both these studies, extracellular matrix deposition increased when fibroblasts were delivered on meshes, but there is a need for long-term *in vivo* studies to assess whether the cells make a difference in extracellular matrix production and strength and longevity of the implant. All these studies highlight that cellular therapy may significantly improve mesh biocompatibility for POP by modifying the host response to the mesh material.

41.7.1 A large animal preclinical model for pelvic organ proplapse

Animal models of POP are vital in providing preclinical data prior to testing any new TE approach in women. There is no perfect animal model to study POP owing to its multifactorial etiology. Humans are bipedal with a unique pelvic orientation, not recapitulated in most mammalian species. Nonhuman primates, including rhesus and squirrel monkeys, are the best animal model for POP study as they develop spontaneous prolapse similar to women [86]. These nonhuman primates are bipedal for some of the time and have similar anatomical and histological pelvic structures to humans. Their reproductive cycle and pregnancy outcome closely match those of humans. However, nonhuman primate models are limited by ethical issues, their short life span, lengthy pregnancy duration before developing POP, and prohibitive costs [86].

The sheep is a quadripedal large animal that has similar pelvic tissue anatomy, histology, size, and vaginal dimensions but a larger fetal head/maternal pelvis ratio than women [86,87]. Ewes have the same three pelvic support structures as women and their pelvises experience increased internal pressures due to their ruminant anatomy, offsetting some of the disadvantage of being quadrupedal [86]. Like humans, sheep have prolonged labors with relatively large fetuses and spontaneously develop antepartum uterovaginal prolapse at the same frequency (8–12%) as women develop chronic POP. Ovine prolapse is associated with age and pregnancy, similar to the situation in women [86]. Regional variation in the biochemical composition of the ovine vagina was discovered in a comparative study of the posterior vaginal wall in postmenopausal women and in a sheep model [88]. Total collagen and elastin tissue-associated protein content in the vaginal wall in the two species were similar. The proximal region near the cervix of the ovine vagina had significantly higher collagen and glycosaminoglycan content compared with the distal region. The proximal ovine vagina was also much

stiffer, stronger, and more elastic than more distal regions, in contrast to human vaginal tissues. There was a significantly increased percentage of type III to type I collagen (40%) in human compared with ovine vagina [88]. The increase in type III collagen in postmenopausal women correlates with their propensity to develop prolapse. The similarity between women and sheep in the biochemical composition and histology of the vaginal wall complements the similarity in the biomechanical properties and therefore makes the sheep a good preclinical model for vaginal studies involving POP (Figure 41.2F).

41.8 Conclusions

Endometrium is a highly regenerative tissue and it is likely that adult stem/progenitor cells are responsible for its cyclical growth, differentiation, and regression. Rare populations of perivascular human MSCs have been identified in both the functional (shed) and germinal layers of the endometrium. These eMSCs are present at all stages of the menstrual cycle, as well as in inactive and atrophic postmenopausal endometrium. eMSCs can be harvested from endometrial biopsies in an office-based procedure with minimal discomfort and without the use of anesthesia, making the endometrium an attractive, although still underrecognized, alternative source of MSCs. Importantly, eMSCs can be enriched by magnetic bead selection using the W5C5 antibody or by flow cytometry sorting using CD140b and CD146 antibodies, removing contaminating stromal cells or leucocytes not possible when using plastic adherence for MSC isolation. eMSCs are clonogenic, can self-renew *in vitro*, display multipotent differentiation into mesodermal lineages *in vitro*, and generate endometrial stromal-like tissues and vascular cells *in vivo*.

Newly fabricated polyamide–gelatin composite meshes are superior to commercially available PP meshes, promoting neovascularization, collagen deposition, and minimizing inflammatory cell infiltration. These properties were further augmented when eMSCs were integrated with PA/G mesh as a TE construct and implanted into a fascial defect in rats. The eMSCs initiated a vigorous M1 macrophage influx and promoted their differentiation to a wound-healing M2 phenotype. eMSCs promoted the deposition of physiological fibrilar, crimped nonscarring collagen fibers, significantly improving the biocompatibility and the biomechanical properties of the explanted mesh–tissue complex. These are favorable and promising profiles of the PA/G–eMSCs TE construct.

Substantial progress has been made in examining the utility of these TE constructs in a small animal model, but to take this approach to the clinic for the treatment of POP, more studies examining the mechanism of how eMSCs exert their anti-inflammatory effects are required. Finally, an autologous large animal model using the sheep will provide the necessary preclinical data for translation as a potential treatment for POP in women.

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RESEARCH ARTICLE

Isolation and Characterisation of Mesenchymal Stem/Stromal Cells in the Ovine Endometrium

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Abstract

Objective

Mesenchymal stem/stromal cells (MSC) were recently discovered in the human endometrium. These cells possess key stem cell properties and show promising results in small animal models when used for preclinical tissue engineering studies. A small number of surface markers have been identified that enrich for MSC from bone marrow and human endometrium, including the Sushi Domain-containing 2 (SUSD2; W5C5) and CD271 markers. In preparation for developing a large animal preclinical model for urological and gynecological tissue engineering applications we aimed to identify and characterise MSC in ovine endometrium and determine surface markers to enable their prospective isolation.

Materials and Methods

Ovine endometrium was obtained from hysterectomised ewes following progesterone synchronisation, dissociated into single cell suspensions and tested for MSC surface markers and key stem cell properties. Purified stromal cells were obtained by flow cytometry sorting with CD49f and CD45 to remove epithelial cells and leukocytes respectively, and MSC properties investigated.

Results

There was a small population $CD271^+$ stromal cells (4.5±2.3%) in the ovine endometrium. Double labelling with CD271 and CD49f showed that the sorted $CD271^+CD49f^-$ stromal cell population possessed significantly higher cloning efficiency, serial cloning capacity and a qualitative increased ability to differentiate into 4 mesodermal lineages (adipocytic, smooth muscle, chondrocytic and osteoblastic) than $CD271^-CD49f^-$ cells. Immunolabelling studies identified an adventitial perivascular location for ovine endometrial $CD271^+$ cells.

Competing Interests: The authors have declared that no competing interests exist.

Conclusion

This is the first study to characterise MSC in the ovine endometrium and identify a surface marker profile identifying their location and enabling their prospective isolation. This knowledge will allow future preclinical studies with a large animal model that is well established for pelvic organ prolapse research.

Introduction

Tissue engineering (TE) is the combination of a range of biological and synthetic material scaffolds with a variety of cell types and has revolutionized treatment options for several clinical conditions. TE approaches have for instance been used to generate new tissues and organs [1] including the bladder and vagina [2], and to improve long-term outcomes of surgical interventions. TE approaches using stem cells and in particular mesenchymal stem/stromal cells (MSC) are most promising because they possess key properties; self-renewal, high proliferative potential and differentiation. However, the main action of MSC whether transplanted with or without material scaffolds appears to be through paracrine action on endogenous cells through their release of numerous factors [3].

Mesenchymal stem cells or mesenchymal stromal cells (MSC), originally identified in the bone marrow are defined as plastic adherent cells with a characteristic surface phenotype, colony-forming ability, and multipotency by differentiating*in vitro*into adipogenic, chondrogenic and osteogenic mesodermally-derived lineages [4]. More recently, MSC have been identified in most human tissues including umbilical cord blood, adipose tissue and endometrium [5–8]. Human endometrium contains a small population of clonogenic stromal cells with typical MSC properties [9–11]. Endometrial MSC (eMSC) have also been identified as a component of endometrial side-population (SP) cells [11–14]. The eMSC are clonogenic and self-renew as demonstrated by serial cloning in culture [10]; they undergo multilineage differentiation into four mesenchymal lineages, including smooth muscle cells*in vitro*, indicating their similarity to bone marrow MSC. Endometrial MSC also generate endometrial stroma in xenograft assays [14,15]. One advantage of human eMSC is the relative ease with which they can be obtained by an endometrial biopsy as an office-based procedure without the use of anaesthesia, which is significantly less painful or invasive than bone marrow aspiration or liposuction [16].

MSC have typically been obtained from bone marrow and menstrual blood by adherence to plastic cultureware [4,16] and from culture of the stromal vascular fraction of unfractionated adipose tissue [17]. These cultures are heterogeneous, comprising stromal, endothelial and perivascular cells and are not particularly enriched for MSC, although a small percentage MSC will be present. The advantage of using specific surface markers for prospective isolation of MSC is that enriched populations are obtained which contain the majority of the clonogenic, self-renewing cells that will produce many more cells upon culture expansion [5]. Several single cell surface markers, distinct from the commonly expressed MSC phenotypic markers, have been used to enrich MSC from various tissue sources, including Stro-1 [18], CD271 also known as NGFR (low-affinity nerve growth factor receptor) or p75 NTR (neurotrophin receptor) [19] and MSCA-1 (Tissue Non-specific Alkaline Phosphatase, TNAP) [20,21] Ovine bone marrow MSC (bmMSC) have been prospectively isolated from bone marrow and adipose tissue using Stro-4 [22].

Specific markers have now been identified that enrich for human eMSC [7, 15]. Almost all clonogenic stromal cells with MSC properties are found in the $CD146^{+}PDGFRB^{+}$ and $W5C5^{+}$ subpopulations of endometrial stromal cells. The epitope for the W5C5 antibody is

Sushi Domain-containing 2 (SUSD2) [23]. These markers revealed that eMSC are located around blood vessels as pericytes in both the functionalis and basalis layer of human endometrium. They also enable the prospective isolation of eMSC from biopsy, curettage or hysterectomy tissue.

The aim of this study was to determine if MSC were present in ovine endometrium, develop methods for isolating MSC from ovine endometrium and characterize their adult stem cell properties of self-renewal, high proliferative potential and differentiation. This is an important step forward in the strategy for developing an autologous preclinical large animal model to assess new generations of TE constructs comprising eMSC and novel materials.

Materials and Methods

Animal Welfare

The animal surgery was done at the Monash University Melbourne, with approval from the Monash Medical Centre Animal Ethics Committee A (Ethics no. MMCA 2013/38) for animal experimentation. All experiments were conducted in strict accordance with good practice for the use of animals as detailed in the National Health and Medical Research Council Guidelines in compliance with the Animal Welfare Act. All animals were humanely euthanized according to the current guidelines by intravenous administration with Pentobarbitone sodium into the jugular vein (150mg/kg).

Ovine Uterine Tissues

Ovine tissues (n = 15) were collected from 3–5 year old parous ewes treated with a progesterone sponge-controlled internal drug release dispenser (CIDR, 30 mg Flugestone acetate, EAZI-BREED) inserted vaginally for two weeks and removed 48 hr prior to tissue collection to initiate estrous. The sheep were placed in dorsal recumbency; a 10 cm lower abdominal midline incision was performed and the uterus exposed. The uterus was removed from the intra-abdominal cavity by ligating the ligaments and then put into ice-cold transport medium (HEPES-buffered DMEM/F-12 medium (Invitrogen) supplemented with 1% antibiotic antimycotic (Invitrogen) [21], stored at 4°C and processed within 18 hours. The uterus was opened via a longitudinal incision along the length of both horns and endometrial tissues were taken from different regions of the uterus both at the caruncular and intercaruncular areas.

Ovine Endometrial Stromal Cell Isolation

Ovine endometrial tissues dissected from the opened uterus were cut into small pieces and minced using a tissue chopper (MacIllwain tissue chopper; Campden Instruments, Loughborough). Minced tissues were dissociated in 5 mg/ml collagenase type I (Worthington Biochemical Corporation), 40 μg/ml deoxyribonuclease type I (Worthington Biochemical Corporation) and 5 mM glucose in PBS for 1.5 hr at 37°C on a rotator. The enzymatic reaction was terminated by adding an equal volume of DMEM/F-12 medium containing 15 mM HEPES (Life Technologies) supplemented with 5% newborn calf serum (Life Technologies) and 1% antibiotic-antimycotic (Life Technologies)(Bench Medium). Dissociated tissues were then filtered through 70-μm cell strainer (BD Biosciences) and stromal cells were collected in the filtrate. To remove red blood cells, Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB) was under-layered beneath the cell suspension and centrifuged at 500g for 15 min at room temperature (RT). Cells at the media/Ficoll-Paque interface were collected and washed with Bench Medium.

Flow Cytometry Sorting

Isolated stromal cells (up to 1 x 10^7 cells/100μl) were incubated with antibody combinations of allophycocyanin (APC)-conjugated CD49f (1:10, clone GoH3, rat IgG2a; Miltenyi Biotec) and Alexa Fluor 488-conjugated CD45 (1: 20; mouse IgG1; Life Technologies) or phycoerythrin (PE)-conjugated CD271 (1:10, mouse IgG1; Miltenyi Biotec) and APC-conjugated CD49f in 2% fetal bovine serum/PBS (FBS/PBS) for 30 min on ice in the dark. Cells were then washed and resuspended in 1μM Sytox Blue to distinguish live and dead cells (Life Technologies) and fluorescence activated cell sorting (FACS) was undertaken on a MoFlow flow cytometer (Beckman Coulter) or an Influx flow cytometer (Becton Dickinson Biosciences).

Cell Culture and*In Vitro*Colony Forming Assay

Freshly sorted cells were cultured in stromal medium containing DMEM/F-12 (Life Technologies), 10% fetal bovine serum (Life Technologies), 2 mM glutamine (Life Technologies), 0.5 mg/ml primocin, 10 ng/ml basic fibroblast growth factor (FGF2) (Peprotech) used for our studies on human eMSC and incubated at 37°C in 5% CO_2. Medium was changed every 2–3 days.

For colony forming assays, freshly sorted cells were seeded at very low seeding densities of 10–50 cells/cm^2 onto fibronectin-coated (10μg/ml) (BD Biosciences 10cm-dishes (BD Biosciences) and cultured in stromal medium with changes at day 6/7. Fibronectin and FGF2 are included in the medium to assist attachment and establishment of clonal cultures. Colonies were monitored to ensure they were derived from single cells. For subcloning, plates were seeded at the lower density to ensure individual clones were clearly separated to avoid contamination. Clonal cultures were fixed in 10% formalin at day 12 and stained with haematoxylin. Cloning efficiency was determined on plates seeded at the higher density to ensure sufficient clones/plate for statistical purposes. Only colonies with>50 cells were counted and colony forming efficiency was then determined [9].

Serial Cloning Assay

Several of the largest individual clones on cloning plates containing<30 colonies in total were collected per sample and cell type by trypsinisation in cloning rings and recloned. Cells were counted visually under a phase contrast microscope using an ocular grid and seeded at 5–10 cells/cm^2 onto fibronectin-coated 10 cm dishes and cultured in stromal medium as above to generate secondary clones. Similarly, several secondary clones were harvested and recloned a second time to generate tertiary clones as previously described [10]. The cloning efficiency at each subcloning was assessed as above.

*In vitro*Differentiation

To induce adipogenic, osteogenic and myogenic differentiation, sorted ovine endometrial stromal cells ($CD271^+CD49f^-$ and $CD271^-CD49f^-$) were seeded separately at 5,000 to 10,000 cells/cm^2 on coverslips (Thermo Scientific) in 4-well plates or 24-well plates (BD Biosciences) and cultured in respective differentiation media for 4 weeks as described previously for human cells [10]. For chondrogenic differentiation, 5 x 10^5 cells were pelleted in 15-mL Falcon tube by centrifugation at 1100 rpm for 5 min at 4°C and cultured in chondrogenic medium to produce a 3D micromass culture [10]. Controls were sorted cells cultured in 1% fetal bovine serum stromal medium. Medium was changed every 2–3 days.

Following 3–4 weeks culture in differentiation or control media, cells on coverslip were fixed and stained with 4% Alizarin Red (pH 4.1), 1% Oil Red O or by immunohistochemistry using an antibody toα-smooth muscle actin (3.6μg/ml, clone 1A4; Dako) for osteogenic,

adipogenic and myogenic differentiation, respectively. Chondrogenic micromass cultures were fixed, processed and paraffin embedded. Sections were stained with 1% alcian blue (Sigma-Aldrich). Stained cells or sections were examined under Olympus BX41 microscope (Olympus), and images taken using Olympus DP25 digital camera (Olympus).

Flow Cytometric Analysis

FACS sorted CD49f$^-$ cells or cultured CD271$^+$CD49f$^-$ cells were stained with primary antibodies PE-conjugated CD271, PE-conjugated PDGFRB (1.25μg/ml, mouse IgG1; R&D Systems), PE-conjugated SUSD2, (W5C5 clone, 1:20, mouse IgG1; Biolegend), APC-conjugated CD90 (25μg/ml, mouse IgG1κ, BD Pharmingen), CD146 (1:2, supernatant, clone CC9, mouse IgG2a; donated by Prof David Haylock, CSIRO, Clayton, Victoria, Australia), CD73 (10μg/ml, mouse IgG1κ; BD Pharmingen) or CD105 (10μg/ml, mouse IgG1ƙ; BD Pharmingen). The CD146 samples were incubated with secondary antibody fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG2a (5μg/ml, clone R19-15; BD Pharmingen). CD73 and CD105 samples were incubated with secondary antibody PE-anti-mouse IgG1 (2μg/ml, clone A85-1, BD Pharmingen). Isotype matched controls or unlabelled controls were included for each antibody and used to set the electronic gates on the flow cytometer. Cells were then incubated with Sytox Blue and analysed by FACS Canto II analyser (BD Biosciences). FACS data were analysed by FACSDiva software (BD Biosciences).

Immunostaining

Frozen OCT sections or cultured cells were fixed in 4% paraformaldehyde for 10 min at RT, permeabilized with 0.2% Triton X-100 for 10 min and used for chromogen immunostaining or immunofluorescence.

For chromogen staining, sections were blocked with 0.3% hydrogen peroxide for 10 min followed by serum-free protein block (Dako) for 10 min. Sections were then incubated with CD49f primary antibody (1:100, clone GoH3; BD Pharmingen) or rat IgG2a isotype control (1:200; BD Pharmingen) overnight at 4°C, followed by biotinylated goat anti-rat secondary antibody (1:200; Vector Laboratories, Burlingame, CA, US) for 60 min at RT followed by streptavidin-horse radish peroxidase (1:200; GE Healthcare) for 60 min at RT and then 3,3'-diaminobenzidine (Sigma-Aldrich) for 5 min. Sections were then counterstained with haematoxylin, dehydrated and mounted with DPX. Sections were examined under Olympus BX41 microscope (Olympus), and images taken using Olympus DP25 digital camera.

For immunofluorescent staining, sections or cultured cells were blocked with Protein Block (Dako) and stained for CD271 in combination with either von Willebrand Factor (vWF) or alpha smooth muscle actin (αSMA). For CD271 and vWF, sections were incubated with a primary antibody against vWF (1:50; clone F8/86 mouse $IgG_{1\kappa}$; Dako) for 1 hr at 37°C, followed by Alexa Fluor 488 conjugated donkey anti-mouse IgG (1:500; Abcam) for 30 min at RT, washed three times and incubated with 10μg/ml mouse IgG to block any residual Alexa Fluor 488 anti-mouse IgG. Sections were then incubated with PE-conjugated CD271 (1:100, mouse IgG1; Miltenyi Biotec) overnight at 4°C. For CD271 andαSMA, sections were incubated with PE-conjugated CD271 (1:100) and rabbit anti-αSMA (1:100, Abcam ab5694) for 1hr at RT, washed then incubated with Alexa Fluor 568 anti-mouse IgG (1:500) and Alexa Fluor 488 conjugated donkey anti-rabbit IgG (1:500; Molecular Probes).

Fixed cultured cells were stained with either CD271 (1:100) orα-SMA (1:100) for 1hr at RT, washed twice with PBS and further incubated in donkey anti-mouse IgG Alexa Fluor 568 (1:500). Immunofluorescence preparations were counterstained with Hoechst 33528 (4μg/ml;

Molecular Probes), mounted with fluorescent mounting media (Dako) and examined using Nikon C1 confocal microscope (Nikon Instruments Inc).

Statistical Analysis

All analyses were done with GraphPad PRISM software (version 6; GraphPad Software Inc., San Diego, CA, US). Data are shown as mean ± SEM. Two-way ANOVA and post hoc test (Tukey's correction) were used for comparisons between groups. Results were considered statistically significant when the P value$<$0.05.

Results

Identification and characterisation of ovine endometrial MSC (eMSC)

The ovine endometrium differs from human in that it has aglandular caruncles and glandular intercaruncles (S1 Fig). We therefore first examined the cellular yield of single cell suspensions derived from caruncular and intercaruncular regions. The total cell numbers were 1.84 x 10^5 ± 0.46 x 10^5 cells/g tissue (n = 5) and 2.86 x 10^5 ± 0.95 x 10^5 cells/tissue (n = 4) respectively and was not significantly different (p$>$0.05). Therefore for this study, cells were isolated from the whole ovine endometrium including from both caruncles and intercaruncles.

Initially, unfractionated cell suspensions containing both endometrial epithelial and stromal cells were seeded at cloning density (50 cells/cm^2). In contrast to cloning or even standard culture of human endometrial cells, ovine epithelial cell clones outgrew the stromal clones (S2 Fig).

In order to isolate pure stromal cells from ovine endometrium we next determined a surface marker for the ovine epithelial cells to enable their removal by sorting using magnetic beads or a cell sorter. By immunohistochemistry, CD49f (α_6 integrin) was expressed on glandular and luminal epithelial cells (Fig 1A). To quantify the stromal population by flow cytometry, we dual-labelled ovine endometrial cells with CD45 and CD49f antibodies to exclude leukocytes and epithelial cells, respectively. This CD49f$^-$CD45$^-$ stromal population comprised 62.7 ± 11.6% (n = 4) of the endometrial cell population (Fig 1B). CD49f$^-$CD45$^-$ stromal cells were clonogenic (Fig 1C) with a mean cloning efficiency of 3.48 ± 1.79% (n = 4) (Fig 1D). CD49f$^-$CD45$^-$ stromal cells also underwent self renewal*in vitro*as shown by their ability to serially clone at least twice (Fig 1D) with the first serially cloned cells having a cloning efficiency of 17.8 ± 5.6% (n = 3). CD49f$^-$CD45$^-$ stromal cells also underwent differentiation into multiple mesodermal lineages; adipogenic, myogenic, osteogenic and chondrogenic (Fig 1E).

Having identified an eMSC population in ovine endometrium, we next sought to determine if several key surface markers used to obtain purified populations of human bmMSC and eMSC were expressed on ovine CD49f$^-$CD45$^-$ stromal cells. However, we found none of the antibodies we had previously used for human eMSC purification, PDGFRB, SUSD2 (W5C5), CD146,or several phenotypic MSC markers, CD90, CD73 and CD105 (Fig 2) were detected, suggesting lack of cross reactivity between human and ovine species. Percentages below 1% were regarded as negative. Since CD271 has been reported on ovine bmMSC [24], we next examined CD49f$^-$CD45$^-$ ovine endometrial stromal cells for expression of CD271. Fig2A and 2B shows that a small population of CD271$^+$ cells were present in the purified endometrial stromal fraction.

Prospective isolation of ovine eMSC

Since there was a small CD271$^+$ population in ovine endometrial stromal cell suspensions, the eMSC were extracted by double labelling with CD271 and CD49f antibodies and sorted as shown inFig 3A–3E. Dual colour immunofluorescence frequently revealed CD271$^+$ cells in the perivascular region surrounding vWF-expressing endothelial cells of arterioles and less

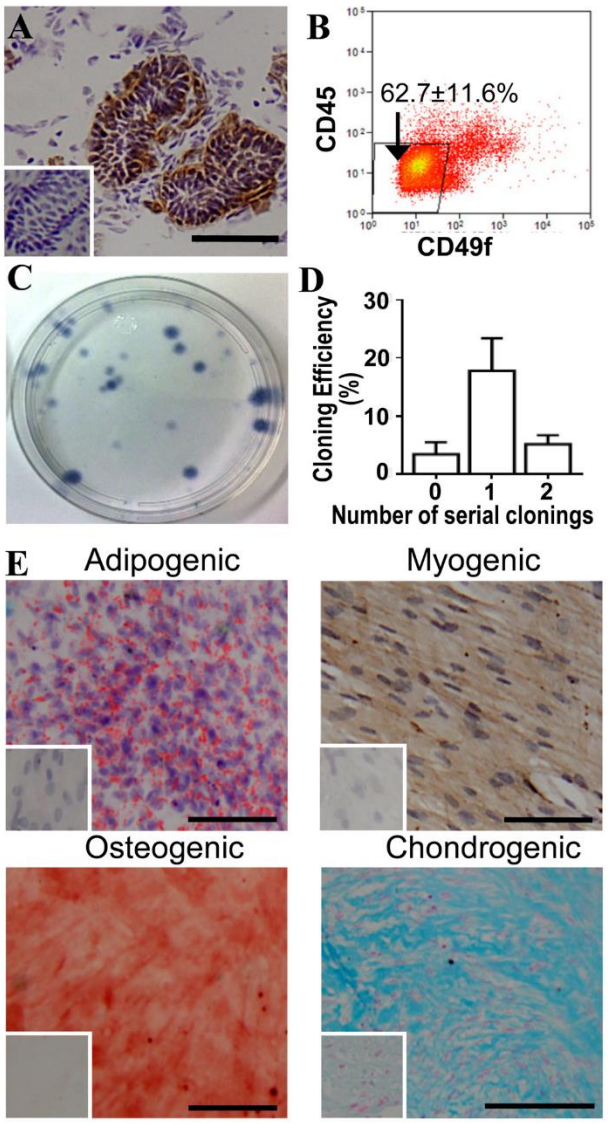

Fig 1. MSC properties of ovine endometrial stromal cells purified with CD49f antibodies to remove epithelial cells.(**A**) Immunohistochemical staining of ovine endometrium showing glandular epithelial cells positive for CD49f (brown). Inset, isotype control. (**B**) Representative flow cytometry dot plot for CD49f and CD45 (leukocyte marker) sorting ovine endometrial cell suspensions. The double negative population are the stromal cells, the percentage shown is mean±SEM (n = 4). (**C**) Culture plate showing colony forming units from $CD49f^{-}CD45^{-}$ sorted endometrial stromal cells. (**D**) cloning efficiency (CE) of serially cloned $CD49f^{-}CD45^{-}$ endometrial stromal cells. Two to three large colonies from each ovine samples (n = 3) were serially cloned twice. (**E**) Differentiation of $CD49f^{-}CD45^{-}$ sorted stromal cells into mesodermal lineages after 4 weeks culture in induction media. Images are representative of n = 3 biological experiments. Insets, controls cultured in 1% serum DMEM media. Scale bar, 50μm.

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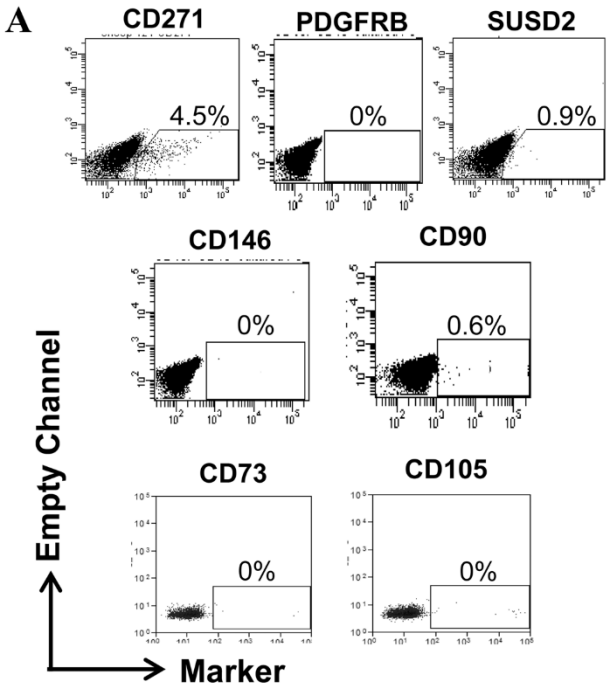

B

Marker	Mean (%)	SEM	n
CD271	4.54	2.33	5
PDGFRB	0.88	0.59	5
SUSD2 (W5C5)	0.93	0.48	3
CD146	0.00	0.00	3
CD90	0.8	0.32	4
CD73	0	nil	2
CD105	0	nil	2

Fig 2. Surface phenotype of CD49f$^-$CD45$^-$ ovine endometrial stromal cells.(**A**) Representative flow cytometry plots for phenotypic data of CD49f$^-$CD45$^-$ cells with their relative percentages of positive cells and (**B**) summarised in the table.

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frequently venules (Fig 4A–4D). Other CD271$^+$ cell were not clearly associated with vWF$^+$ vessels but were often arranged in a linear manner suggesting they may be associated with capillaries (Fig4A,4Cand4D). None of the CD271 $^+$ cells detected in the ovine endometrium expressed the pericyte markerαSMA (Fig 5A–5D) although some were closely associated with theαSMA $^+$ region surrounding venules (Fig 5C) and arterioles (Fig 5D), suggesting their location in the adventitia of arterioles and venules.

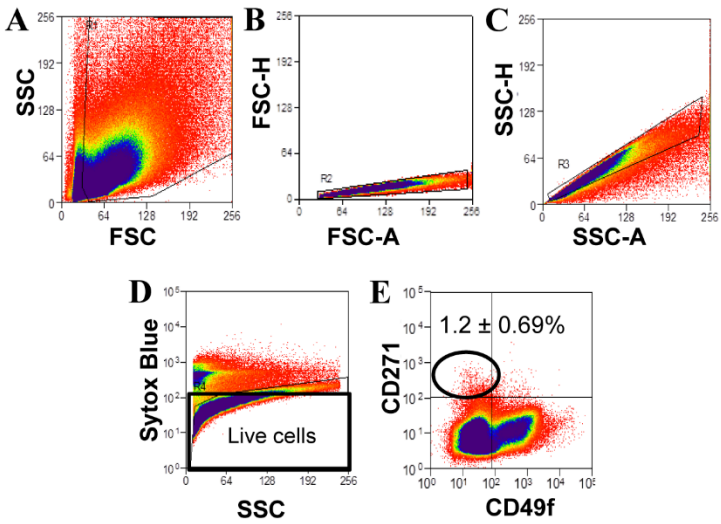

Fig 3. CD271 identifies a stromal subpopulation in ovine endometrium.(**A-E**) Flow cytometry cell sorting of freshly isolated ovine endometrial cells double labelled with CD271 and CD49f antibodies. (**A**) Scatter dot plots to set gates for viable cells and (**B-C**), for single cells, (**D**) dead cells were removed by Sytox Blue gating. (**E**) Representative dot plot for dual staining of stromal cells with CD271 and CD49f antibodies of n = 10 biological samples.

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Cloning efficiency of ovine eMSC

The cloning efficiency of $CD271^{+}CD49f^{-}$ and $CD271^{-}CD49f^{-}$ cells, as shown in Fig 6A–6C, was significantly higher in the $CD271^{+}CD49f^{-}$ cells (5.46 ± 1.14%, n = 10) compared with the $CD271^{-}CD49f^{-}$ population (1.58 ± 0.48%, n = 8) (p = 0.007). Serial cloning was further assessed for $CD271^{+}CD49f^{-}$ and $CD271^{-}CD49f^{-}$ cells, showing significantly higher cloning efficiencies at the first and second subcloning for the $CD271^{+}CD49f^{-}$ cells compared with $CD271^{-}CD49f^{-}$ cells ($p<0.0001$ and 0.001 respectively) (Fig 6C).

Differentiation of $CD271^{+}CD49f^{-}$ and $CD271^{-}CD49f^{-}$ cells

Multilineage differentiation was achieved by culturing the sorted $CD271^{+}CD49f^{-}$ and $CD271^{-}CD49f^{-}$ cells in specific culture induction media for four weeks. The $CD271^{+}CD49f^{-}$ cells differentiated into adipogenic, myogenic, osteogenic, and chondrogenic cells (Fig 6D, upper panel), staining strongly with Oil Red O,αSMA antibody, Alizarin Red and Alcian Blue (matrix) respectively, fulfilling another MSC criterion. The $CD271^{-}Cd49f^{-}$ cells showed weak differentiation into adipocyte, smooth muscle cell and chondrogenic lineages and failed to differentiate into the osteoblast lineage as there was no staining with Alizarin Red (Fig 6Dlower panel). No differentiation was induced when cells were cultured in control medium.

Characterising $CD271^{+}CD49f^{-}$cells

Fig 7shows that 92.0 ± 5.0% of $CD271^{+}CD49f^{-}$ sorted cells still expressed CD271 after two to four passages in culture (n = 4) by flow cytometry and verified by immunofluorescence (Fig 7A). However continued passaging of $CD271^{+}CD19f^{-}$ results in their differentiation into $CD271^{-}$ cells as the percentage $CD271^{+}$ reduces to 51% at passage 5 (n = 2) and 40% at passage 6 (n = 2). A small number of $CD271^{-}CD49f^{-}$ cells become $CD271^{+}$ during culture expansion; 15% and 12% at passages 5 and 6 respectively (data not shown). Cultured $CD271^{+}CD49f^{-}$ cells

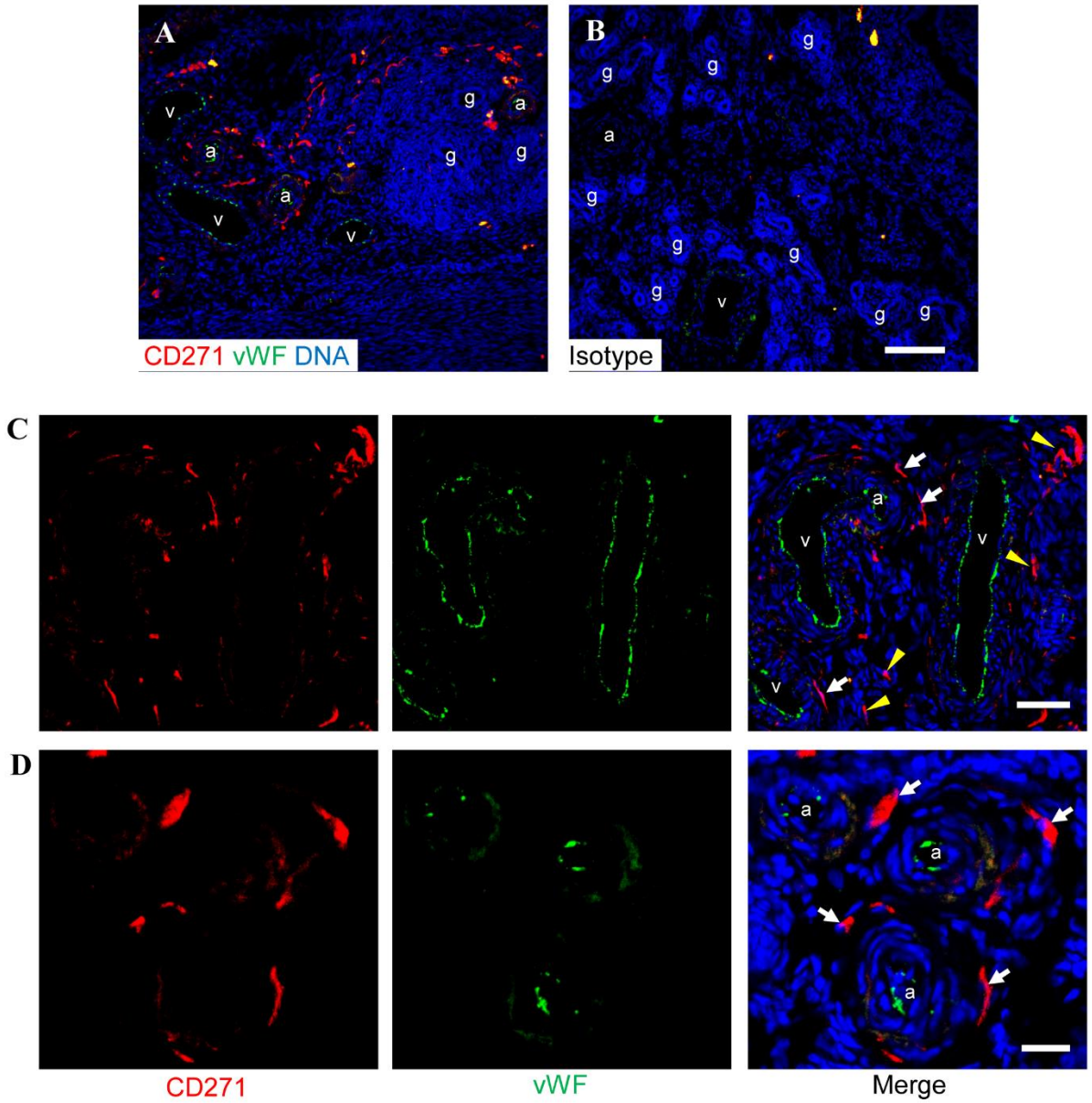

Fig 4. Immunofluorescence staining of ovine endometrium for CD271 and vonWillebrand Factor. (A)A low power micrograph of endometrium shows CD271 (red) relative to venules (v) and arterioles (a) that express vonWillebrand Factor (vWF, green).**(B)**Isotype control stained endometrium.**(C&D)**Some $CD271^+$ cells were associated with vWF^+ vasculature (white arrows) while others were not (yellow arrowheads). Nuclei are stained with Hoechst 33258 (blue). Scale bars: B = 100μm, C = 50μm, D = 20μm, A is at the same magnification as B. a, arteriole; g, gland; v; venule.

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proliferate rapidly in culture with a population doubling time of 18.7 hr at passage 5 (n = 2) while $CD271^-CD49f^-$ cells had a slower rate of growth with a population doubling time at passage 5 of 30.0 hr (n = 2).

While $CD271^+$ cells do not express αSMA *in vivo* (Fig 5), in culture they upregulate αSMA by passage 1 (Fig 7A). Other human MSC phenotypic markers were not expressed in cultured

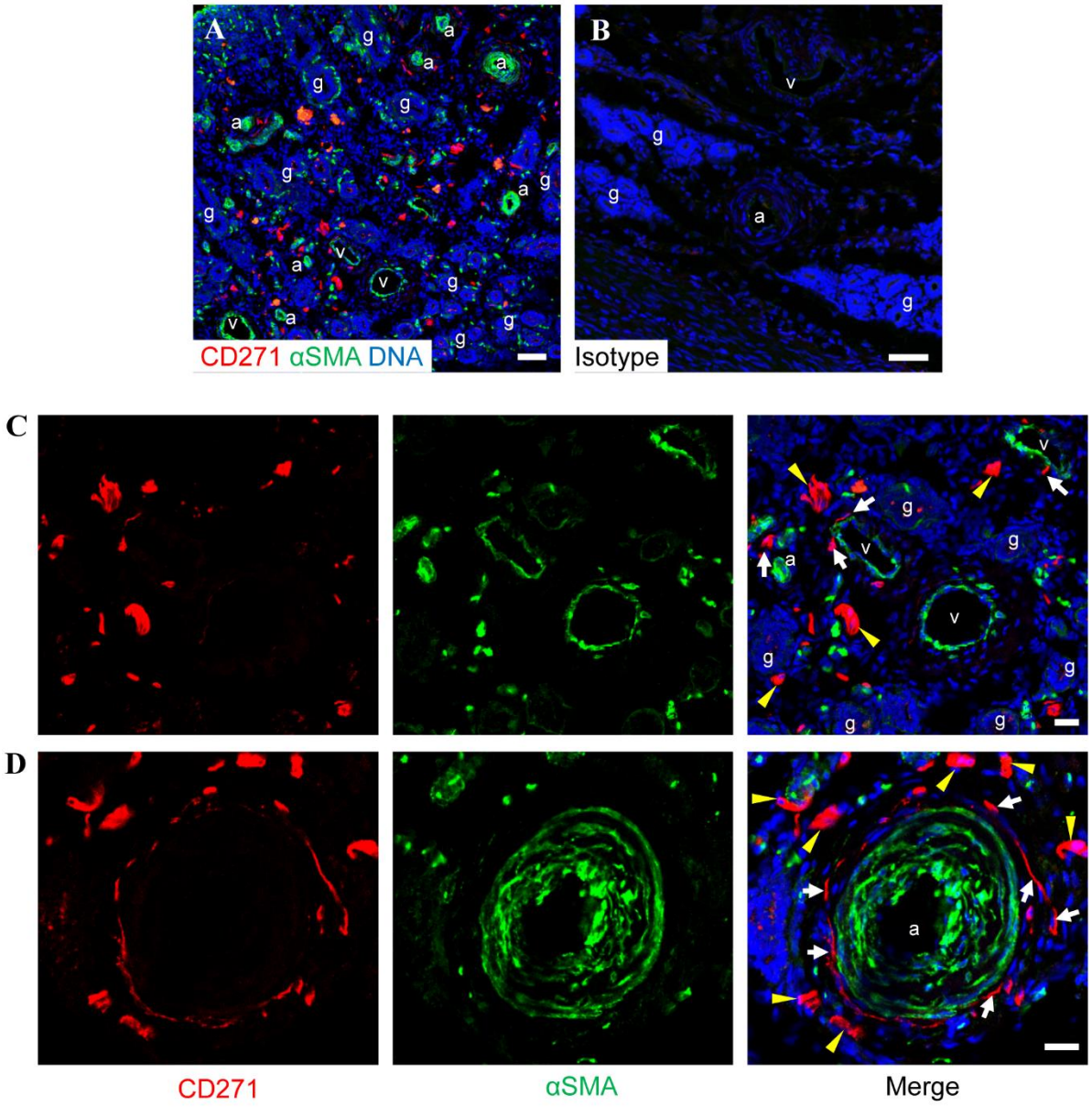

Fig 5. Immunofluorescence staining of ovine endometrium with CD271 and alpha-smooth muscle actin.(A) A low power micrograph of endometrium shows CD271(red) relative to cells expressing alpha-smooth muscle actin (αSMA, green) in the region surrounding venules (v), arterioles (a) and in the stroma between. (B) Isotype control stained endometrium. (C&D) Flattened $CD271^{+}$ cells were associated withαSMA^{+} regions surrounding the vasculature (white arrows) but did not expressαSMA, suggesting their location in the adventitia. $CD271^{+}$ cells not closely associated with large vessels or arterioles (yellow arrowheads) also did not expressαSMA. Nuclei are stained with Hoechst 33258 (blue). Scale bars: A&B = 50μm, C&D = 20μm. a, arteriole; g, gland; v; venule.

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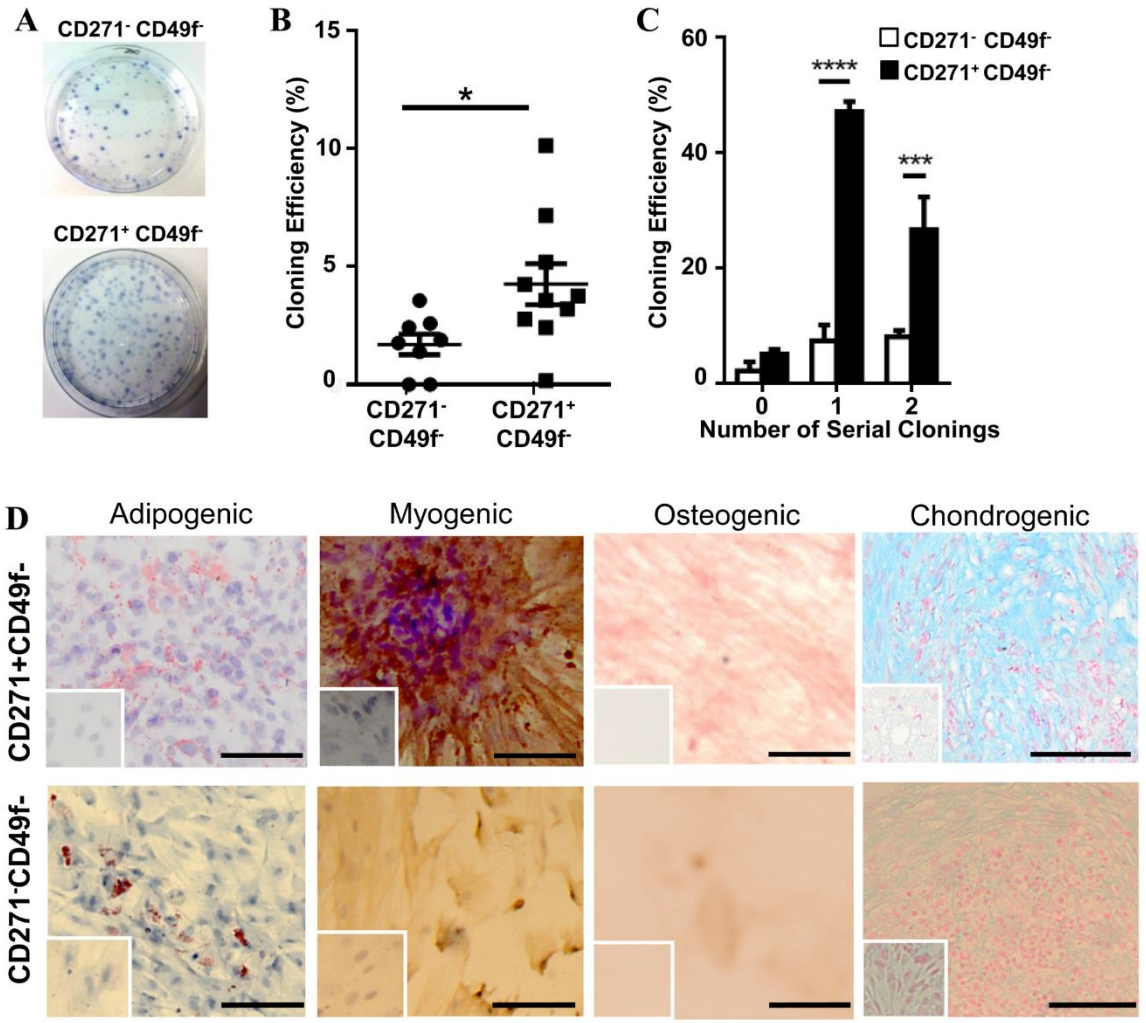


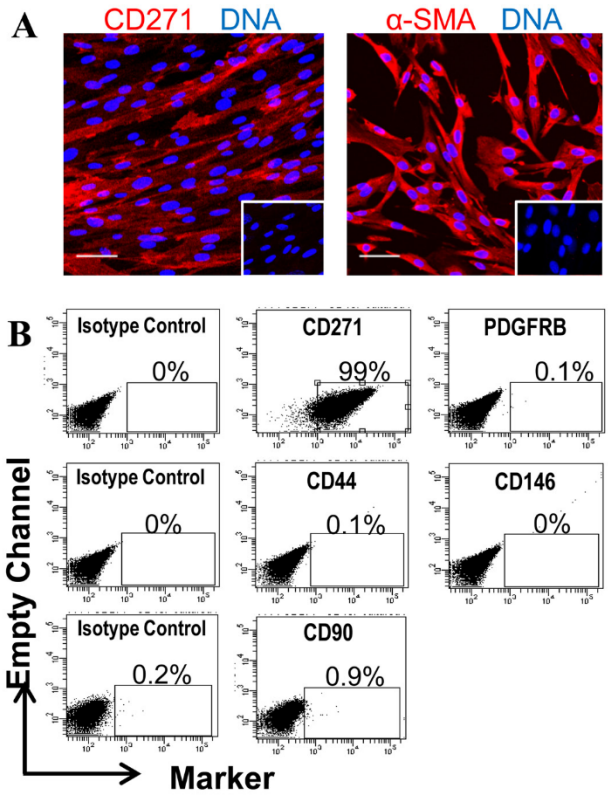
Fig 6. CD271$^+$CD49f$^-$ selects for ovine endometrial MSC.(**A**) Cloning plates showing representative examples from CD271 $^+$CD49f$^-$ and CD271$^-$CD49f$^-$ stromal cells and (**B**) cloning efficiency of CD271$^+$CD49f$^-$cells (n = 10) and CD271$^-$CD49f$^-$ cells (n = 6). (**C**) Serial cloning of CD271$^-$CD49f$^-$ (white bars) endometrial stromal cells and CD271$^+$CD49f$^-$ (black bars) candidate eMSC. Two to three large colonies from each ovine sample (n = 3) were serially cloned 2 times. (**D**) CD271$^+$CD49f$^-$ and CD271$^-$CD49f$^-$ cells were induced to differentiate into mesodermal lineages by culturing in induction media for 4 weeks. Images are representative of n = 3 biological samples. Insets are controls cultured in 1% FBS DMEM media.*P<0.05,***P<0.001,****P<0.0001. Bars are mean±SEM. Scale bar, 50μm.

doi:10.1371/journal.pone.0127531.g006

CD271$^+$CD49f$^-$ cells (Fig7Band7C), similar to ovine endometrial stromal cells (Fig 2). While CD271 proved to be effective in isolating eMSC from ovine endometrium, the human markers CD73, CD90, CD105, PDGFRB, or SUSD2 were not detectable on eMSC from the ovine endometrium (Fig7Band7C). A small percentage of CD271 $^+$CD49f$^-$ cells (3.15 ± 1.65%, n = 4) expressed the MSC phenotypic marker CD44 (Fig 7), much lower than for human eMSC.

Discussion

In this study we demonstrated for the first time that the ovine endometrium contains a small subpopulation of mesenchymal stem/stromal cells fulfilling several relevant MSC criteria;



C

Marker	Mean (%)	SEM	n
CD271	92.03	5.01	4
CD90	1.63	0.91	4
CD44	3.15	1.65	4
CD146	0.03	0.03	4
PDGFRB	0.33	0.23	4

Fig 7. Phenotype of CD271$^+$CD49f$^-$ ovine endometrial cells by immunofluorescence and flow cytometric analysis.(**A**) CD271 andα-SMA immunostaining on passage 1 cultured ovine CD271 $^+$CD49f$^-$ cells (red). Nuclei are stained with Hoechst 33258 (blue). Insets, Isotype controls. Scale bar, 50μm. (**B**) Representative flow cytometry dot plots for MSC markers representative of n = 4 biological samples and (**C**) table showing percent positive cells for each marker.

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clonogenicity and multilineage mesodermal differentiation. We also demonstrated that CD271 is a selective marker enriching for the ovine eMSC subpopulation, as CD271$^+$CD49f$^-$ cells had a higher percentage of clonogenic cells, showed greater self-renewal*in vitro*and better ability to differentiate into mesodermal lineages compared to CD271$^-$CD49f$^-$ stromal cells. Similar to bmMSC and to human eMSC, the ovine eMSC were able to differentiate into adipogenic, myogenic, osteogenic and chondrogenic lineages.

Many of these CD271$^+$ stromal cells were found in a perivascular location around arterioles and some venules in ovine endometrium, although they were not in close apposition to the endothelial cells as is the case for human eMSC. CD271$^+$ cells were also located along presumptive vascular structures morphologically resembling capillaries. These presumptive capillaries did not immunostain with vWF antibody, which is more selective for endothelial cells of larger vessels [25]. Unfortunately other endothelial markers are unavailable for ovine endothelial cells. None of the CD271$^+$ cells detected expressedαSMA suggesting that they are not pericytes. CD271$^+$ cells surrounding arterioles may represent perivascular adventitial cells, a population previously reported to have MSC properties [26,27].

CD271 has been previously used as a marker to isolate MSC from human bone marrow and umbilical cord blood [28]. Human umbilical cord CD271$^+$ cells had slower proliferation rates than bone marrow CD271$^+$ cells, although bone marrow CD271$^+$ cells were more osteogenic than umbilical cord CD271$^+$ cells [28]. Another comprehensive study examining the cross reactivity of the CD271 marker in multiple mammalian species found that CD271 enriched for clonogenic ovine, monkey, goat, canine, and porcine bmMSC concluding that it was a MSC marker for 6 large animal species useful for preclinical research [24]. Our study also identifies CD271 as a useful marker to purify ovine eMSC for our preclinical studies on their use in tissue engineering approaches for treating urological and gynecological disorders.

While all MSC have the accepted MSC properties, there are differences in some properties dependent on the tissue source of the MSC, particularly in their capacity to differentiate into the various lineages [29]. Cloning efficiencies for ovine eMSC and bmMSC are similar; 5.5% versus 4.6% [30]. The endometrium provides an additional valuable tissue from which MSC with high proliferative and broad differentiation capacity can be obtained.

The lack of specific MSC surface markers in tissues from non-human species has been described, i.e. for ovine bmMSC [30]. In line with the findings from this study, currently available human MSC phenotypic markers CD73, CD105, PDGFRβ, or CD146 were also not detectable on ovine bmMSC. In this study, we found that our selective markers for prospective isolation of human eMSC were either not expressed on ovine eMSC or the SUSD2 (W5C5), PDGFRB and CD146 antibodies did not cross react with the ovine epitopes. Neither were the antibodies to several of the phenotypic surface markers of bmMSC (CD73, CD90, CD105) immunoreactive with ovine endometrial stromal cells. The identification of CD271 as a specific marker of a small subpopulation of ovine endometrial stromal cells with eMSC properties allows the prospective isolation of ovine eMSC. Isolating pure eMSC is important to avoid a heterogeneous cell population in the stromal fraction [29], particularly for tissue engineering applications where cells with maximum proliferative potential are required.

It was not possible to culture ovine eMSC without first removing the epithelial population, which unlike human endometrial epithelial cells, possessed high proliferative capacity, outcompeting the stromal population. The sheep for this study were all parous and several years old. It has already been described previously that, unlike in humans, there was no correlation between age and bmMSC proliferative potential or age and MSC numbers in the ovine model [31].

The ease of access of endometrial tissue is a significant advantage of using this cell source from humans. Characterisation of ovine eMSC allows this source of MSC to be investigated in a large pre-clinical animal model. For example we are investigating their use as a cell based therapy for pelvic organ prolapse and the ovine model is ideal for testing this prior to clinical trials [32,33]. eMSC were recently discovered in human endometrium [9,10], where they likely have a role in endometrial regeneration each menstrual cycle. They are also believed to play a role in non-menstruating species and have been identified as label retaining cells in mice [34, 35]. More recently eMSC were also described in a larger animal, the pig [36], however the cloning efficiency was low in this species. This could be because the authors examined the unsorted

stromal fraction. In non menstruating species it is suggested that eMSC have a role in endometrial remodelling that occurs each estrus cycle, and in particular in regenerating the stromal vascular component of the endometrium following delivery of the placenta and foetuses.

Tissue engineering has become popular during the last decade with increasing numbers of successful clinical applications, particularly in the urology and gynecology fields as exemplified in the reconstruction of the bladder and the vagina [37]. Due to similarities in anatomy to humans, sheep have been frequently used as a large preclinical animal model, for example the implantation of autologous bmMSC combined with ceramic to generate bone tissue [38]. The identification and availability of eMSC in the ovine uterus can facilitate further research in the gynecology and urology field using an autologous model. Tissue engineering attempts combining eMSC and novel materials for pelvic organ prolapse repair have provided promising data in a small animal model [39] and now need to be examined in a large preclinical animal model. The sheep is an excellent model for preclinical studies [40]. Both Gynecology and Urology disciplines are investigating novel treatment options using cell seeded scaffold devices as tissue engineering constructs for either fascia repair or the restoration of damaged and under developed organs. The availability of autologous eMSC in a large animal model will facilitate future research in this field, having applications for very common conditions such as pelvic organ prolapse and stress urinary incontinence.

Conclusion

Ovine endometrium contains a small population of mesenchymal stem/stromal cells which are enriched in the $CD271^+$ $CD49f^-$ $CD45^-$ subpopulation.

Supporting Information

S1 Fig. H&E staining of ovine uterus. Dotted line delineates the endometrium and myometrium. C, caruncle; IC intercaruncle. Scale bar, 200μm.
(PDF)

S2 Fig. Ovine endometrial epithelial contamination on stromal cell cloning plate. The well was seeded with 50 unsorted ovine endometrial stromal cells/cm^2 and cultured for 14 days. Contaminating epithelial clones (dotted) competed with and overgrew stromal clones (square) on the cloning plate.
(PDF)

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Author Contributions

Conceived and designed the experiments: CEG VL KST. Performed the experiments: VL KST DU JD SG YRO. Analyzed the data: VL KST JD SG YRO CEG. Wrote the paper: VL KST DU CEG.

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