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**THE ROLE OF DECIDUAL MESENCHYMAL
STEM/STROMAL CELL AGEING IN LABOUR**

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ABSTRACT

Ageing and parturition share many common pathways, but whether these two processes are associated, or even dependent on each other, is poorly understood. This study focuses on the decidua, where there is evidence that decidual cells undergo ageing as parturition approaches term, and these ageing-related changes of the decidua may trigger labour. Mesenchymal stem/stromal cells (MSCs) are a type of stem cell that reside in the decidua. Ageing of decidual MSCs (DMSCs) may contribute to the functional and molecular changes in decidual tissue required for spontaneous onset of labour (SOL) at term. The objective of this study was to determine whether DMSCs from patients experiencing SOL show evidence of molecular and functional loss and/or changes compared with DMSCs from patients, not in labour (NIL), undergoing Caesarean section (C-section) delivery.

Placentae were collected from full-term (39-40 week gestation), SOL (n = 8) and NIL (n = 7) patients with non-complicated pregnancies. DMSCs were isolated from *decidua basalis*, which remains attached to the placenta following delivery. DMSCs were characterised to ensure their decidua-origin and stem cell properties. Important cell functions, including cell proliferation, cell migration, cell death, lipid peroxidation, aldehyde dehydrogenase (ALDH) expression, and pro-inflammatory cytokine secretion were then compared between SOL- and NIL-DMSCs. SOL-DMSCs demonstrated a significant increase in necrosis, lipid peroxidation, migration, and IL-6 production compared with NIL-DMSCs ($p < 0.05$). SOL-DMSCs also showed an increase in apoptosis as well as a decrease in proliferation, ALDH expression and IL-8 production compared with NIL-DMSCs, however, the differences were not statistically significant ($p > 0.05$). These findings suggested that SOL-DMSCs underwent advanced ageing at term.

The lipid contents of DMSCs from both patient groups were extracted using Folch's method, separated using High Performance Liquid Chromatography (HPLC) and identified using tandem mass spectrometry (MS/MS). The quantity of each lipid species from DMSCs of both patient groups was measured and compared. SOL- and NIL-DMSCs contained the same lipid species, however the expression level of particular lipid species significantly differed. DMSCs may undergo changes in lipid

metabolism during labour at term, possibly to provide the energy source for the labour process.

In conclusion, this study provides novel evidence that DMSCs undergo ageing-related functional and molecular changes associated with labour and these changes may play a significant role in promoting spontaneous labour at term.

DECLARATION

This is to certify that

- (i) this thesis comprises only my original work towards the MPhil (Medicine) except where indicated in the preface,
- (ii) due acknowledgement has been made in the text to all other material used,
- (iii) this thesis is fewer than 50,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Date: 26th August 2019

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PREFACE

This preface is to clarify that all research described in this MPhil thesis was undertaken by the author, Joan Christie Wijaya, at the Department of Maternal-Fetal Medicine Pregnancy Research Centre, Royal Women's Hospital and the Department of Obstetrics and Gynaecology of the University of Melbourne during her MPhil candidature, unless stated otherwise. Specifically, the author acknowledges the following people for their direct involvement in this study:

1. Dr. Mark Pertile and Dr. Melissa Glass for performing fluorescence *in situ* hybridisation (FISH) of DMSCs at Victorian Clinical Genetics Services, Melbourne, Australia
2. Dr. Shuai Nie for performing mass spectrometry on the DMSC lipid extract samples at the Mass Spectrometry and Proteomics Facility (MSPF) of Bio21 Molecular Science & Biotechnology Institute, Melbourne, Australia.

This MPhil thesis gave rise to one original review article (publication status: submitted for publication) and one original research article (publication status: submitted for publication). The author contributed as the principal author in both articles. Dr. Bill Kalionis (principal supervisor), Dr. Harry M. Georgiou (co-supervisor), Prof. Shaun P. Brennecke (co-supervisor), Dr. Maria I. Kokkinos (co-supervisor), and Dr. Ramin Khanabdali are the co-authors. The supervisors contributed in the experimental design, data analyses, and manuscript editing. Some relevant data from Dr. Ramin Khanabdali's work were included in the research article. He also assisted in manuscript editing.

A substantial portion of the review and research journal articles (see appendices VI and VII) has been included in various parts of this MPhil thesis supplemented with additional details to conform with the relevant thesis requirements. The work pertaining to this study was approved by the Royal Women's Hospital Human Research Ethics Committees (HREC) (Projects 10/49 and 12/42).

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Khanabdali, R., **Wijaya, J.C.**, Georgiou, H. M., Xia, S., Abumaree, M.H. & Kalionis, B. (2019). The Effect of Ageing on Mesenchymal Stem Cells and their Secreted Extracellular Vesicles.

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LIST OF ABBREVIATION

5'-NT	Ecto-5'-nucleotidase
AcCA	Acyl Carnitine
Akt	Protein kinase B
ALCAM	Activated leukocyte cell adhesion molecule
ALDH	Aldehyde dehydrogenase
AMA	Advanced maternal age
AMPK	5' AMP-activated protein kinase
APC	Allophycocyanin
APC-Cy7	Allophycocyanin-Cy7 (tandem conjugate)
ARM	Artificial rupture of membrane
AV	Annexin V
BCA	Bicinchoninic acid
BMI	Body mass index
CD	Cluster of differentiation
Cer	Ceramide
CL	Cardiolipin
CMSCs	Chorionic villous-derived MSCs
Co	Coenzyme Q
COX	Cyclooxygenase
CRH	Corticotrophin-releasing hormone
C-section	Caesarean section
CXCR4	Chemokine receptor type 4
DAMPs	Damage-associated molecular pattern molecules
DAPI	4',6-diamidino-2-phenylindole
DEAB	N,N-diethylaminobenzaldehyde
DMSCs	Decidual mesenchymal stem/stromal cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSCs	Decidualised stromal cells
ECS-DMSCs	DMSCs from placentae of emergency C-section patients
ELISA	Enzyme-Linked Immunosorbent Assay
ESCs	Embryonic stem cells
EVs	Extracellular vesicles
EVTs	Extravillous trophoblasts
FCS	Fetal calf serum
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor

GSHPx	Glutathione peroxidase
HAT	Histone acetylase
HBSS	Hanks' balanced salt solution
HCAM	Homing cell adhesion molecule
hCG	Human chorionic gonadotropin
Hex1Cer	Hexosyl ceramide
Hex2Cer	Dihexosyl ceramide
Hex3Cer	Trihexosyl ceramide
HLA	Human leukocyte antigen
HMGB1	High-mobility group box 1
HPA	Hypothalamic pituitary adrenal
HSF	Heat shock factor
Hsp	Heat shock protein
ICM	Inner cell mass
IFN- γ	Interferon- γ
IGFBP-1	Insulin-like growth factor binding protein 1
IL	Interleukin
ISCT	International Society for Cell & Gene Therapy
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPI	Lysophosphatidylinositol
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MIF	Migration inhibitory factor
miR	MicroRNAs
MMPs	Matrix metalloproteinases
MSCs	Mesenchymal stem/stromal cells
mTORC1	Mammalian target of rapamycin complex 1
NF κ B	Nuclear factor κ -light-chain-enhancer of activated B cells
NIL	Not in labour
p21	Cyclin-dependent kinase inhibitor
PAI-1	Plasminogen activator inhibitor
PC	Phosphatidylcholine
PCOS	Polycystic ovary syndrome
PE ⁽¹⁾	Phycoerythrin
PE ⁽²⁾	Phosphatidylethanolamine
PG ⁽³⁾	Prostaglandin
PG ⁽⁴⁾	Phosphatidylglycerol

PI ⁽⁵⁾	Propidium iodide
PI ⁽⁶⁾	Phosphatidylinositol
PR	Progesterone receptor
PS	Phosphatidylserine
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
SAHF	Senescence-associated heterochromatin foci
SASP	Senescence associated secretory phenotype
SESN	Sestrin
sFlt-1	Soluble FMS-like tyrosine kinase-1
SM	Sphingomyelin
SOD	Superoxide dismutase
SOL	Spontaneous onset of labour
SROM	Spontaneous rupture of membrane
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TG	Triglyceride
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumour necrosis factor
Trp53	Transformation-related protein 53
uNK	Uterine natural killer cells
VEGF	Vascular endothelial growth factor
α -MEM	Minimum essential medium eagle - alpha modification

These particular abbreviations were applied in: ⁽¹⁾ Section 4.2.2 and Appendix II, ⁽²⁾, ⁽⁴⁾ and ⁽⁶⁾ Section 4.4 (i.e., 4.4.1 and 4.4.2), Section 5.4, Section 6.1, and Appendix I and V, ⁽³⁾ Section 1.3.1., Section 1.4., and Section 5.3.4., ⁽⁵⁾ Section 3.3.3., Section 4.3.3., Section 5.3.3., Appendix I and III.

1. LITERATURE REVIEW

1.1. General overview

During the secretory phase of each menstrual cycle, the maternal endometrium develops a specialised lining of tissue, called the decidua, which is an important requirement for preparing, establishing and maintaining the pregnancy (Vinketova, Mourdjeva, & Oreshkova, 2016). The main roles of the decidua are to facilitate proper embryo reception (i.e., recognition, selection, and acceptance), develop immune tolerance to protect the allogeneic embryo from possible attacks by maternal immune cells, and to provide a physical scaffold as well as nutrients for the developing embryo prior to the development of the mature placenta (Mori, Bogdan, Balassa, Csabai, & Szekeres-Bartho, 2016; Vinketova et al., 2016).

Recent studies show that the decidua progressively ages as pregnancy approaches term parturition (Hirota, Cha, Yoshie, Daikoku, & Dey, 2011), while premature and accelerated ageing of decidua are associated with pre-term parturition (Cha et al., 2013; Hirota et al., 2010). In these studies, the ageing-related changes were observed particularly on the decidualised stromal cells (DSCs), which are the major resident cells in the decidua. This supports the notion that the ageing-related changes of decidua at parturition originate at the cellular level. Ageing of the resident cells of decidua is thought to play an essential role in the initiation of labour, and thereby, may be involved in determining the timing of parturition (Cha & Aronoff, 2017; Deng et al., 2016). Nonetheless, the current evidence that ageing, particularly of the decidua, is beneficial or even essential for natural parturition at term, is still limited. Thus, there are many opportunities for further investigation.

Given that stem cell exhaustion is a hallmark of ageing, this study investigated whether decidual mesenchymal stem/stromal cells (DMSCs), which are the predominant stem cell type that resides in the decidua, may also undergo ageing processes associated with spontaneous onset of labour (SOL) at term. This may offer a novel insight into the molecular mechanisms of the natural, non-pathological processes of parturition at term, which is a crucial baseline to determine the direction of further prospective studies in parturition and its-related disorders.

1.2. General concept of biological ageing

1.2.1. Definition and evolutionary perspective of ageing

Human ageing is a complex process. It is difficult to define as it encompasses multiple processes operating at many levels from the single cell to the whole organism (e.g., molecular, cellular, physiological and psychosocial). Ageing is defined as the accumulation of various physiological changes that lead to the decline of function over time (Magalhaes, 2011; McDonald, 2013). From an evolutionary perspective, ageing is considered to result from the accumulation of permanent damage as a consequence of the body's decision to limit energy and resources devoted to tissue repair at the level necessary for restoring the ability to reproduce (Izaks & Westendorp, 2003; Rattan, 2006). This strategy is important to ensure successful continuation of generations during a limited lifespan (Rattan, 2000). The perceptible outcomes of this survival strategy are loss of vigour and increased risk of diseases due to the overwhelming build-up of permanent damage over time (Izaks & Westendorp, 2003). Studies show that various common human chronic diseases including neurodegenerative diseases, cardiovascular disorders,

cancer, and diabetes are strongly associated with advancing age (Fulop et al., 2010; Kenyon, 2005). Therefore, although ageing occurs throughout life, it manifests most intensely at post-reproductive age, which is about the age of 50 and older in humans (Rattan, 2006).

1.2.2. The multiple features of ageing: the impetus of the clock of life

Ageing is proposed to initiate, drive, and ultimately terminate a person's life by exerting different effects at various development stages (**Figure 1**). In contrast to the detrimental effects of advanced adult ageing [e.g., loss of vigour, increased morbidity and mortality risks (Harman, 1998)], recent evidence shows that ageing may play beneficial roles in early human development by facilitated pregnancy and parturition (Cox & Redman, 2017; Menon, Bonney, Condon, Mesiano, & Taylor, 2016). For example, at the early stages of gestation, ageing of syncytiotrophoblast layer is essential to maintain normal placental development and function required to sustain pregnancy (Cox & Redman, 2017). At the later stages of gestation, ageing of various gestational tissues, including the fetal membranes (Menon, 2016; Poletini et al., 2015) and decidua (Hirota et al., 2011; Hirota et al., 2010), may weaken various physical supports for the fetus (Menon, Bonney, et al., 2016) and promote the secretion of various cellular signalling molecules (e.g., pro-inflammatory cytokines) required for labour and parturition (Hirota et al., 2011; Norwitz et al., 2015). After birth, the ageing features accumulate over time and begin to intensify post-adulthood as the dysfunctional burden of accumulated aged cells/tissues outpaces stem cell rejuvenation capacity. The stem cell population may also undergo age-related exhaustion (i.e., a decrease in cell number and function)

(Collado, Blasco, & Serrano, 2007; Jones & Rando, 2011). This concept of stem cell exhaustion may also apply when ageing occurs to promote parturition in healthy term pregnancies. This particular area, however, is yet to be established. The beneficial effects of ageing at the early stages of life, particularly in association with parturition, are rarely explored despite many shared common features.

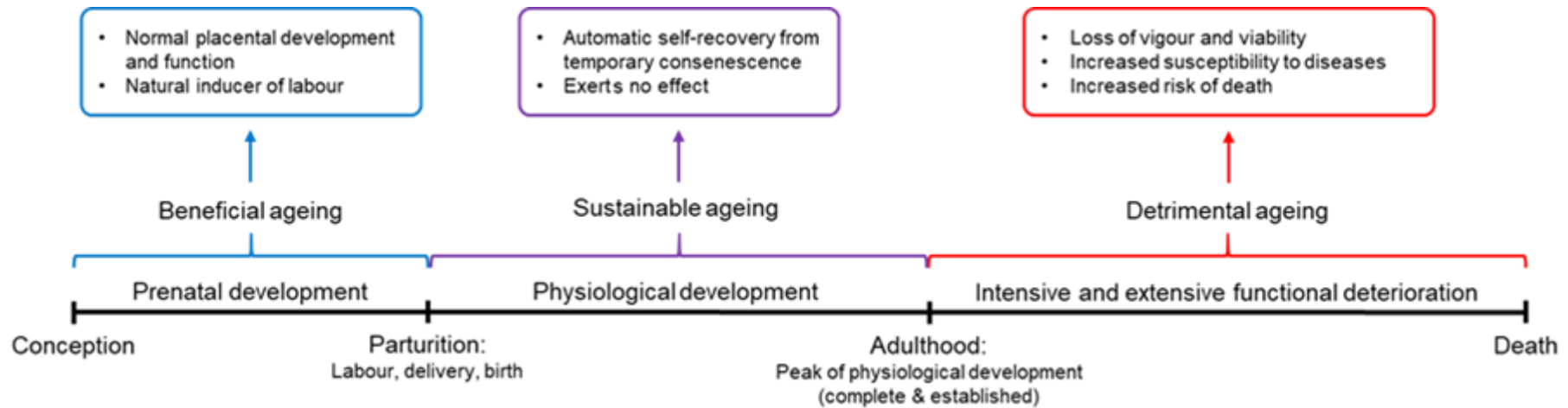


Figure 1. Various features of ageing throughout human's clock of life

Ageing can be beneficial, sustainable, or detrimental depending on the development stage of life. At the beginning of life, ageing is beneficial for sustaining pregnancy and promote parturition. During childhood and adulthood, ageing exerts no perceptible effect as the tissue repair system functions at its peak to support successful reproduction. Post-adulthood, the detrimental effects of ageing begin to take place with death being the endpoint.

1.2.3. The hallmarks of ageing

Lopez-Otin, Blasco, Partridge, Serrano, and Kroemer (2013) proposed nine hallmarks of ageing, which are classified into three groups, i.e., primary, antagonistic, and integrative hallmarks, based on the similarity of their characteristics (**Figure 2**). These hallmarks are commonly expressed in aged organisms and were determined based on the following criteria: manifested during normal ageing, cause acceleration of ageing if experimentally exacerbated, and hinder normal ageing process if experimentally alleviated (Lopez-Otin et al., 2013).

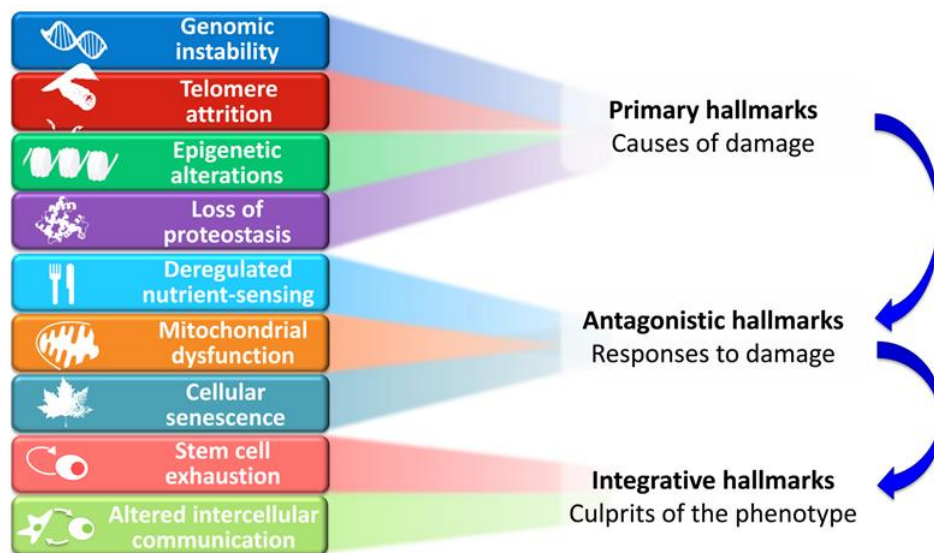


Figure 2. The hallmarks of ageing

Image is adapted from (Lopez-Otin et al., 2013).

The primary hallmarks are ageing phenomena that have undeniably adverse impacts and are the causes of age-related damage. The antagonistic hallmarks are ageing phenomena that generate opposite effects depending on the intensity of their responses to the damages caused by the primary hallmarks. When controlled, these ageing phenomena are beneficial for maintaining normal tissue function, but when uncontrolled, these phenomena are harmful. The integrative hallmarks are ageing

phenomena that directly affect tissue homeostasis and functions, thus contribute to ageing phenotypes (Lopez-Otin et al., 2013).

The work in this thesis relates to stem cell exhaustion from the integrative hallmarks. Some leading causes of stem cell exhaustion are increased oxidative stress and DNA damage, disturbed proteostasis and mitochondrial dysfunction in somatic stem cells, or combinations of these factors (Oh, Lee and Wagers, 2014). Studies suggest aged stem cells are unresponsive to extrinsic signals, have lower self-renewal capability, and undergo abnormal differentiation, senescence or apoptosis, which contribute to the overall decline in the tissue functionality [as reviewed in (Jones & Rando, 2011)].

1.3. General concept of human parturition

1.3.1. Human Parturition: Labour and Delivery

Parturition is the action or process of giving birth to offspring. This natural process comprises labour and delivery, and these terms are often used interchangeably with parturition. Labour is characterised by the regular and painful myometrial contractions that increase in duration, intensity, and frequency as the time of birth approaches (The National Health Service, 2009). Labour follows the withdrawal of inhibitory factors that maintain myometrial quiescence during pregnancy e.g., progesterone, prostacyclin, relaxin, and nitric oxide (Norwitz, Robinson, & Challis, 1999). Once initiated, myometrial contractility is stimulated in response to the secretion of uterotropins (i.e., estrogen) and uterotonins (e.g., oxytocin) by endocrine glands (Norwitz et al., 1999), and by the production of pro-inflammatory factors e.g., prostaglandins (PGs), mainly PG-F_{2α} and PG-E₂, by the decidua and

fetal membranes, respectively (Kredentser, Embree, & McCoshen, 1995; Okazaki, Casey, Okita, MacDonald, & Johnston, 1981; Skinner & Challis, 1985), which act through the cyclooxygenase (COX)-2 pathway (Loudon, Groom, & Bennett, 2003). The main feature of myometrial stimulation is the upregulation of specific contraction-associated myocytes and major gap components (i.e., connexin 43), which are required to transmit the contractile signals between myocytes (Kota et al., 2013; Norwitz et al., 1999). Besides the myometrial contractility, labour is also characterised by ⁽¹⁾ cervical ripening (i.e., cervical dilatation and effacement, loss of the mucus plug, fetal membrane rupture, and leakage of amniotic fluid), leading to ⁽²⁾ the delivery of the neonate and ⁽³⁾ placental detachment. These phenomena are clinically referred to as the three stages of labour (Steer & Flint, 1999). Besides mechanical stretching, endocrine regulation, and inflammation (Kamel, 2010), various studies also associate labour with other molecular and biochemical pathways e.g., lipid metabolism (Mosher et al., 2014), remodelling of the uterine and cervical extracellular matrix (Roh, Oh, Yoon, & Lee, 2000), oxidative stress (Fainarua et al., 2002), and apoptosis (Kumagai et al., 2001).

1.3.2. Timing of parturition: the key to pregnancy success and perinatal outcome

Human parturition normally occurs between 37–42 weeks of gestation, a period that is commonly called “term”. Term is further re-defined into several specified periods i.e., early-term (37 weeks 0 days to 38 weeks 6 days), full-term (39 weeks 0 days to 40 weeks 6 days), late-term (41st week) and post-term (42 weeks and beyond) (The American College of Obstetricians and Gynecologists Committee, 2013). The re-definition of term was necessary to address the differences in the risk of adverse

perinatal outcomes throughout the ‘term’ period (e.g., respiratory morbidity, stillbirth, and fetal growth restriction), with a higher risk was observed in early and late/post-term (Engle & Kominiarek, 2008; Maiti et al., 2017).

The timing of parturition varies between species and among individuals within the same species. The discrepancy between species indicates differences in the time to accommodate complete fetal growth before birth. Discrepancies within the same species (e.g., pre, early, full, late, and post-term period in human) indicate the existence of more than one regulatory mechanism, widely known as “gestational clocks”, that determine end-point of pregnancy. The gestational clocks work in diverse and redundant manners to compensate for the possibility that one or more regulatory pathway(s) may fail to occur at the appropriate time [as reviewed in (Diemert & Arck, 2018; Menon, Bonney, et al., 2016)].

Various types of gestational clocks have been proposed. These clocks are classified based on the tissue compartments (i.e., fetal membrane, decidual, myometrial, placental, and fetal clocks) or the molecular pathways involved (i.e., endocrine, immune, proteomic, and epigenetic clocks) (**Figure 3**). In brief, the fetal membrane clock refers to the initiation of parturition by fetal membrane senescence (Menon, 2016). The decidual clock signifies the initiation of parturition by the upregulation of inflammatory signals secreted by the decidua (Norwitz et al., 2015). The myometrial clock refers to the initiation of parturition by an increased inflammatory load on the gravid myometrium leading to progesterone withdrawal (Menon, Bonney, et al., 2016). The placental and fetal clocks signify an increase in corticotrophin-releasing hormone (CRH) secretion by placental trophoblasts (Smith, Mesiano, & McGrath, 2002) and an upregulation of the fetal hypothalamic

pituitary adrenal (HPA) axis to stimulate various hormonal cascades required for the maturation of fetal organs e.g., lungs (Kitterman et al., 1981) as pregnancy approaches term. The endocrine clock refers to the roles of fetal and placental hormones e.g., CRH and HPA axis during the onset of labour (Menon, Bonney, et al., 2016). The immune clock is based on the chronology of immune cell behaviours in peripheral blood, their interconnected pathways and changes as pregnancy progresses (Aghaeepour et al., 2017). The proteomic clock refers to the dynamic changes of the plasma proteome during pregnancy (Aghaeepour et al., 2018). The epigenetic clock is based on the regulation of DNA methylation in association with labour (Mitsuya, Singh, Sooranna, Johnson, & Myatt, 2014; Wang et al., 2019). Despite the abundant evidence of various gestational clocks, the mechanisms by which these gestational clocks sequentially or simultaneously work to initiate parturition remain unclear.

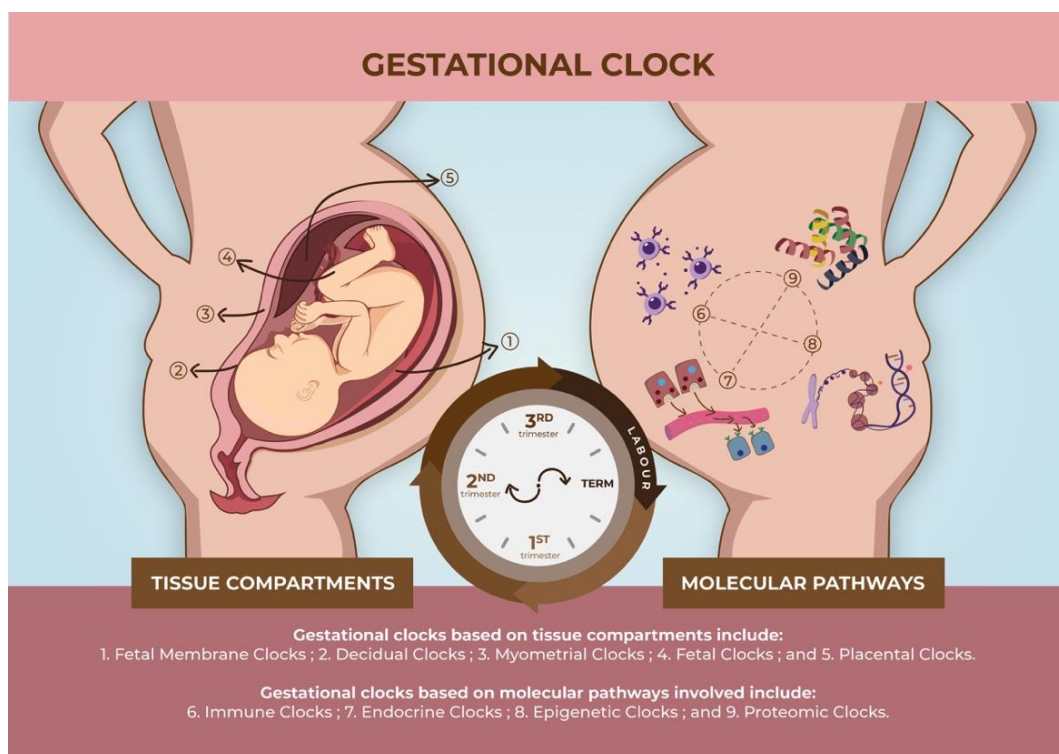


Figure 3. Various types of proposed gestational clocks

1.4. Building the link between parturition and ageing

Ageing and parturition share common features. For example, increased sterile inflammatory responses are evident in both. The onset of labour involves the release of the endogenous sterile inflammatory stimuli [e.g., the nuclear protein high-mobility group box 1 (HMGB1) (Menon, Behnia, et al., 2016), and cell-free fetal DNA (Phillippe, 2014)] by various gestational tissues. These stimuli activate Toll-like Receptor (TLR) pathways, which in turn trigger nuclear factor NF κ B to increase the transcription of numerous genes, particularly those encoding the inflammatory cytokines (Noguchi et al., 2010; Scharfe-Nugent et al., 2012), such as interleukin (IL)-1 β , IL-6, IL-8, IL-10, granulocyte macrophage colony-stimulating factor (GM-CSF), and tumour necrosis factor (TNF)- α (Neal et al., 2015; Sennstrom et al., 2000). A significant increase of IL-1 β , IL-6, and IL-8 was observed in the cervix and myometrium, interferon- γ (IFN- γ), IL-6 and IL-8 in the chorio-decidua, as well as IL-1 β and IL-8 in the amnion during labour at term (Osman et al., 2003; Veith & Rice, 1999). The elevation of pro-inflammatory cytokines in turn triggers the three stages of labour by stimulating the production of PGs and matrix metalloproteinases (MMPs). Cervical ripening involves the increase of MMP-1, MMP-3, MMP-8, MMP-9, cathepsin S, COX-2, and PG-E₂ (Challis et al., 2009; O'Brien, 1995). Membrane rupture involves the increase of MMP-9, collagenases, and PGs and the concomitant decrease of tissue inhibitor of metalloproteinase (TIMP)-2 (Challis et al., 2009; O'Brien, 1995). Myometrial contractility involves the increase of COX-2, PG-E₂, and oxytocin receptor that stimulate myocyte contraction (Challis et al., 2009; O'Brien, 1995).

In relation to ageing, accumulating evidence shows the chronic and progressive increase of pro-inflammatory factors, known as “inflamm-ageing” (Xia et al., 2016), is one of the main features of ageing. With regard to the hallmarks of ageing, “inflamm-ageing” belongs to the integrative hallmarks, particularly in the altered intercellular communication category. Furthermore, most of the parturition-associated pro-inflammatory mediators mentioned above are also members of the senescence associated secretory phenotype (SASP) (Coppe, Desprez, Krtolica, & Campisi, 2010; Freund, Orjalo, Desprez, & Campisi, 2010). SASP is a group of pro-inflammatory cytokines, chemokines, growth factors, and proteases commonly secreted by senescent cells. Senescent cells are characterised by the permanent growth arrest, morphological changes (e.g., the overall increase in cell size and distorted nuclei), the presence of senescence-associated heterochromatin foci (SAHF), resistance to apoptosis, as well as increased nuclear factor NFκB, which, as explained above, contribute to the parturition-related inflammation pathway (Campisi & d'Adda di Fagagna, 2007). Recent studies show that senescent cells accumulate in tissue with increasing age (Jeyapalan, Ferreira, Sedivy, & Herbig, 2007; Michaloglou et al., 2005). Senescence is also implicated as a primary cause of age-related diseases (Childs, Durik, Baker, & van Deursen, 2015) since experimental clearance of senescent cells in mouse models delays the appearance of ageing-related phenotypes and extends the health span of mice [as reviewed in (Naylor, Baker, & van Deursen, 2013)]. In association with “inflamm-ageing”, it is proposed that chronic inflammation during the ageing process is promoted through the secretion of SASP by the senescent cells (Freund et al., 2010; Olivieri, Prattichizzo, Grillari, & Balistreri, 2018). Based on this understanding of ageing

and parturition, it is likely that ageing of various gestational tissues contributes to increased production of pro-inflammatory factors with impending parturition, with cellular senescence as a possible underlying mechanism. Nevertheless, further investigation is still required on the type of aged cells or gestation tissues that contribute to the increase of the pro-inflammatory signalling during parturition. The threshold level of pro-inflammatory mediators required for normal parturition or their aberrant level associated with major pregnancy disorders may also be important.

Another common feature shared by ageing and parturition is the weakening of physical support. This occurs in parturition, during labour, and indicates programmed tissue disintegration by the resident cells to promote the delivery of the fetus. In many studies, most notably those of muscle tissues, the loss of tissue integrity/strength is strongly associated with stem cell exhaustion during the ageing process (Goldspink, 2007, 2012; Mendelsohn & Larrick, 2016). Therefore, it is likely that as pregnancy approaches term, stem cell exhaustion is naturally scheduled to reduce gestation tissue integrity and allow parturition. There are various possible causes of the gestation tissue-derived stem cell exhaustion. Recent evidence shows that inflammation, through the prolonged secretion of SASP, may impair normal stem cell function and behaviour, and thus hamper tissue repair and regeneration in aged organisms (Doles, Storer, Cozzuto, Roma, & Keyes, 2012; Kovtonyuk, Fritsch, Feng, Manz, & Takizawa, 2016; Lepperdinger, 2011). As parturition is strongly related to inflammatory pathways (Gomez-Lopez, StLouis, Lehr, Sanchez-Rodriguez, & Arenas-Hernandez, 2014), these findings provide indirect support to inflammation as the possible cause. Nonetheless, additional

studies are required to confirm whether the exposure period and level of pro-inflammatory mediators circulated during pregnancy affect stem cell function leading to labour.

In addition to inflammation, stem cell exhaustion can also result from DNA damage and/or increased exposure to reactive oxygen species (ROS) [i.e., by exposure to oxidative stress], as commonly occurs in aged organisms (Oh, Lee, & Wagers, 2014). Similarly, high exposure to oxidative stress is also observed at term pregnancy. The labouring gestational tissues are constantly exposed to high oxidative stress due to increased pro-inflammatory stimuli and prolonged exposure to circulating maternal factors. During delivery, various physiological changes in the uterine environment further elevate ROS production. These changes include alternating ischemia and reperfusion due to suppressed utero-placental blood flow, a rapid transition from hypoxic intra-uterine to oxygen-rich extra-uterine environment, and higher oxygen consumption resulting in increased mitochondrial respiration (Diaz-Castro et al., 2015; Poletini et al., 2015). Higher lipid peroxidation and protein oxidation as well as lower expression of antioxidant enzymes e.g., glutathione peroxidase (GSHPx) are also detected in labour-myometrial samples, indicating an increased susceptibility of the labouring myometrium to oxidative damage (Khan, Matharoo-Ball, & Shaw, 2010). With regard to stem cells, it is possible that gestation tissue-derived stem cells are subjected to the similar oxidative stressors approaching parturition. Nevertheless, additional studies, particularly on the type of stem cell function affected, are required for a clearer understanding on the role of stem cells in labour. In summary, the proposed association between ageing and parturition is shown in **Figure 4** below.

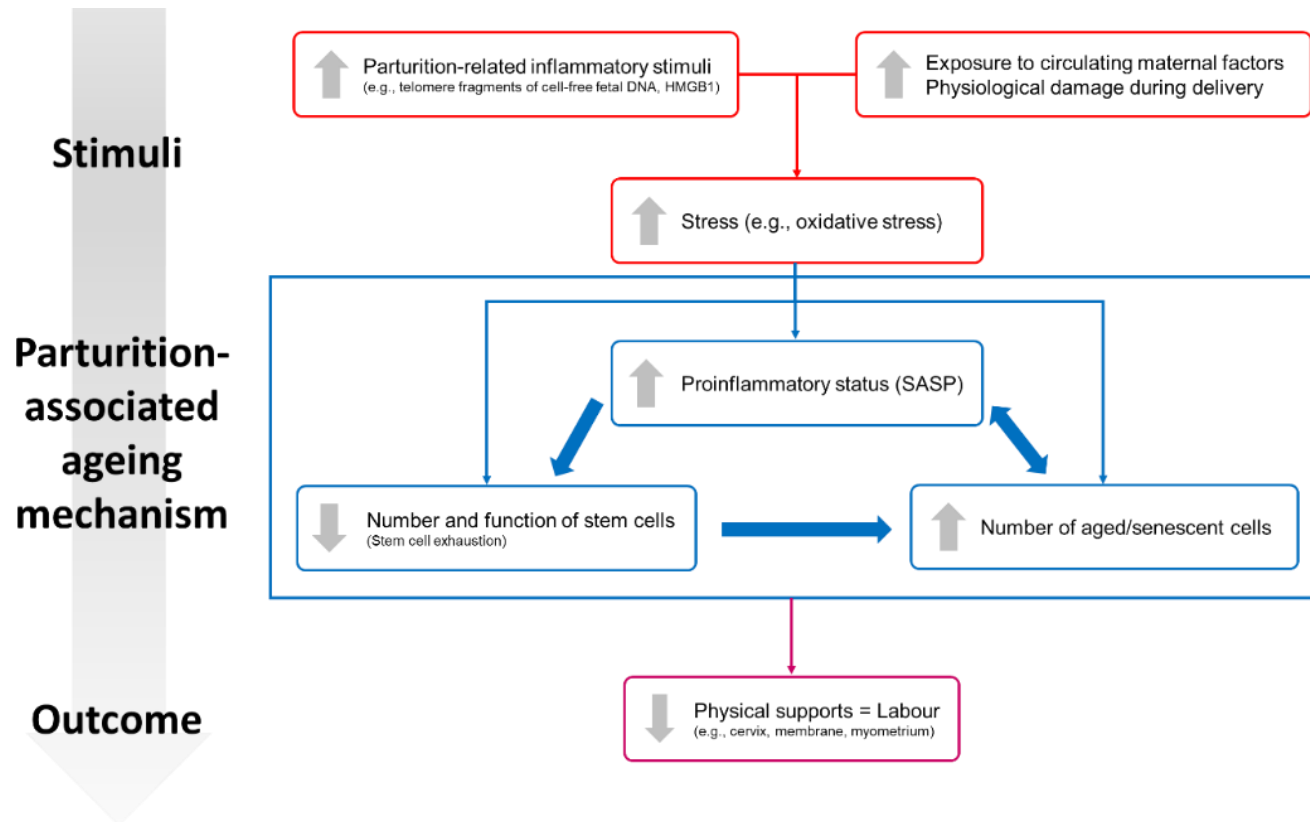


Figure 4. Proposed association between ageing and parturition

Approaching parturition, increased exposure to inflammatory stimuli, prolonged exposure to circulating maternal factors, and various physiological events during delivery induce oxidative stress within various gestation tissues. As a result, the production of pro-inflammatory factors, the number of senescent cells, and decreased number and function of stem cells within the tissue increase. These factors work in a cycle-like mechanism with a similar outcome, which is the weakening of physical support (clinically referred as labour).

1.5. Decidua, decidual ageing, and parturition

1.5.1. General features of human decidua

The human endometrium routinely undergoes cyclical changes. The cycle begins with the **proliferative phase** where there is a rapid proliferation of endometrial epithelium and stromal cells, resulting in a thickened endometrial lining. This is followed by the **secretory phase**, which is characterised by the differentiation of the glandular epithelium into its highly secretory state, the differentiation of the stromal cells from elongated, fibroblast-like mesenchymal cells into rounded, epithelioid-like cells, and vascular remodelling. The cycle ends with **menstruation**, which features the shedding of the thickened and differentiated endometrial lining, and **menstrual repair** where endometrial tissue and blood vessels are restored (Okada, Tsuzuki, & Murata, 2018; Salamonsen, Nie, Hannan, & Dimitriadis, 2009).

The decidua is a specialised tissue lining resulting from the changes occurring during the secretory phase of the human endometrium. In pregnancy, the decidua is characterised by three regions: *decidua capsularis*, *decidua parietalis*, and *decidua basalis* (Pansky, 1982). The decidual region that surrounds the conceptus is the *decidua capsularis*. By week 22, *decidua capsularis* will fuse with *decidua parietalis*, which was the remaining decidual lining of the uterus, and attach to the fetal membranes, thus eliminating the uterine cavity (Pansky, 1982). *Decidua basalis* is the portion of the decidua that remains attached to the maternal side of the placenta during placental detachment (Gellersen & Brosens, 2014). The boundary area where the decidua is separated from the uterine wall at parturition is known as the **zone of separation** (Samuel, Jack, & Nathanielsz, 1975).

The formation of the decidua, called decidualisation, from the human endometrium is one of the essential events for a successful pregnancy. The process begins in response to elevated levels of estradiol (bioactive estrogen) and progesterone during the mid-secretory phase of the endometrial cycle, approximately 6 days after ovulation (Gellersen & Brosens, 2014; Vinketova et al., 2016). Since the final structure of the decidua is highly receptive to the allogeneic embryo, decidualisation is essential for implantation and placenta formation. Impaired cyclic decidualisation, as shown with decreased expression of the decidual marker – prolactin – results in recurrent pregnancy loss (Salker et al., 2010). Impaired decidualisation may encourage miscarriage in subsequent pregnancies by disrupting the natural process of embryo recognition and selection as well as the maternal responses to embryonic signals during implantation (Salker et al., 2010). This evidence suggests that the decidua is essential as the physical scaffold during pregnancy and the loss of decidua or its physical support properties may result in the termination of pregnancy.

The properties of the decidua may be attributed to its major resident cells. One major resident cell type in the decidua is the decidualised stromal cells (DSCs) (García-Pacheco, Oliver, Kimatrai, Blanco, & Olivares, 2001). DSCs have large round nuclei, high numbers of nucleoli, accumulated glycogen and lipid droplets in the cytoplasm, dense membrane-bound secretory granules, and expanded rough endoplasmic reticulum and Golgi complex (Okada et al., 2018). These cells are characterised by their expression of progesterone receptors (PR) and specific surface markers (e.g., Zn-dependent metallo-proteinases such as CD10, CD13, and CD21). DSCs also secrete a characteristic set of markers [e.g., prolactin,

plasminogen activator inhibitor (PAI-1), insulin-like growth factor binding protein 1 (IGFBP-1), and vascular endothelial growth factor (VEGF)] as well as various cytokines (e.g., IL-1, IL-11, and IL-15) (Dunn, Kelly, & Critchley, 2003). Despite their well-defined characteristics, an understanding of DSC functions, particularly during parturition, is still limited.

1.5.2. Current understanding of the roles of decidual ageing in parturition

Current studies provide evidence that decidua undergoes functional and molecular changes similar to those seen in the ageing process as term parturition approaches. This suggests a potential role for decidual ageing in the initiation of labour at term (Cha & Aronoff, 2017; Deng et al., 2016). These functional and molecular changes are likely to originate at the cellular level in DSCs or decidual mesenchymal stem/stromal cells (DMSCs), which make up the majority of resident cells in the decidua. However, insight into a potential role for decidual ageing in the initiation of labour is currently based primarily on studies of DSCs.

At parturition, degenerated DSCs and their fibrous debris accumulate at the "zone of parturition", which in turn, lessens the contact between endometrium and placenta and promotes placental detachment (Larsen, 1963). As parturition approaches, molecular changes accumulate in DSCs, including DNA damage-induced apoptosis (Gu et al., 1994) and telomere attrition (Bonney et al., 2016). Epigenetic alterations associated with various molecular pathways of parturition were observed with advancing gestation as well. These include reduced DNA methylation associated with increased expression pro-inflammatory receptors e.g., TLR-2 and TLR-9 (Walsh et al., 2017), reduced histone acetylation associated with decreased expression of PR and progesterone withdrawal (Condon, Jeyasuria,

Faust, Wilson, & Mendelson, 2003), and increased nuclear localisation of p65, one of the components of transcription factor NFκB (Yan, Sun, & Gibb, 2002). These molecular changes of DSCs at parturition are regarded as hallmarks of ageing and classified under the primary hallmark category (Lopez-Otin et al., 2013).

Changes in protein homeostasis of DSCs, particularly at the expression level of heat shock protein (Hsp), promote labour at term. Increased levels of Hsp-70 and Hsp-90 are proposed to inhibit uterine PR leading to progesterone withdrawal and stimulation of estrogen receptor, which consequently initiate labour (Wu, Derks, Zhang, & Nathanielsz, 1996). Increased levels of Hsp70 are also associated with a lack of autophagy (Sisti, Kanninen, Ramer, & Witkin, 2015), which was reported to be essential to promote the onset of labour (Gawriluk, Ko, Hong, Christenson, & Rucker, 2014; Signorelli et al., 2011). The upregulation of mammalian target of rapamycin complex 1 (mTORC1) signalling pathways was thought to mediate this inverse relationship of Hsp70 and autophagy (Sisti et al., 2015). With regard to ageing, elevated Hsp70 levels are associated with increased oxidative stress, which was often observed in tissues affected by ageing or ageing-related diseases (Ikwegbue, Masamba, Oyinloye, & Kappo, 2017; Rodgers, Ford, & Brunk, 2009). At parturition, the release of cell-free telomere fragments in the fetal membranes exacerbates oxidative stress in the tissue environment (Poletini et al., 2015). Accumulation of defective organelles, particularly mitochondria, due to decreased autophagy may also gradually increase the level of oxidative stress in the system, which in turn increases the level of Hsp70. This serves as a positive loop for the signal to propagate labour at term.

The onset of labour is also associated with the senescence of DSCs (Cha & Aronoff, 2017). Premature senescence of DSCs promoted preterm birth in murine models (Deng et al., 2016; Hirota et al., 2010). Inactivation of Trp53, the gene that encodes p53, in murine models resulted in the upregulation of mammalian target of rapamycin complex 1 (mTORC1), cyclin-dependent kinase inhibitor (p21), and protein kinase B (Akt) signalling pathways leading to DSC senescence (Hirota et al., 2011; Hirota et al., 2010). The upregulation of the mTORC1 signalling pathway in these studies is consistent with parturition-related pathways of Hsp-70 and mTORC1 described above. Moreover, reduced expression of p53 was reported to downregulate sestrin 2 (SESN2) and 5' AMP-activated protein kinase (AMPK) signalling pathways (Cha et al., 2013; Deng et al., 2016), which subsequently also upregulate the mTORC1 signalling pathway and promote the onset of labour. Senescent DSCs accumulate in the labouring decidua and secrete numerous SASPs, which mostly comprise inflammatory cytokines required for the initiation of labour e.g., IL-6, IL-8, and various MMPs (Cha & Aronoff, 2017). With regard to ageing, cellular senescence, the upregulation of mTORC1 and the downregulation of AMPK-SESN2 signalling pathways are frequently observed in aged organisms and reversing or blocking these changes was reported to delay or revert the ageing process (Giannakou et al., 2004; Hay, 2011; Lee, Budanov, & Karin, 2013; van Deursen, 2014).

Nevertheless, although stem cell exhaustion is also a hallmark of ageing and despite some evidence of their possible involvement, no studies have investigated the potential role of stem cells, particularly DMSCs, in parturition. Therefore, this MPhil study aims to provide initial evidence to answer this research gap. The

proposed mechanisms in which the ageing of stem cells, particularly of DMSCs, may affect labour at term are described in details in the subsequent sections.

To conclude, a summary of the features of decidual ageing and its role in parturition as described above is depicted in **Figure 5**. More details on this topic are also available in the appended review article (**Appendix VI**).

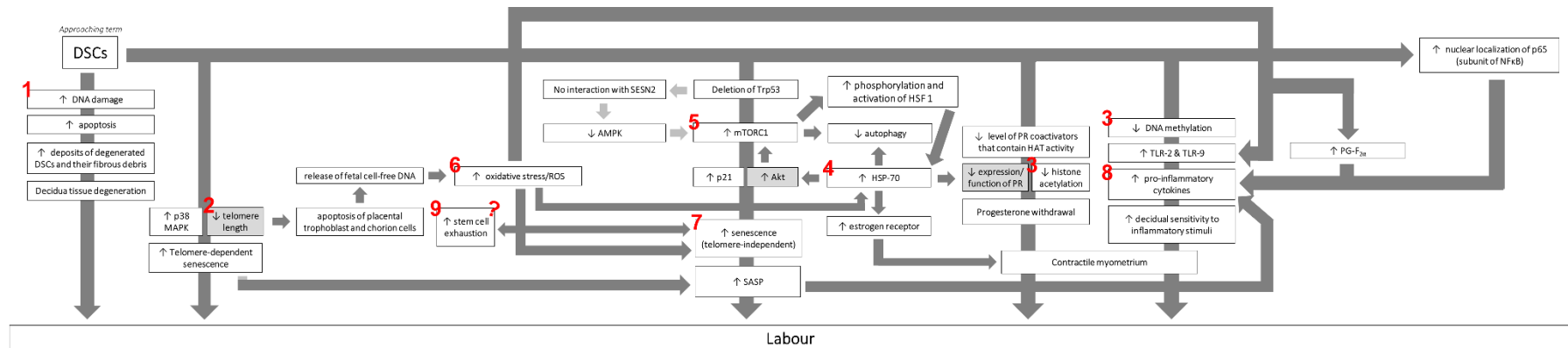


Figure 5. Labour-associated ageing pathway in decidua

Red-coloured numbers mark the hallmarks of ageing: 1: DNA damage; 2: telomere attrition; 3: epigenetic alterations; 4: loss of proteostasis; 5: deregulated nutrient sensing; 6: mitochondrial dysfunction; 7: cellular senescence; 8: altered intercellular communication; 9: stem cell exhaustion. Arrows indicate the sequence of the phenomena. Abbr. DSCs: decidual stromal cells, MAPK: mitogen-activated protein kinase, ROS: reactive oxygen species, SESN2: sestrin 2, AMPK: 5' AMP-activated protein kinase, mTORC1: mammalian target of rapamycin complex 1; p21: cyclin-dependent kinase inhibitor; Akt: protein kinase B, SASP: senescence-associated secretory phenotype, HSF: heat shock factor, HSP: heat shock protein, PR: progesterone receptor. HAT: histone acetylase, TLR: toll-like receptors, PG-F_{2α}: prostaglandin 2α, NFκB: nuclear factor κ-light-chain-enhancer of activated B cells.

1.6. Stem cells, ageing, and parturition: their possible association in the decidual gestational clock

1.6.1. Stem cells: their definition and classification

Stem cells are generally defined as populations of unspecialised cells that are capable of self-renewal through symmetric and/or asymmetric division and of differentiation into various types of specialised cells in response to intrinsic and extrinsic signals from their microenvironment (Bongso & Lee, 2005; Cai, Weiss, & Rao, 2004; Reya, Morrison, Clarke, & Weissman, 2001). In addition to these minimum criteria, stem cells are distinguishable from other dividing cells by their high expression of telomerase, DNA repair enzymes, aldehyde dehydrogenase (ALDH), and chemokine receptor type 4 (CXCR4), a lack of lineage markers, and low uptake of the nuclear dye Hoechst 33342 (Cai et al., 2004). These properties are exhibited by stem cells regardless of their origin.

Stem cells are classified based on either their differentiation potential (i.e., totipotent, pluripotent, and multipotent) or their tissue of origin (i.e., embryonic, fetal, and adult). Zygotes up to the 8-cell stage of morula are totipotent as they are able to form any type of cell of an organism (Fortier, 2005). Cells of the inner cell mass (ICM) of blastocyst (5-7 days) are pluripotent as these cells are able to develop into any type of embryonic cell, but not extraembryonic cells. Stem cells derived from ICM are called embryonic stem cells (ESCs) (Bongso & Lee, 2005; Fortier, 2005). Fetal and adult stem cells are mostly multipotent as their differentiation potential is restricted to several specific cell lineages depending on their local microenvironment (Bongso & Lee, 2005; Fortier, 2005). Fetal stem cells are isolated from various tissues of aborted fetus. Adult stem cells are found in virtually

all tissues and organs e.g., hematopoietic (bone marrow, peripheral blood), mesenchymal (bone marrow stroma, decidua, placenta), liver, neuronal, epidermal, eye, and gut stem cells (Bongso & Lee, 2005).

Current trends in stem cell research show a progressive increase in studies using adult stem cells rather than ESCs (National Research Council (US) and Institute of Medicine (US) Committee on the Biological and Biomedical Applications of Stem Cell Research, 2002; Prentice, 2019). Moral and ethical concerns regarding the isolation of ESCs primarily contributes to the hindrance of studies using ESCs in numerous countries (Wert & Mummery, 2003). Mesenchymal stem cells (MSCs) are a group of adult stem cells that receive considerable research interest due to their superior properties for therapeutic application. MSCs are easy to isolate and culture, multipotent, immunosuppressive, allow both autograft and allograft, are unlikely to form malignancy due to their limited replicative lifespan, and have less ethical concerns compared with ESCs (Kim & Park, 2017).

1.6.2. MSCs: their characteristics, sources, and evidence of ageing-related functional changes in gestational tissues

MSCs are multipotent stem cells that are able to differentiate into various mesenchymal cell lineages, such as adipocytes, osteoblasts, and chondrocytes. In addition to their differentiation capability, MSCs are characterised based on their expression of MSC-specific surface protein markers and adherence to plastic as described by the International Society for Cell & Gene Therapy (ISCT) (Dominici et al., 2006). In gestational tissues, MSCs are found in tissues of fetal-origin including cord blood, umbilical cord, amniotic fluid, and the fetal-side of the placenta (i.e., amnion, chorion, and chorionic villi) and in tissues of maternal-origin

including *decidua parietalis* and *decidua basalis* (Kusuma, Murthi, & Kalionis, 2016). Based on current understanding of the origin of the placenta, MSCs are proposed to act as precursors of all non-trophoblast cells of the villous core (James, Srinivasan, Alexander, & Chamley, 2014). In the decidua, MSCs may also be responsible for giving rise to DSCs since the decidua is mostly composed of stromal cells, and DSCs also express mesenchymal cell characteristics (Oliver, Montes, Galindo, Ruiz, & Olivares, 1999).

The abundant numbers of MSCs in various gestational tissues and their role in the formation and function of the placenta indicate the importance of MSCs during pregnancy. However, the role of MSCs in parturition remains poorly understood. Currently, studies of the association between MSCs and pregnancy mostly focus on the decline of MSC functions, properties, and differentiation capability in pregnancy-related disorders that are associated with placental ageing. For example, Rolfo et al. (2013) reported that chorionic villous-derived MSCs (CMSCs) from patients with preeclampsia have reduced proliferation, increased senescence, and increased levels of various pro-inflammatory cytokines (e.g., TNF- α , IL-6, IL-8) and chemotactic factors (e.g., migration inhibitory factors/MIF, and soluble FMS-like tyrosine kinase-1/sFlt-1) compared with CMSCs from normotensive patients. On the maternal side, Mayne et al. (2017) reported that preeclampsia-affected placentae show signs of premature senescence and ageing, including increased SASPs, increased apoptosis and increased expression of ageing-related biomarkers. Furthermore, Kusuma, Abumaree, Perkins, Brennecke, and Kalionis (2017) showed that preeclampsia-DMSCs have decreased resistance to oxidative stress. Preeclampsia-DMSCs were also reported to have different microRNAs (miRNAs)

expression (e.g., miR-16, miR-181a, miR-494), particularly those that regulate the immunosuppressive, proliferation, and angiogenesis-regulating potential of DMSCs, when compared with normotensive control (Kusuma et al., 2016). miRNAs are short non-coding regulatory RNAs consisting of about 22 nucleotides that function in mRNA silencing (by cleaving the target mRNAs) and post-transcriptional regulation of gene expression (by suppressing gene translation) (Bartel, 2004). In MSCs, miRNAs are located within the cells or in the secretory, membrane-derived vesicles known as extracellular vesicles (EVs), which are known to facilitate the regenerative properties of MSCs via paracrine function (EL Andaloussi, Mager, Breakefield, & Wood, 2013; Ferguson et al., 2018). In pathological situations e.g., preeclampsia, the secretion of EVs containing aberrant miRNA expression by MSCs may contribute to the pathogenesis of preeclampsia (Kusuma et al., 2016). In relation to term parturition, these findings may serve as indirect evidence that MSC functions may change at parturition, specifically in the decidua, since the decidua demonstrates strong evidence of ageing and senescence at parturition.

1.6.3. DMSCs: their characteristics and possible roles in parturition

DMSCs are a major stem cell type in the decidua. Like other type of stem cells, DMSCs have the ability to regenerate senescent cells, particularly senescent DSCs. During pregnancy, the removal of senescent DSCs is essential to sustain the receptive properties of the decidua towards the growing fetus. This hypothesis is supported by a recent study showing senescent DSCs undergo immunological clearance mediated by uNK to maintain decidual homeostasis in the cycling endometrium since endometrial MSCs cannot differentiate into new DSCs in the

presence of senescent DSCs (Brighton et al., 2017). In contrast, the inability of MSCs to differentiate in the presence of senescent DSCs is important for parturition. It is likely that senescent DSCs are not rejuvenated to weaken the physical support of the decidua for the fetus and the placenta during labour. As described in **Section 1.4**, the deterioration of tissue physical support due to stem cell exhaustion has been reported in studies using muscle stem cells where their exhaustion was associated with the loss of tissue integrity and strength (Goldspink, 2007, 2012). Since DMSCs in this MPhil study were isolated from the *decidua basalis* that provided physical support to the placenta during pregnancy, the evidence of DMSC ageing-related functional loss associated with SOL may indicate a potential role in facilitating placental detachment during labour.

DMSCs are also known for their high resistance to oxidative stress and inflammatory stimuli because of their proximal location with the maternal blood circulation (i.e., located close to the endothelial cells that line the maternal blood vessels). As discussed earlier, high oxidative stress and pro-inflammatory stimuli are well-established components of parturition. Moreover, DMSCs are removed from the lining of the blood vessels by extravillous trophoblast (EVT) during blood vessel remodeling as pregnancy progresses (Kusuma et al., 2015). The removal of DMSCs from the blood vessel lining as parturition approaches indicates that DMSCs need to stop functioning and/or be removed from the zone of separation to allow progression of the pregnancy to parturition.

In addition, ageing of DMSCs may also stimulate myometrial contraction at term by increasing the secretion of labour-associated SASPs. This increase in SASPs may result from higher numbers of senescent DSCs or originate from the senescent

DMSCs themselves. Nevertheless, all the above DMSC-related labour mechanisms are poorly understood and require further investigation.

1.7. Human placenta and decidua as a study model of ageing-associated parturition features: their advantages and disadvantages

Most studies of decidual ageing in parturition were performed in animal models, e.g., rabbit and rodents (Hirota et al., 2010; Samuel et al., 1975; Sladek & Roberts, 1996). However, human parturition is a complex and unique process and thus, most animals are the inadequate models of human parturition. The findings in animals cannot be readily extrapolated to humans because of the many environmental and functional differences between humans and animal models, in terms of placentation, parturition and ageing (Mitchell & Taggart, 2009; Mitchell, Scheibye-Knudsen, Longo, & de Cabo, 2015).

The human placenta is ideally suited to study ageing-associated parturition features as it contains both of the maternal (*decidua basalis*) and fetal (*chorionic villi*) components of the gestational tissues upon parturition (Huppertz, 2008) (**Figure 6**). In general, the placenta is a transient feto-maternal organ that develops and is maintained only during pregnancy to sustain fetal growth (Caruso, Evangelista, & Parolini, 2012). During pregnancy, it serves as a physical barrier that protects the developing fetus from toxic xenobiotics, infection and maternal illnesses (Gude, Roberts, Kalionis, & King, 2004). It facilitates transportation and metabolism of nutrients and waste between the mother and the fetus (Zakowski & Herman, 2004), and enables the evasion of maternal immune rejection against the fetus, which is genetically different to the mother (Lewis, Coulam, & Moore, 1986). The placenta

also secretes various hormones crucial for pregnancy and labour e.g. progesterone, human chorionic gonadotropin (hCG), estrogen, and lactogen (Costa, 2016; Zakowski & Herman, 2004).

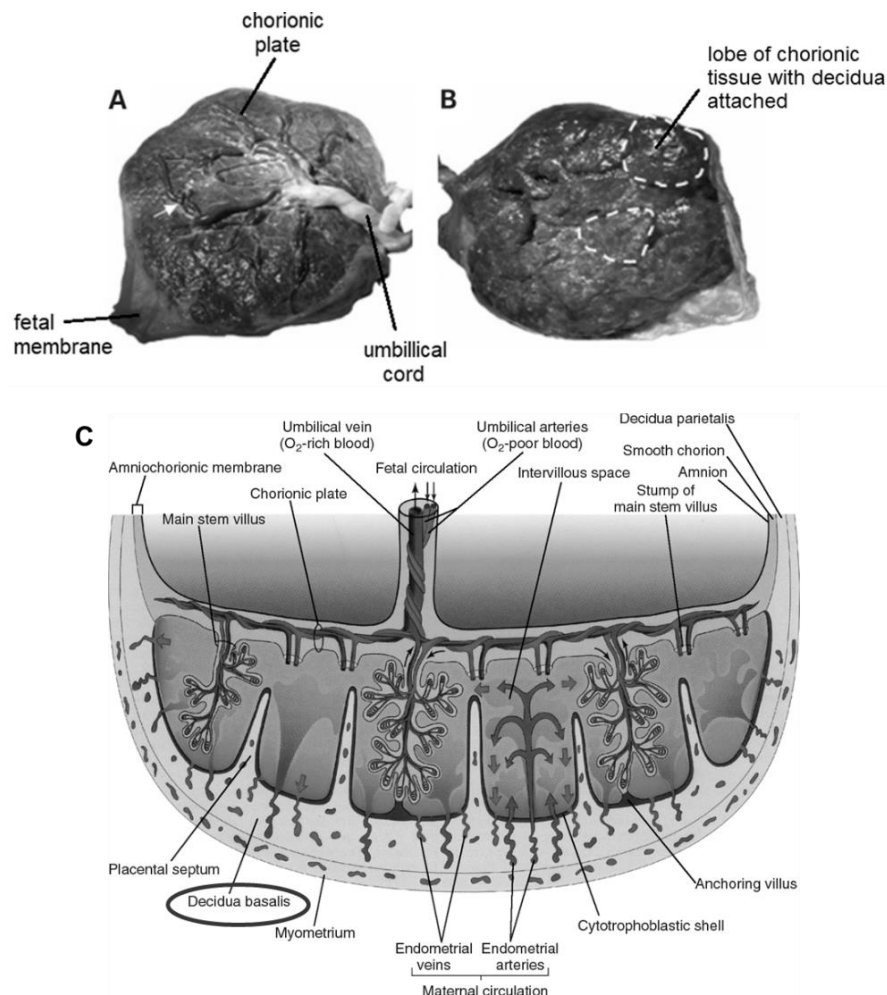


Figure 6. The structure of human placenta

The macroscopic morphology of (A) the fetal component of placenta and (B) the maternal component of placenta. Images were adapted from Huppertz (2008). (C) The schematic of a cross section of a term human placenta. Image was adapted from Saunders (2009).

There are many advantages for using human placenta to study ageing-associated parturition features in the decidua. Firstly, it allows studies directly on human decidual tissue that remains attached to the placenta. Therefore, the molecular and functional changes of the decidua can be directly measured in the tissue, making it

more reflective to the *in vivo* condition. Human placentae are abundantly available and their collection does not require invasive procedures or raise any significant ethical constraints (compared with tissue biopsy of human decidua) as it is naturally expelled at the end of pregnancy (Zhu et al., 2014). Moreover, human placentae also serve as an abundant source of DMSCs (Abomaray et al., 2016; Kusuma et al., 2015), which as previously explained, may play significant roles in initiating parturition.

However, the use of human placentae in ageing-parturition studies has some limitations. The use of human placenta only allows for a narrow range of time-point observations as it is impractical and unethical to obtain the samples from non-pathological pregnancies before the delivery of the neonate. Placentae from spontaneous preterm delivery can be used for this purpose, but their potential pathological properties should be taken into consideration as possible confounding factors in assessing spontaneous labour mechanisms. Moreover, patient-to-patient variations as well as high numbers of medical interventions performed to ensure the safety of the mother and neonate during parturition are inevitable and difficult to control. Nonetheless, ageing-parturition studies on human-derived decidua should be encouraged to validate the findings from animal-based studies.

2. HYPOTHESES AND AIMS

2.1. Hypotheses

1. That DMSCs isolated from placentae of patients undergoing SOL (SOL-DMSCs) show evidence of ageing-related functional changes compared with DMSCs isolated from placentae of patients, not in labour (NIL), undergoing Caesarean section (C-section) delivery (NIL-DMSCs).
2. That SOL-DMSCs show evidence of differences in lipid contents and quantities compared with NIL-DMSCs.

2.2. Aims

1. To compare SOL-DMSCs with NIL-DMSCs using various functional assays, including cell proliferation, migration, and death, oxidative stress (lipid peroxidation and ALDH), and pro-inflammatory cytokine production (IL-6 and IL-8),
2. To compare the lipid species and quantities of SOL-DMSCs with those of NIL-DMSCs by mass spectrometry-based lipid analyses.

3. METHODS

3.1. Tissue collection

Full-term placentae (39 - 40 weeks gestation) from patients undergoing SOL + vaginal delivery (n = 8) and NIL + C-section delivery (n = 7) were collected by research midwives at the Royal Women's Hospital, Melbourne, VIC, Australia and processed immediately, or stored at 4°C before processing. Labour was defined as spontaneous if the contractions naturally occur without induction and complete without pharmacological augmentation. NIL samples should show no sign of spontaneous rupture of membrane. Further exclusion criteria related to factors that affect the function of the placenta and stem cells. Placental samples were not collected from donors ≥ 35 years of age and with a body mass index (BMI) ≥ 30 kg/m². Patients with multiple gestations, miscarriage of the current pregnancy, polycystic ovary syndrome (PCOS), hyperthyroidism, gastric medicines and aspirin consumption, major fetal abnormalities, placental disorders, meconium-stained placentae, or severely calcified placentae were omitted from this study. Samples from patients with breech birth and alcohol consumption were excluded from the lipid peroxidation assay. Tissue collection was approved by Royal Women's Hospital Research and Ethics Committee, VIC, Australia (Projects 10/49 and 12/42). All patients provided written consent prior to donating their samples.

3.2. Isolation, culture maintenance, and characterisation of primary cells

3.2.1. Isolation of primary cells

Primary cells were isolated from *decidua basalis* that remained attached to the maternal side of the placenta according to the published protocols (Kusuma,

Abumaree, Pertile, & Kalionis, 2018). Briefly, two eight-gram tissue sections were dissected from the maternal side of placenta, finely minced, and washed four times with phosphate buffer saline (PBS, Gibco™). The tissue was digested overnight with 2.5% trypsin (Gibco™) and 0.5% DNase I (Worthington) in HBSS [-] (Gibco™) at 4°C. On the next day, the supernatant was removed by centrifugation and the tissue was digested with 3 mg/mL collagenase type 1 and 0.05 mg/mL DNase I (both Worthington) in HBSS [+] (Gibco™) at 37°C for 10 min. The digest was sifted through a 100-μM sieve and the resulting filtrate was fractionated by gradient centrifugation using Histopaque®. The top fraction containing plasma and cells was centrifuged and the resulting cell pellet was washed with red blood cell lysis buffer (Merck) and HBSS [-]. Cells were grown in α-MEM complete medium (Sigma-Aldrich) on plastic cultureware. The summary of the isolation workflow of primary cells from *decidua basalis* is shown in **Figure 7** below.

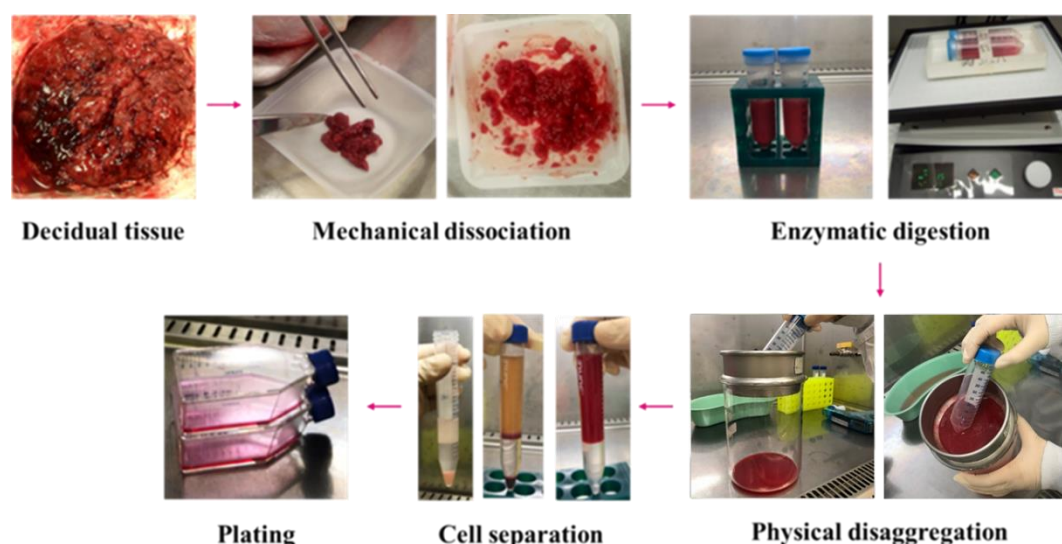


Figure 7. Isolation workflow of primary cells from *decidua basalis*

More details on the components of the reagents used in the method sections are available in **Appendix I**.

3.2.2. *Culture maintenance of the isolated primary cells*

3.2.2.1. *Tissue culture*

All tissue culture activities were conducted aseptically in a Biological Safety Cabinet (BSC) class II located in cell culture rooms. Before and after use, the working area and equipment used in BSC were sterilised using 80% ethanol and/or autoclaved (if appropriate). After use, the BSC working area was radiated with UV light for 20 minutes for further sterilisation. Incubator and water bath were routinely cleaned to prevent contamination. Cell culture medium was prepared under sterile condition, changed every 3-4 days, and stored at 4°C when necessary. All reagents were pre-warmed before use. Cell cultures were maintained at 37°C in a 5% CO₂ incubator.

3.2.2.2. *Passaging isolated primary cells*

The primary cells were passaged as described in published protocols (Kusuma et al., 2018). Briefly, cells were passaged at 70-80% confluency. The first passage typically took 1-3 weeks after initial plating (P0). The subsequent passages for cell propagation typically occurred every 1-2 weeks. Cells were washed twice with HBSS [-] and dissociated by incubation in TrypLE at 37°C for 4 min. Microscopy was used to verify that cells had detached upon TrypLE treatment and gentle tapping. Trypsin digestion was stopped by adding FCS (1:10, v/v) to TrypLE. Cells were pelleted at 300 g for 5 min. The resulting pellet was resuspended in α -MEM complete medium and plated on plastic cultureware. All experiments in this study were performed at passage 3-5.

3.2.2.3. Cell counting

Cell viability was measured using Trypan Blue exclusion assay as described in (Strober, 1997) with modifications. Briefly, cells were mixed with Trypan Blue (Life Technologies) at 1:1 (v/v), and then pipetted into the counting chambers. The numbers of viable, non-viable, and total cells were counted using a Countess Automated Cell Counter (Life Technologies), which then calculated the percentage of viable cells in the suspension.

3.2.2.4. Cell storage: freezing the primary cells

P2 primary cells at 70-80% confluency were stored in liquid nitrogen as long-term stocks. After dissociation using TrypLE, the cell pellet was resuspended in the freezing medium (see **Appendix I**). The cell suspension was then aliquoted into cryovials (Corning). Cryovials were stored in a Mr Frosty (Life Technologies) or in a Cool Cell (Merck) freezing container at - 80°C. After 24 hours, the cryovials were transferred to the liquid nitrogen.

3.2.2.5. Thawing the primary cells from liquid nitrogen stock

Cells retrieved from liquid nitrogen were immediately thawed in a 37°C water bath. Cells were gently mixed with 3 mL α -MEM complete medium and pelleted by centrifugation at 200 g for 5 min. The cell pellet was resuspended in α -MEM complete medium and then, plated. Cell viability was determined as described in **Section 3.2.2.3** if necessary. Cells were cultured for an additional passage before being used in experiments.

3.2.3. *Characterisation of the isolated primary cells*

Primary cells that were consistent with the ISCT minimum criteria for defining MSCs (Dominici et al., 2006), as well as the additional criteria for placental MSCs suggested by Parolini et al. (2008) were then referred to as decidual MSCs (DMSCs). These criteria were assessed using the following assays.

3.2.3.1. *Flow cytometry analyses*

Primary cells were assessed for their positive expression of MSC markers and negative expression of hematopoietic markers specified by ISCT (Dominici et al., 2006) with variations as listed in **Appendix II**. Flow cytometry analyses were conducted as described by Kusuma et al. (2018) at the Flow Cytometry and Imaging Facility, Murdoch Children's Research Institute (MCRI), Australia under the supervision of Dr. Matthew Burton and Dr. Eleanor Jones. Briefly, P3 cells were rinsed with 3 mL HBSS [-] + 2% FCS (Gibco™) and resuspended in 4 mL of the same buffer. Cells were counted as described in **Section 3.2.2.3**. Twelve aliquots, each contained 1×10^5 cells/100 μ L, were prepared in microtubes. Non-specific binding was blocked by adding 5 μ L of human serum blocking buffer per aliquot. Cells were stained with antibodies specific to markers listed in **Appendix II** (except DAPI) and incubated at 4°C for 30 min in the dark. The stained cells were washed with HBSS [-] + 2% FCS (Gibco™) and resuspended in 500 μ L of the same buffer containing 1 μ g/mL DAPI (Merck). Cells were filtered to a 5 mL tube fitted with a cell strainer cap to achieve a single cell suspension. Flow cytometry analyses were performed using LSR Fortessa X-20 with BD FACS Diva software. Representative gating adjustment is shown in **Appendix III – Supplementary figure 1**.

Histograms illustrating the expression level of each markers were created using Weasel software (WEHI Cytometry Laboratory).

3.2.3.2. Differentiation assay

Primary cells were assessed for adipogenesis and osteogenesis potential as described by Kusuma et al. (2018) with minor modifications. Briefly, P3 cells were seeded into the eight-well Nunc Lab-Tek II glass chamber slides (Life Technologies) at 1×10^4 cells/well and 3×10^4 cells/well for osteogenesis and adipogenesis, respectively. Cells were cultured in 500 μ L α -MEM complete medium per well. The induction medium (StemXVivo) were then added to the cells at 50 - 70% confluency for 21 days (osteogenesis) or at 100% confluency for 14 days (adipogenesis). Cells were fixed with 300 μ L/well formaldehyde (Merck) prior to staining. Additional treatment with 300 μ L/well of 60% isopropanol (Merck) for 5 min at RT was performed for adipogenesis. Adipogenesis was confirmed by the formation of lipid droplet stained with Oil Red O (Merck), while osteogenesis was confirmed by the production of calcium deposits stained with Alizarin Red (Merck). Experiments were conducted in duplicate with negative controls. Cells were mounted in 80% glycerol (Chem-Supply) prior to visualization under an Axioskop 2 microscope equipped with a Nikon DXM 1200C camera.

3.2.3.3. Colony forming unit-fibroblast (CFU-F) assay

The CFU-F assay was performed according to a published protocol (Kusuma et al., 2018). In general, this assay assessed the primary cell's ability to form colonies when seeded at low density, referred as self-renewal capacity or clonogenicity, which is an important MSC characteristic (Castro-Malaspina et al., 1980). Briefly,

P3 cells were grown in 6-well plates in triplicate at 400 cells per well at 37°C, 5% CO₂ for 14 days without a medium change. Cells were washed with PBS (Gibco™) and fixed with 10% formalin for 10 min at RT. Cells were again washed, then stained with Crystal Violet (LabChem™) for 10 min at RT. After removal of excess dye, cells were air-dried. Cell clusters (colonies consisting of less than 50 cells) and CFU-F (colonies consisting of 50 cells or more) were counted and their percentages were calculated using the formula below.

$$\text{Clusters or CFU-F (\%)} = \frac{\text{The number of clusters or CFU-F}}{400 \text{ cells}} \times 100 \%$$

3.2.3.4. *Fluorescence in situ Hybridization (FISH)*

Primary cells were assessed for their maternal origin and possible contamination with male fetal cells using FISH analyses as recommended by Parolini et al. (2008). FISH analysis was performed on a minimum of 200 cell nuclei per sample of primary cells from placentae of male babies by Dr. Mark Pertile and Dr. Melissa Glass at Victorian Clinical Genetics Service (VCGS), MCRI, Australia. The slides were prepared according to the published protocols (Kusuma et al., 2018). Briefly, 20 µL cell suspension containing ± 200 cells (P3) was pipetted on a polylysine slide (Life Technologies) in duplicate. Cells were fixed with methanol: acetic acid (3:1, v/v). When necessary, the slides were stored at 4°C prior to the analysis. Cells were hybridised using the fluorescent 18/X/Y chromosome probe set: spectrum Green for X chromosome and Orange for Y chromosome (Abbott Laboratories). The nuclei were counterstained with DAPI (Blue), then observed with Olympus BX51 fluorescence microscope equipped with JAI M300 CCD camera at 600X

magnification. Interphase FISH images were processed using Metasystems ISIS image analysis software v5.3.16 (MetaSystems).

3.3. Functional assays

The function of SOL-DMSCs (n = 8) and NIL-DMSCs (n = 7), unless stated otherwise, were compared using the following assays.

3.3.1. Proliferation assays

3.3.1.1. xCELLigence real-time proliferation assay

xCELLigence system (ACEA Biosciences, Inc.) constantly monitored and recorded real-time changes in electrical impedance resulting from various cellular events detected on the microelectrode of the xCELLigence E-plate. These changes were reported as arbitrary cell indices. In this study, the growth profile of DMSCs was evaluated using the xCELLigence system as previously described (Abumaree et al., 2017; Alshabibi et al., 2017; Alshabibi et al., 2018) with following modifications. Briefly, 100 μ L/well α -MEM complete medium was added to a 96-well E-plate and equilibrated at RT for 30 min. The background was measured. DMSCs at P4 were collected and counted after dissociation with TrypLE (Life Technologies). Cells were then plated into a 96-well E-plate in triplicate (5×10^3 cells/100 μ L α -MEM complete medium/well) and incubated at 37°C without a medium change. Cell index was measured every 5 min for the first 24 hours, and subsequently, every 1 hour by RTCA Software 1.2.1. Cell indices of SOL- and NIL-DMSCs were compared at various time points within attachment (0-5 hour), proliferation (5-80 hour), and survival phase (> 80 hours). For proliferation and survival data analyses,

cell indices were firstly normalised to the indices at 5 and 80 hours, respectively. Prior to the assay, the seeding density was optimised by comparing the growth profile of DMSCs plated at different seeding density.

3.3.1.2. CCK-8 end-point proliferation assay

This assay was performed as per the manufacturer's instructions (Sigma-Aldrich). Briefly, 100 μ L/well α -MEM complete medium was added into the 96-well plates. The plates were equilibrated at RT for 30 min. DMSCs at P4 were collected and counted after dissociation with TrypLE (Life Technologies), and then plated into a 96-well E-plate in triplicate (5×10^3 cells/100 μ L α -MEM complete medium/well). Cells were incubated at 37°C without a medium change and stained with 10% CCK-8 reagent for 4 hours at 37°C after 24, 48, 72, and 165 hours. The absorbance of CCK-8 proportional to the number of living cells in each well was measured at 450 nm at each time point using a SpectraMax Plus 384 microplate reader with SoftMax Pro 5 software. The absorbance of SOL- and NIL-DMSCs was then compared.

3.3.2. Migration assays

3.3.2.1. xCELLigence real-time migration assay

The xCELLigence system (ACEA Biosciences, Inc.) with a 16-well CIM plate was used to measure migration. The CIM plate consists of two chambers (upper and lower) separated by a polyethylene terephthalate membrane with porous size of 8 μ m. Microelectrodes are embedded to the membrane at the lower chamber side. The migration of DMSCs was evaluated using the xCELLigence system as previously described (Abumaree et al., 2017; Alshabibi et al., 2017; Alshabibi et al., 2018) with following modifications. Briefly, 165 μ L/well of α -MEM complete

medium (chemoattractant) and 50 μ L/well of α -MEM alone (negative control) were added to the lower and upper chamber of the 16-well CIM-plate, respectively. After 1-hour equilibration at 37°C, the background was measured. DMSCs were harvested, counted, then plated into the upper chamber of the 16-well CIM-plate (4×10^4 cells/100 μ L α -MEM alone/well). Cells were allowed to settle for 30 min at RT prior to incubation at 37°C for 24 hours. The cell index was measured in real time every 15 min using RTCA Software 1.2.1. A cell index of 0.1 was determined as the migration starting point as per the manufacturer's recommendation.

3.3.2.2. *Scratch-wound assay*

The scratch-wound assay was performed as described by Liang, Park, and Guan (2007) with minor modifications. Briefly, DMSCs at P4 were collected and counted after dissociation with TrypLE (Life Technologies), then 1×10^5 cells/well of SOL-DMSCs (n=1; pool of n = 8 patient samples) and NIL-DMSCs (n=1; pool of n = 7 patient samples) were prepared in triplicate in 6-well plates. Cells were incubated at 37°C. Once a monolayer of cells was formed, a scratch was made across the cell population using a 200 μ L pipette tip. Cells were gently washed with HBSS [-] and incubated for a further 24 hours in fresh α -MEM complete medium. Images of the scratch area at two different positions were captured at 0 and 24 hours using the EVOS® XL Cell Imaging System. Images were processed using Image J software. The percentage of scratch area covered by migrating DMSCs relative to the total scratch area was proportional to the migration level of the cells. Resulting values between SOL- and NIL-DMSCs were compared.

3.3.3. *FITC Annexin V assay*

The FITC Annexin V assay (Trevigen, Inc.) was conducted as previously described by Kusuma et al. (2017). Briefly, DMSCs at P4 were collected and counted after dissociation with TrypLE (Life Technologies). Cell aliquots, each containing 1×10^5 cells/100 μ L, were prepared in duplicate and washed with ice cold PBS. Cells were pelleted at 300 g for 5 min then stained with 100 μ L/sample Annexin V - propidium iodide (AV-PI) reagent mix. Controls were prepared by resuspending the cells in binding buffer, AV, or PI only. Cells were incubated in dark for 15 min at RT then mixed with 400 μ L binding buffer. Cells were filtered into a 5 mL tube fitted with a cell strainer cap to obtain a single cell suspension. Flow cytometry analyses of the AV and PI binding signals in DMSCs were conducted using BD LSRII flow cytometer with BD FACS Diva software. Representative gating adjustment is shown in **Appendix III - Supplementary figure 2**. Cells were classified into four quadrants based on their expression of AV and PI: Q1 represents necrotic cells (AV⁻/PI⁺), Q2 represents late apoptotic cells (AV⁺/PI⁺), Q3 represents healthy/viable cells (AV⁻/PI⁻), and Q4 represents early apoptotic cells (AV⁺/PI⁻). The percentages of SOL- and NIL-DMSCs in each quadrant were compared.

3.3.4. *Oxidative stress assay*

3.3.4.1. *ALDEFLUOR aldehyde dehydrogenase (ALDH) detection assay*

The aldefluor assay (STEMCELL Technologies, Inc.) was conducted as previously described by Kusuma et al. (2017). Briefly, DMSCs at P5 were collected and counted after dissociation with TrypLE (Life Technologies). Cell aliquots, each contained 1×10^6 cells in 1 mL ALDH assay buffer, were prepared. Cells were then

incubated with Aldefluor substrate at 37°C for 30 min. Negative controls were prepared by adding 5 µL ALDH inhibitor N,N-diethylaminobenzaldehyde (DEAB) to the cells. Cells were centrifuged at 250 g for 5 min. The resulting pellet was resuspended in 500 µL ALDH assay buffer containing 1µg/mL DAPI (Merck). Cells were filtered into a 5 mL tube fitted with a cell strainer cap to obtain a single cell suspension. Flow cytometry analyses of the intracellular fluorescence signals resulting from ALDH activity were performed using a BD LSRII flow cytometer with BD FACS Diva software. Representative gating adjustment is shown in **Appendix III - Supplementary figure 3**. The percentages of SOL- and NIL-DMSCs expressing ALDH (ALDH^{br} cells) were then compared.

3.3.4.2. *TBARS (TCA method) lipid peroxidation assay*

This assay was conducted as per the manufacturer's protocol (Cayman Chemical). Briefly, DMSCs at P5 were collected and counted after dissociation with TrypLE (Life Technologies). Cell aliquots, each contained 2x10⁶ cells in 50 µL ice-cold PBS (Gibco™), were prepared. Cells were homogenised in ice. A serial dilution of malondialdehyde (MDA) standards was prepared. Homogenates and standards were mixed with 50 µL of 10% trichloroacetic acid (TCA) and 400 µL of thio-barbituric acid (TBA). Both standards and samples were incubated at 100°C for 1 hour, followed by 4°C for 10 min, and centrifugation at 1,600 g for 10 min at 4°C. The resulting supernatant was placed in duplicate into a 96-well plate (200 µL/well) and its absorbance was measured at 535 nm using a SpectraMax Plus 384 microplate reader with SoftMax Pro 5 software. The lipid peroxidation levels of SOL-DMSCs (n = 7) and NIL-DMSCs (n = 5), proportional to the absorbance, were then compared.

3.3.5. *Pro-inflammatory cytokine (IL-6 and IL-8) detection assays*

3.3.5.1. *Serum-starvation and cell lysate collection*

DMSCs (70% confluent) were serum-starved for 48 hours by reducing FCS concentration in α -MEM complete medium from 10% to 1%. The volume of serum-starved medium was also reduced to 75% to concentrate DMSC secretome. The stressed condition of DMSCs after being cultured in serum starvation medium was validated using the xCELLigence proliferation system. After 48 hours, the serum-starved medium was collected, then centrifuged at 500 g for 5 min and 2,000 g for 10 min to remove cell debris. DMSCs were collected after dissociation with TrypLE (Life Technologies). Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Life Technologies) supplemented with 0.002% AEBSF (MP Biomedicals), and centrifuged at 14,000 g, 4°C for 15 min. The supernatant (cell lysate) was collected and stored.

3.3.5.2. *Bicinchoninic acid (BCA) assay for protein concentration measurement*

The protein concentration of cell lysates was measured using Pierce BCA kit (Thermo Fisher Scientific), with the protocol described by Noble and Bailey (2009) with minor modifications. Briefly, a serial dilution of bovine serum albumin (BSA) standards was prepared. Standards and cell lysates (10 μ L each, prepared in duplicate) were mixed with 200 μ L BCA working reagent in the 96-well plate. The plate was incubated at 37°C for 30 min. The absorbance of both samples and standards was measured at 562 nm using a SpectraMax Plus 384 microplate reader with SoftMax Pro 5 software.

3.3.5.3. *Enzyme-Linked Immunosorbent Assays (ELISAs)*

ELISAs (Crux Biolab) were performed as previously described by Reen (1994) with modification. Briefly, 100 μ L standards, samples (collected medium), and blank were added into the wells pre-coated with human capture IL-6 or IL-8 antibody, and incubated for 2 hours at RT. After washing (4x), 100 μ L biotin labelled IL-6 or IL-8 antibody was added, followed by 1 hour incubation at RT. The plate was again washed (4x). Streptavidin-HRP conjugate was added to each well, followed by 45 min incubation at RT. The plate was again washed (5x). 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added to each well and the plate was incubated for 15 min at RT. The reaction was stopped once the colour development was complete using 0.16 M sulfuric acid solution. The absorbance was measured at 450 nm with wavelength correction at 570 nm and processed using SoftMax Pro 5 software. IL-6 and IL-8 concentrations secreted by SOL- and NIL-DMSCs were normalised to the protein concentration as described in **Section 3.3.5.2.**, before being compared.

3.4. **Mass-spectrometry based lipid analyses**

3.4.1. *Sample preparation*

DMSCs at P5 were serum starved for 48 hours as described in **Section 3.3.5.1**. Prior to cell lysis, cells were resuspended in 400 μ L PBS (Gibco™) and only 50 μ L of this cell suspension was lysed and homogenized in RIPA buffer as described in **Section 3.3.5.1**. Cell lysates were used to measure the protein concentration of the samples using BCA method as described in **Section 3.3.5.2**. RIPA buffer contains

polyethylene glycol (PEG) polymers that interfere the mass spectrometry analysis and thus, was not used to resuspend the mass spectrometry samples.

3.4.2. *Folch's lipid extraction protocol*

Cells resuspended in PBS were aliquoted into microtubes at a volume that would give the equivalent of 12 µg protein/sample. Lipid components of these aliquot were extracted using Folch's lipid extraction protocol (Folch, Lees, & Sloane Stanley, 1957) with minor modifications. Briefly, samples were initially spiked in with 5 µL SPLASH® LIPIDOMIX® Mass Spec Standard (Avanti Polar Lipids, Inc.) each, as the internal standard. Samples were then mixed with 400 µL of chloroform:methanol (2:1, Merck) each, vortexed for 1 min, and centrifuged for 10 min at 825 g. The organic fraction was transferred to a new tube and a further 400 µL/sample of 100% chloroform (Merck) was added to the initial tube. The tube was vortexed for 1 min, then again centrifuged for 10 min at 825 g. The resulting organic fraction was combined with the formerly extracted fraction and then, dried in a vacuum centrifuge. The pellet was resuspended in 10 µL of 100% chloroform (Merck) then diluted with 90 µL of 100% methanol (Merck) immediately prior to the analyses. The list of lipid mixtures contained in the standard is presented in **Appendix I**. The amount of sample (12 µg) and standard (5 µL) required were determined prior to the analyses of individual samples by evaluating the MS responses of a serial dilution of the pool of SOL- and NIL-DMSCs (a pool of n = 7 per group) and two standard concentrations (5 and 10 µL) to ensure that the lipid samples were measured within the linear response ranges.

3.4.3. Mass spectrometry analyses

Mass spectrometry analyses were conducted by Dr. Shuai Nie at the Mass Spectrometry and Proteomics Facility (MSPF), Bio21 Molecular Science & Biotechnology Institute, Australia. Sample analyses were performed in positive and negative mode on a Thermo Fisher Orbitrap Lumos (Thermo Fisher, Scoresby, Vic, Australia) combined with a Thermo Fisher U3000 nanoHPLC (Thermo Fisher, Scoresby, Vic, Australia). Further instrument details were as described by Wang et al. (2018). The resulting raw mass spectrometry data files were searched, aligned into their corresponding group (SOL-DMSCs and NIL-DMSCs), and filtered using LipidSearch software v.4.2.20 SP1 (Thermo Scientific) with parameters as defined by Wang et al. (2018). The ID and quantities of each lipid species identified from SOL- and NIL-DMSCs were compared. For the lipid species belonged to the lipid class that had the representative standard included in the SPLASH® LIPIDOMIX® mixture (**Appendix I**), the absolute amount of each lipid species (µg) per 100 µL sample was determined using the formula:

$$\frac{\text{Peak area of the lipid species}}{\text{Peak area of the lipid class standard}} \times \text{the amount of the standard spiked into the samples (}\mu\text{g)}$$

For the lipid species belonging to the lipid class that was not included in the SPLASH® LIPIDOMIX® mixture, the relative quantification was determined directly using the peak area of the lipid species, with the assumption of total lipid analysed was equal for all samples as it had been normalised based on the protein concentration of the samples (12 µg/sample).

3.5. Statistical analyses

The sample size (n) tested in the statistical analyses was defined based on the criteria described by Lazic, Clarke-Williams, and Munafò (2018). Specifically, “n” is equal to the number of biological replicates (i.e., the number of patients) in the experiments with individual samples. Data distribution was determined based on the graphical assessment of skewness. The data were also subjected to the D'Agostino & Pearson omnibus normality test when the sample size was sufficient. The data following Gaussian distribution were analysed using unpaired student t-test, while the data following non-Gaussian distribution were analysed using Mann–Whitney U test, which is the non-parametric equivalent of unpaired student t-test, unless stated otherwise. Statistical analyses were performed using GraphPad Prism 5.01 software. The statistical significance was set at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. For the functional assays, the data were generated from the combination of two different batches of patient samples. One common sample was tested in both batches of samples as a replication control. Prior to data merger, data were normalised using the average value of each group.

4. RESULTS

4.1. Clinical characteristics of placental donor

The clinical characteristics of the placental donors are summarised in **Table 1** below. All clinical characteristics of patients undergoing SOL and NIL were not significantly different ($p > 0.05$).

Table 1. Summary of patient characteristics

Patient characteristics	Labour status		p-value
	SOL (n = 8)	NIL (n = 7)	
Maternal			
Age (years)	31.1 ± 1.2	30.9 ± 1.7	0.86 ^a
BMI (kg/m ²)	21.9 ± 0.7	21.7 ± 1.7	0.70 ^a
Labour			
Total length of labour (h)	7.10 ± 1.41	N/A	N/A
Membrane status	5 SROM 3 ARM 0 Intact	0 SROM 1 ARM 6 Intact	N/A
Birth and Neonate			
Gestational age (weeks)	40.14 ± 0.16	39.59 ± 0.22	0.08 ^a
Placental weight (gram)	666 ± 46	632 ± 53	0.69 ^a
Infant birth weight (gram)	3762 ± 63	3759 ± 142	0.46 ^a
Infant gender	1 Male 7 Female	4 Male 3 Female	0.12 ^b

Numerical data were presented as mean ± SEM. P-value was calculated using (a) Mann Whitney U test and (b) Fisher's exact test. The differences between groups are considered statistically significant when $p\text{-value} < 0.05$. ARM: artificial rupture of membrane; SROM: spontaneous rupture of membrane.

4.2. Isolation and characterisation of the primary cells

The following sections show representative images of each characteristic of isolated primary cells from SOL and NIL patients.

4.2.1. *In vitro* morphology of the isolated primary cells

The isolated primary cells from both SOL and NIL patients adhered to plastic cultureware and showed the typical fibroblast-like morphology (**Figure 8**).

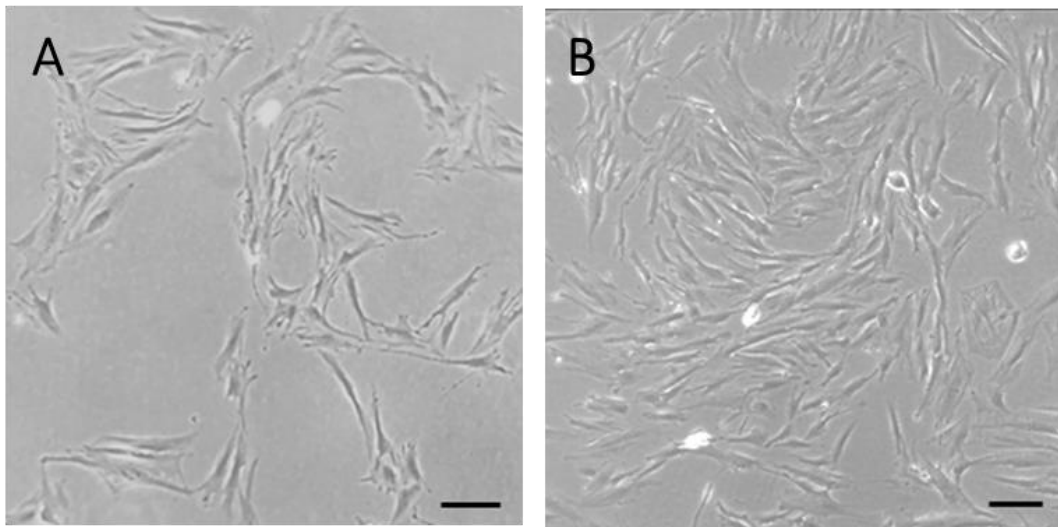


Figure 8. *In vitro* morphology of the representative primary cells

Primary cells were isolated from term placentae of patients undergoing **(A)** SOL and **(B)** NIL. Cells from both patients demonstrated typical fibroblastic morphology of MSCs. Morphology was observed at passage 2, using a bright field inverted microscope (Leica DM E) at a magnification of 100X. Scale bar = 50 μ m.

4.2.2. *Flow cytometry analyses of MSC-specific surface antigens*

Based on flow cytometry analyses, the primary cells from both SOL and NIL patients expressed MSC-specific surface markers, where $\geq 95\%$ of the cells were positive for CD90, CD 166, CD 73, CD44, and CD 105 and $\leq 5\%$ of the cells were positive for hematopoietic markers: CD 45, CD 19, and HLA-DR (**Figure 9**).

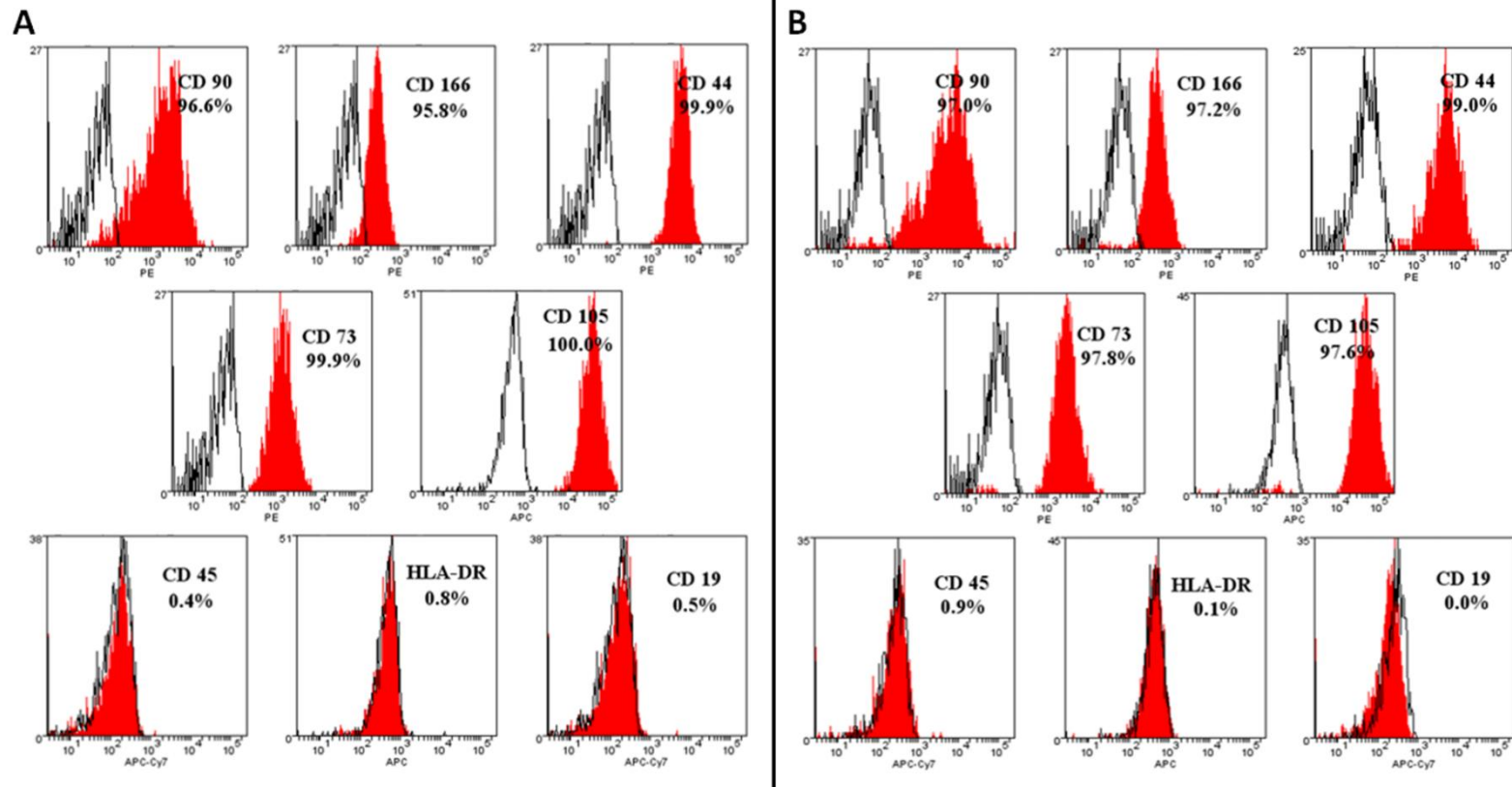


Figure 9. Flow cytometry analyses of the representative primary cell surface marker expression

Primary cells isolated from both **(A)** SOL and **(B)** NIL patients were consistent with ISCT criteria for defining MSCs by positively expressed CD90, CD166, CD44, CD73, and CD105 ($\geq 95\%$ positive) and negatively expressed the hematopoietic markers CD45, HLA-DR, and CD19 ($\leq 5\%$ positive). Red and white histograms represent the expression of MSC-specific surface markers and their associated isotype, respectively. X-axis: fluorescence intensity. Y-axis: number of recorded events, set to 10^4 cells per run. The percentages of cells positively expressed the surface markers are shown. PE: Phycoerythrin; APC: Allophycocyanin; Cy7: tandem conjugate.

4.2.3. Differentiation potential of the isolated primary cells

Under appropriate stimulation, the primary cells from both SOL and NIL patients were able to differentiate into the adipogenic and osteogenic lineages, as shown through the formation of lipid droplets (**Figure 10**) and calcium deposits (**Figure 11**), respectively.

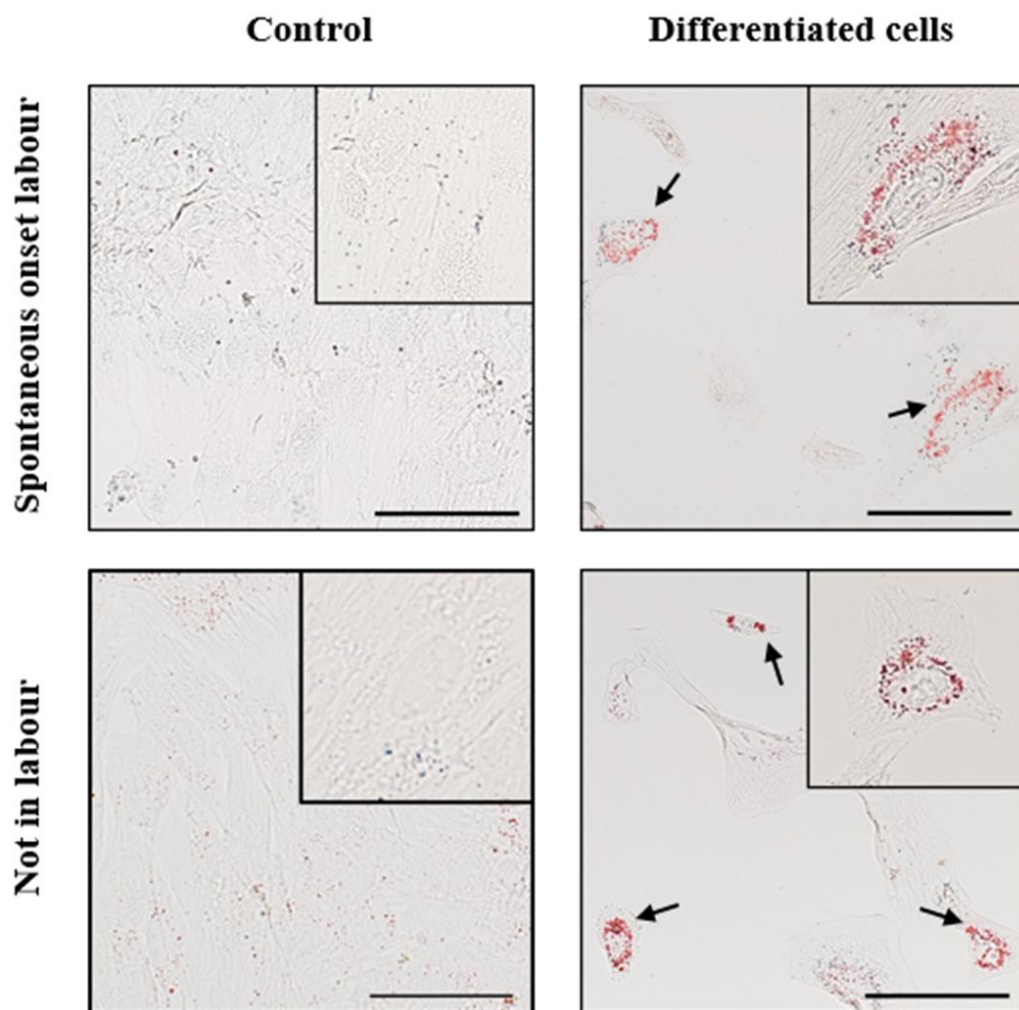


Figure 10. Adipogenic potential of the representative primary cells

Primary cells from both SOL and NIL patients showed potential to differentiate into the adipogenic lineage, as shown through the formation of lipid droplets (indicated by arrows) after 14 days incubation with adipogenic induction media. Cells were stained with Oil Red O prior to the microscopic observation at 200X magnification (Axioskop 2 microscope equipped with Nikon DXM 1200C camera). Scale bar = 50 μ m. Insets show the differentiated cells at 400X magnification.

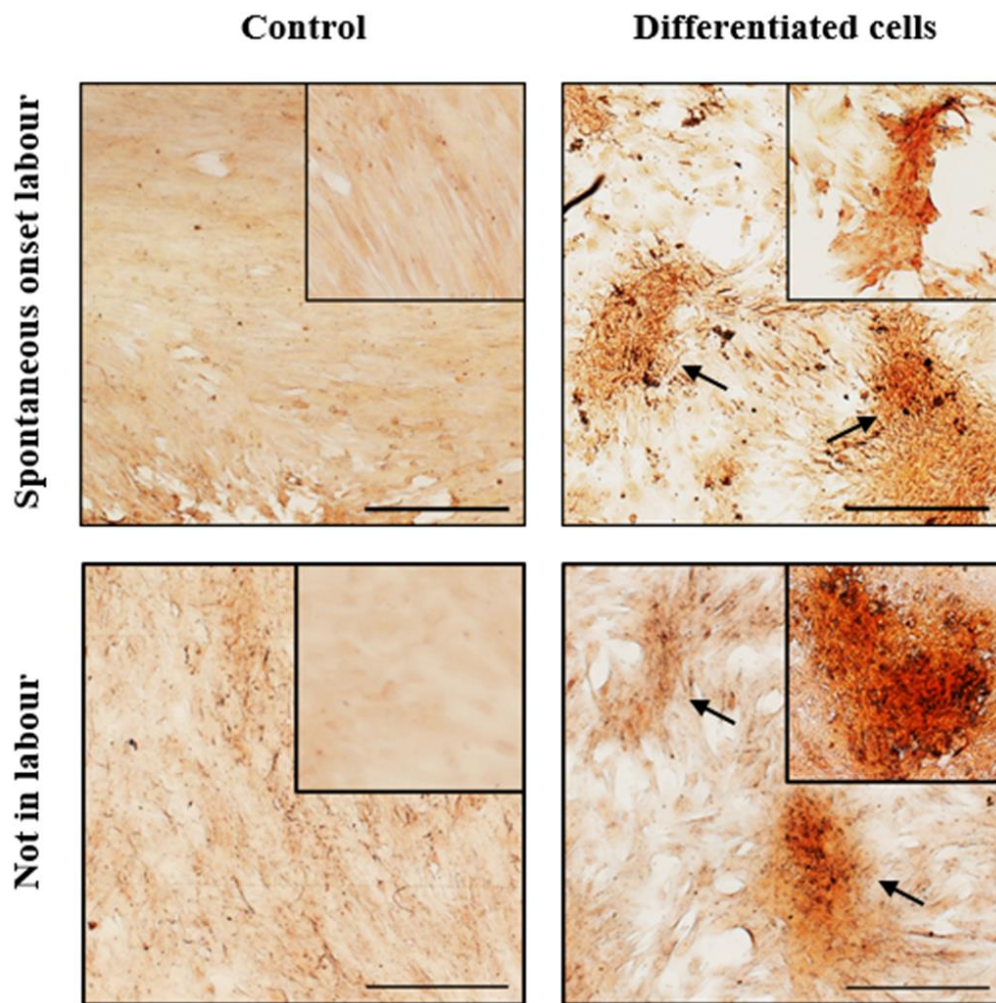


Figure 11. Osteogenic potential of the representative primary cells

Primary cells from both SOL and NIL patients showed potential to differentiate into the osteogenic lineage, as shown through the formation of calcium deposits (indicated by arrows) after 21 days incubation with osteogenic induction media. Cells were stained with Alizarin Red prior to the microscopic observation at 40X magnification (Axioskop 2 microscope equipped with Nikon DXM 1200C camera). Scale bar = 100 μ m. Insets show the differentiated cells at 100X magnification.

4.2.4. *Clonogenic potential of the isolated primary cells*

The isolated primary cells from both SOL and NIL patients were able to form colonies when seeded at low density, as expected of MSCs (**Figure 12A-D**). However, the differences of the clonogenic efficiency between SOL- and NIL-DMSCs were not statistically significant ($p > 0.05$, **Figure 12E**). The descriptive

statistics of the clonogenic efficiency for the isolated primary cells from both patients are shown in **Appendix V - Supplementary table 1**.

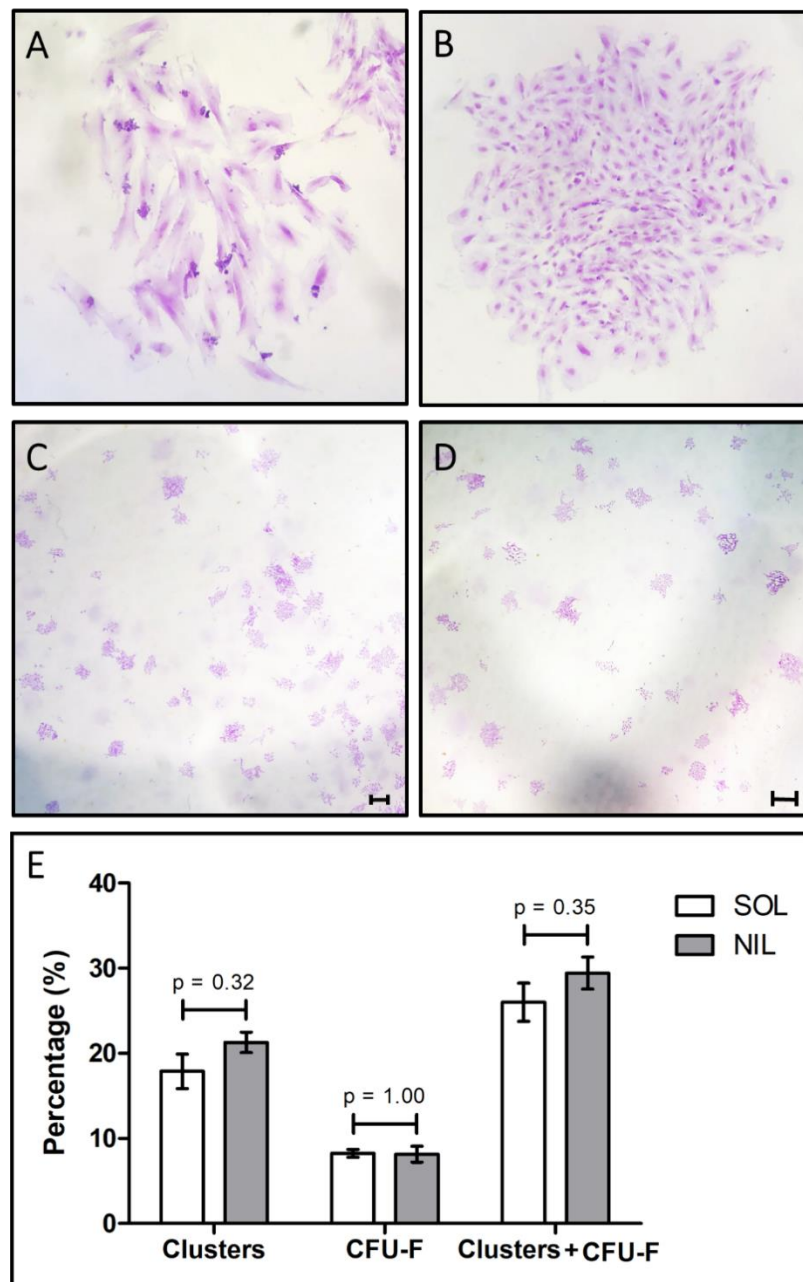


Figure 12. Clonogenicity of the isolated primary cells

Morphology of a representative **(A)** cell cluster containing fewer than 50 cells and **(B)** CFU-F colony of greater than 50 cells at 400X magnification. Clonogenicity of the isolated primary cells from patients undergoing **(C)** SOL and **(D)** NIL at 64 X magnification. Cells were observed with a stereo microscope (Leica/Wild M3C). Scale bar: 1 mm. **(E)** Clonogenic efficiency of the isolated primary cells from both patients were not significantly different ($p > 0.05$). Data were presented as mean $\pm$ SEM. P-value was calculated by Mann-Whitney U test. Samples were tested in triplicate.

4.2.5. FISH analyses of the isolated primary cells

Based on FISH analyses of the primary cells prepared from placentae of male newborn, the cells from SOL and NIL patients were of maternal origin, as shown by the presence of two X chromosomes signals and the absence of Y chromosome signals (**Figure 13**).

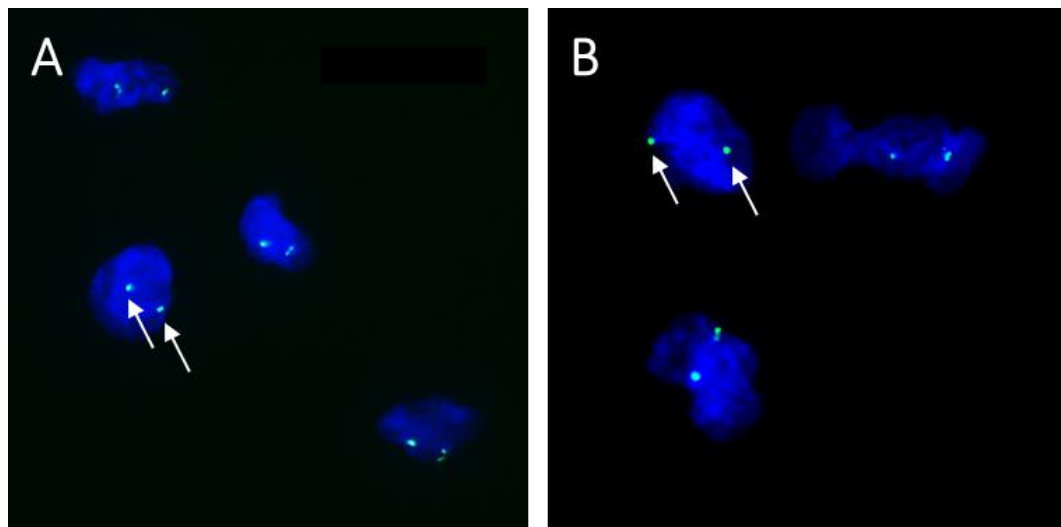


Figure 13. FISH analyses of the representative primary cells

FISH analyses were performed on a minimum of 200 cell nuclei per sample of primary cells from placentae of male babies. Primary cells from **(A)** SOL and **(B)** NIL patients were confirmed as maternal in origin by the presence of two Spectrum Green X chromosome signals (arrows) and the absence of Spectrum Orange Y chromosome signals. Cell nuclei were counterstained with DAPI. Nuclei were observed with a fluorescence microscope (Olympus BX51 microscope equipped with JAI M300 CCD camera) at 600X magnification. Interphase FISH images were processed using MetaSystems ISIS image analysis software v5.3.16.

Since the primary cells isolated from SOL and NIL patients were consistent with the ISCT criteria for defining MSCs (Dominici et al., 2006) and were of maternal origin, they were subsequently referred to SOL-DMSCs and NIL-DMSCs, respectively.

4.3. Functional changes of DMSC associated with SOL

The following sections listed the evidence of functional loss and/or changes of SOL-DMSCs (n = 8) compared with NIL-DMSCs (n = 7) from full-term patients (39 weeks 0 days to 40 weeks 6 days) unless stated otherwise.

4.3.1. Proliferation capacity of SOL- and NIL-DMSCs

Prior to the individual sample analyses, the seeding density of DMSCs was optimised by comparing the xCELLigence growth profile of DMSCs plated at different seeding density (1×10^4 , 7.5×10^3 , 5×10^3 , and 2.5×10^3 cells/well). DMSCs seeded at 5×10^3 cells/well showed the longest exponential growth without a prolonged attachment phase (**Appendix IV - Supplementary figure 4**). Therefore, this seeding density was used in all subsequent proliferation assays.

4.3.1.1. xCELLigence real-time proliferation assay

Since xCELLigence is a real-time assay, the complete growth profile of DMSCs, as reflected through the changes of cell indices over time, can be monitored and recorded (**Appendix IV - Supplementary figure 5**). **Figure 14A** below showed the individual graphical representation of cell attachment, proliferation and survival phase of SOL- and NIL-DMSCs. Statistical analyses were conducted on the cell indices at various specific time points in each phase. The descriptive statistics of the cell indices in each designated time point are shown in **Appendix V - Supplementary table 2**. There were no statistically significant differences in the cell indices observed between SOL- and NIL-DMSCs at all phases ($p > 0.05$, **Figure 14B**). Nevertheless, there was a trend towards lower cell index of SOL-

DMSCs at proliferation and survival phase, which suggested a reduced proliferation and survival capacity of SOL-DMSCs compared with NIL-DMSCs.

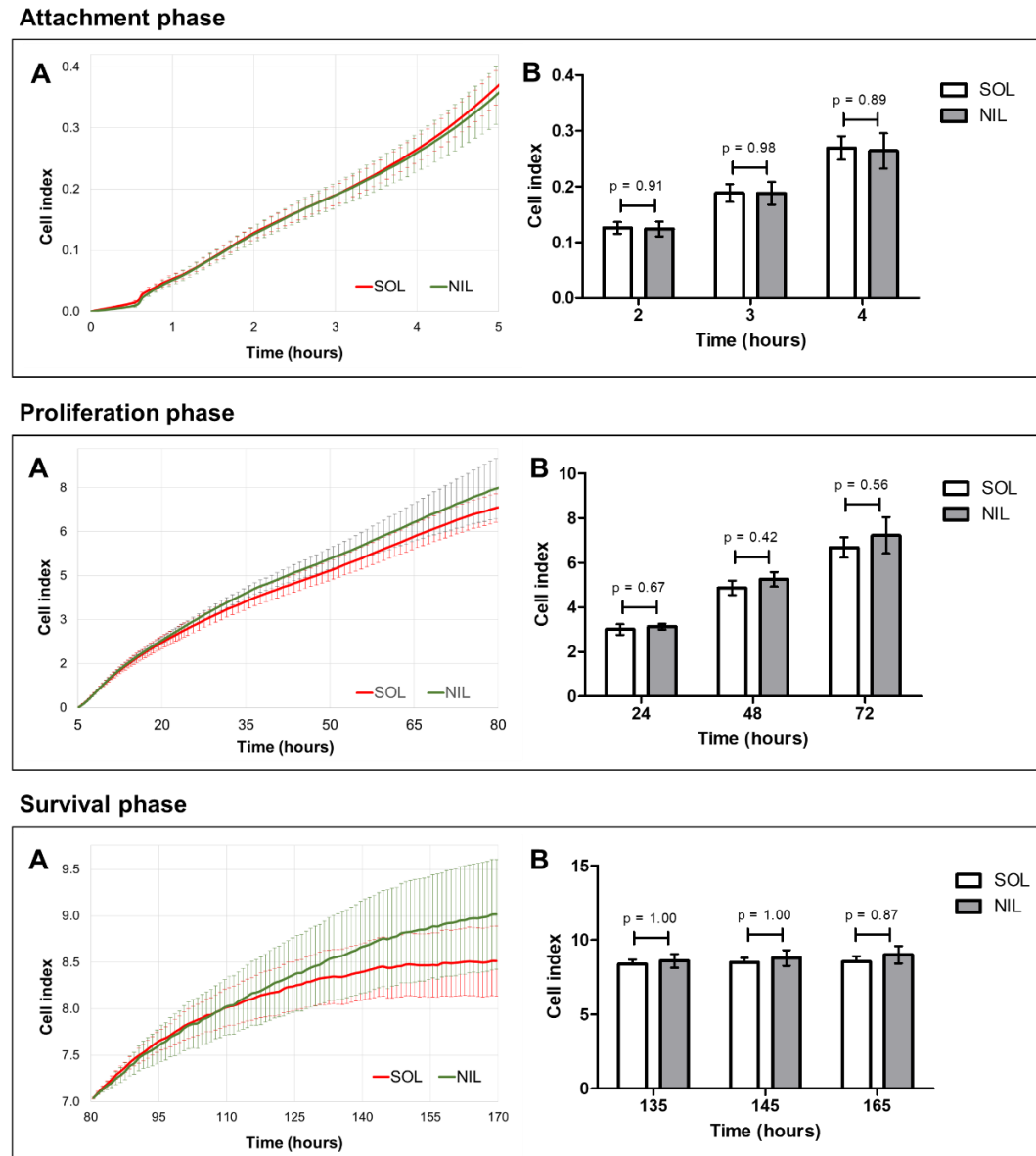


Figure 14. xCELLigence real time proliferation assay

Graphs (A) showed the cell indices of SOL- and NIL-DMSCs, presented as mean $\pm$ SEM, against time during attachment (0-5 hours), proliferation (5-80 hours), and survival phase (> 80 hours). Graphs (B) showed the statistical analyses performed at certain time points in each phase. Data were presented as mean $\pm$ SEM. There were no statistically significant differences of the cell indices observed between SOL- and NIL-DMSCs at all phases ($p > 0.05$). P-value was calculated using unpaired student t-test (attachment and proliferation phase) and Mann-Whitney U test (survival phase). Each sample was tested in triplicate.

4.3.1.2. CCK-8 end-point proliferation assay

Since CCK-8 measurement is an end-point assay, it is not possible to normalise the starting point of the proliferation and survival phase. Nevertheless, the outcomes from this CCK-8 assay remained consistent with the outcomes of the xCELLigence real-time proliferation assay. SOL-DMSCs were shown to have lower proliferation indices compared with NIL-DMSCs as reflected through lower absorbance of CCK-8 reagent of SOL-DMSCs at 450 nm. However, the differences were not statistically significant ($p > 0.05$; **Figure 15**). The descriptive statistics of the absorbance at each time point are shown in **Appendix V - Supplementary table 3**.

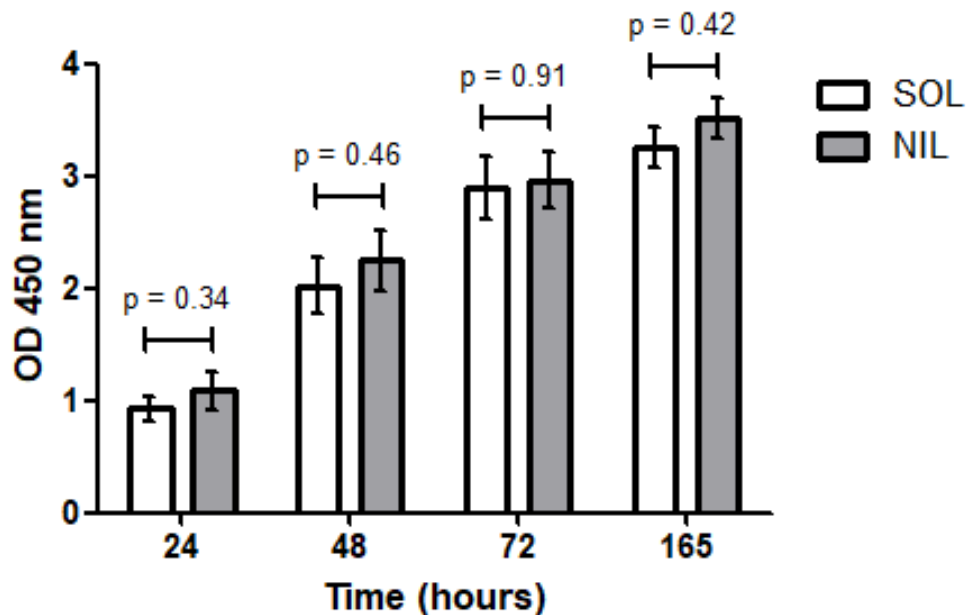


Figure 15. CCK-8 end-point proliferation assay

The absorbance of CCK-8 reagent at 450 nm was proportional to the numbers of viable DMSCs present at observation time points. The proliferation indices of SOL-DMSCs were generally lower compared with NIL-DMSCs. However, the differences were not statistically significant ($p > 0.05$). Data were presented as mean $\pm$ SEM. P-value was calculated using Mann-Whitney U test. Each sample was tested in triplicate.

4.3.2. Migration capacity of SOL- and NIL-DMSCs

4.3.2.1. xCELLigence real-time migration assay

SOL- and NIL-DMSCs resuspended in α -MEM alone (0% FCS) at the upper chamber of the CIM plate were shown to migrate to the lower chamber of the CIM plate filled with α -MEM complete medium (10% FCS) as the chemoattractant. The migration capacity of SOL-DMSCs was higher compared with NIL-DMSCs. This was reflected by higher cell indices of SOL-DMSCs detected at the lower chamber of CIM plate throughout the time course compared with NIL-DMSCs (**Figure 16A**). The differences were statistically significant at 16 hours after migration starting point ($p < 0.05$, **Figure 16B**), which was set as cell index ≥ 0.1 as per manufacturer's recommendation.

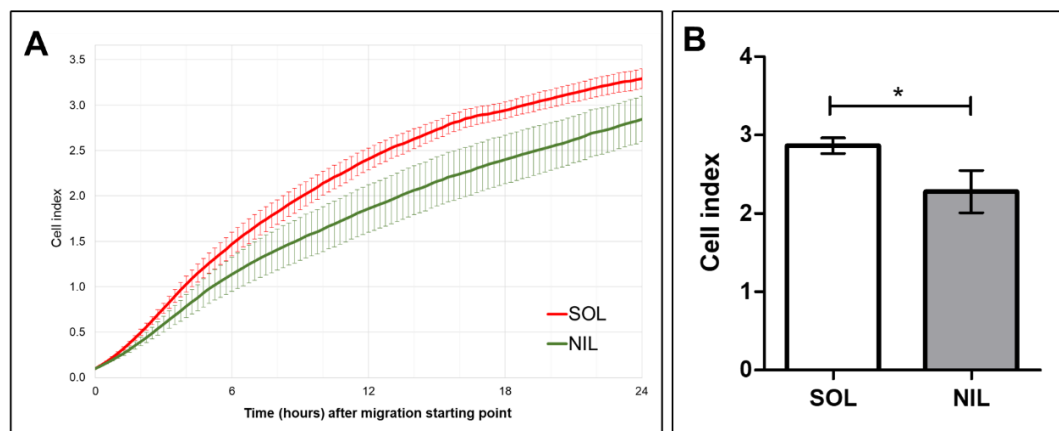


Figure 16. xCELLigence real-time migration assay

Graph (A) showed the cell indices of SOL- and NIL-DMSCs that migrated to the lower chamber of CIM plate over 24 hours. Data were presented as mean $\pm$ SEM. (B) SOL-DMSCs showed a statistically significant higher level of migration compared with NIL-DMSCs ($p = 0.04$) at 16 hours after migration starting point (cell index ≥ 0.1). This was reflected by a higher cell index of SOL-DMSCs (2.86 ± 0.10) compared with NIL-DMSCs (2.28 ± 0.27). Data were presented as mean $\pm$ SEM. P-value was calculated using Mann Whitney U-test. * $p < 0.05$.

4.3.2.2. Scratch-wound assay

This assay provided a qualitative confirmation of the migration capacity of SOL- and NIL-DMSCs to support the outcomes from the xCELLigence migration assay, which was based on the arbitrary cell indices. Consistent with the outcomes from the xCELLigence assay, the scratch-wound assay showed both SOL-DMSCs (n = 1, a pool of n = 8 patient samples) and NIL-DMSCs (m = 1, a pool of n = 7 patient samples) migrated to the wound area in responses to higher FCS concentration in α -MEM complete medium (**Figure 17**).

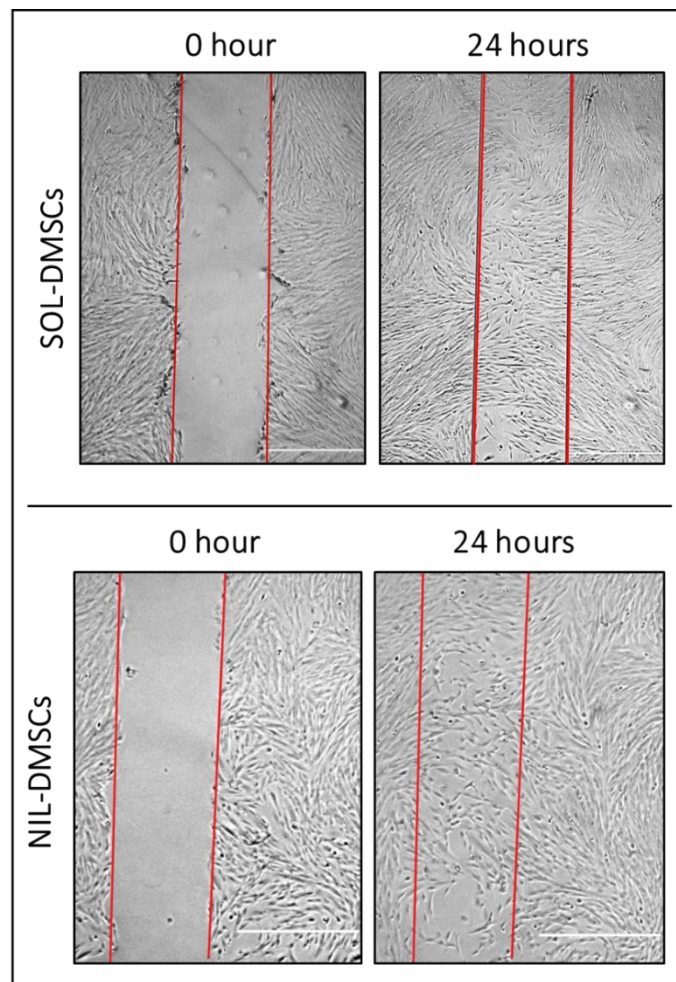


Figure 17. Representative images of the migrating DMSCs in the scratch assay

Microscopy observation at 24h showed SOL-DMSCs and NIL-DMSCs to migrate to the wound area. Cells were observed using an EVOS® XL Cell Imaging System at a total magnification of 25X. Scale bar represents 1 mm.

SOL-DMSCs also exhibited higher migration capacity compared with NIL-DMSCs as reflected through higher percentages of wound area covered by the migrating DMSCs (**Figure 18**). However, the statistical test was not performed since each group only consists of n =1 pooled sample with 6 technical replicates.

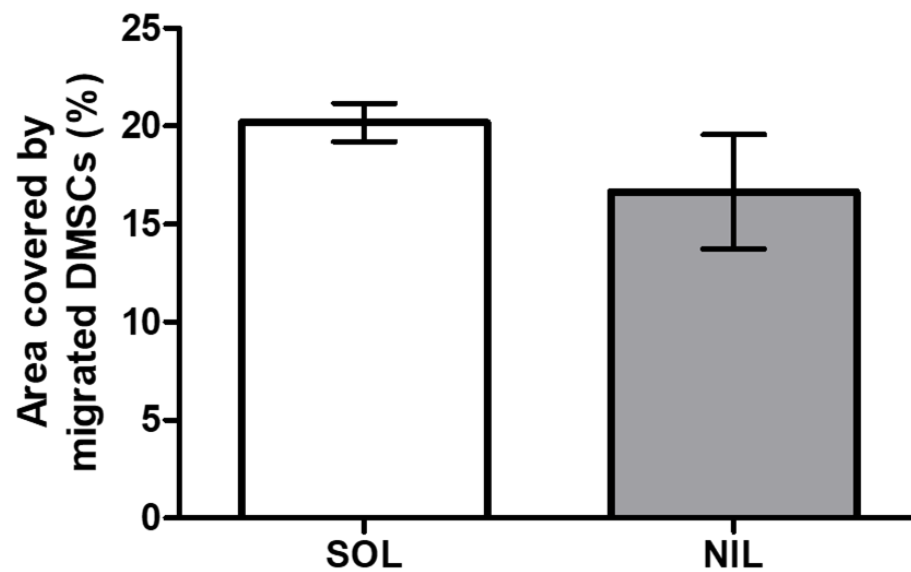


Figure 18. Quantification of wound area covered by the migrating DMSCs

The percentages of wound area covered was higher in SOL-DMSCs ($20.20 \pm 0.97\%$) compared with NIL-DMSCs ($16.65 \pm 2.92\%$). Data were presented as mean $\pm$ SEM.

4.3.3. Cell death profile of SOL- and NIL-DMSCs

The FITC Annexin V assay showed a statistically significant higher percentage of necrotic cells (Q1) in SOL-DMSCs compared with NIL-DMSCs ($p < 0.05$; **Figure 19**). SOL-DMSCs also showed a higher percentage of apoptotic cells i.e., early (Q4), late (Q2), and total apoptotic cells (Q2 + Q4) and a lower percentage of viable/healthy cells (Q3) compared with NIL-DMSCs. However, the differences were not statistically significant ($p > 0.05$, **Figure 19**). The descriptive statistics of the percentages of cell population in all quadrants are shown in **Appendix V - Supplementary table 4**.

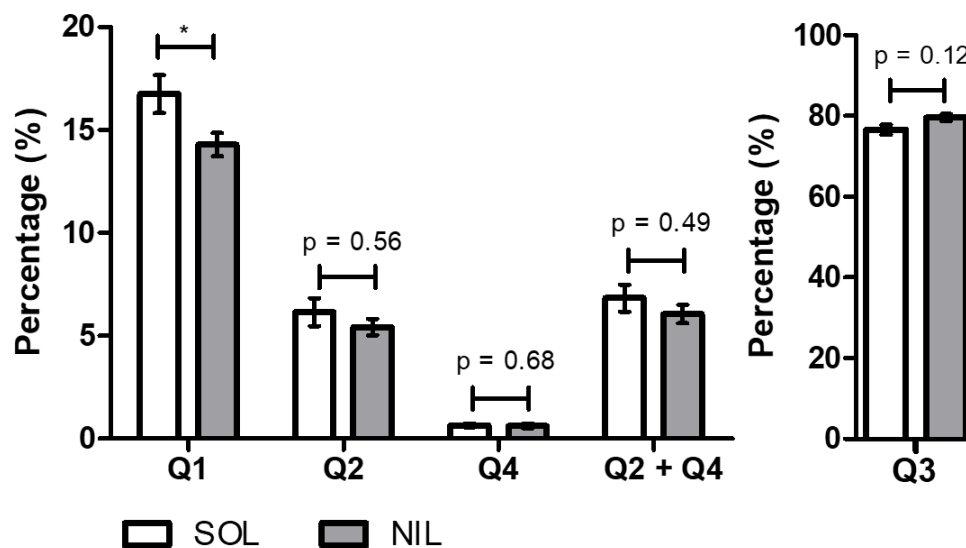


Figure 19. FITC Annexin V profile of SOL- and NIL-DMSCs

SOL-DMSCs showed a statistically significant higher percentage of necrotic cells compared with NIL-DMSCs ($p = 0.03$). An increase in the percentage of apoptotic cells (Q2, Q4, Q2+ Q4) and a decrease in the percentage of viable/healthy cells (Q3) were detected in SOL-DMSCs compared with NIL-DMSCs. However, the differences were not statistically significant ($p > 0.05$). Data were presented as mean $\pm$ SEM. P-value was calculated using Mann Whitney U-test. * $p < 0.05$.

4.3.4. Oxidative stress profile of SOL- and NIL-DMSCs

SOL- and NIL-DMSCs were assessed for their ability to resist oxidative stress based on the percentage of ALDH^{br} cells in the population using the Aldefluor assay. The oxidative stress level of both DMSCs was also evaluated by the potential for lipid peroxidation. This was determined by measuring the production of MDA, which is a by-product of lipid peroxidation.

4.3.4.1. Aldefluor assay

The Aldefluor assay showed a lower percentage of ALDH^{br} cells in SOL-DMSCs compared with NIL-DMSCs but, the differences were not statistically significant ($p > 0.05$) (**Figure 20**).

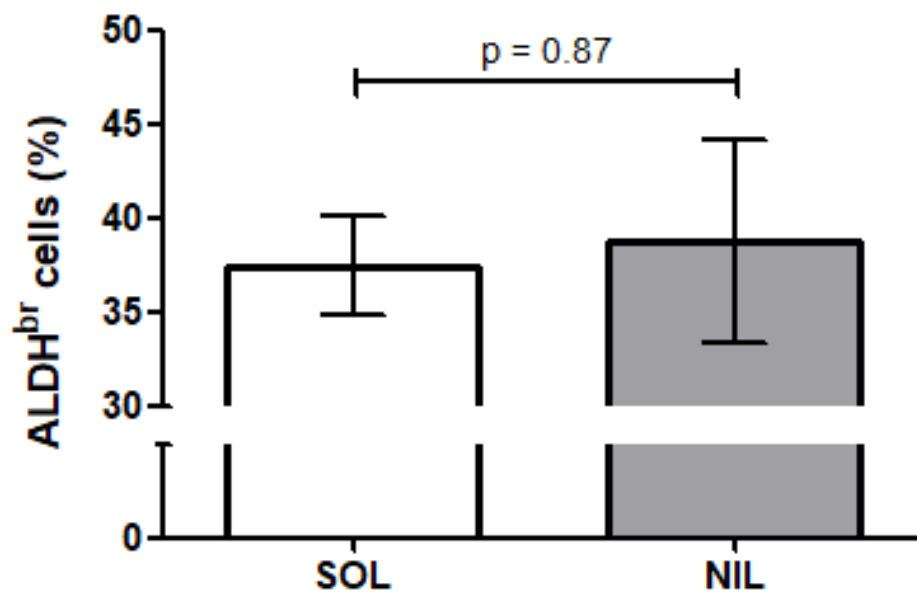


Figure 20. Aldefluor assay

SOL-DMSCs showed a lower percentage of ALDH^{br} cells ($37.45 \pm 2.60\%$) compared with NIL-DMSCs ($38.74 \pm 5.38\%$). However, the differences were not statistically significant ($p > 0.05$). Data were presented as mean $\pm$ SEM. P-value was calculated using Mann Whitney U-test.

4.3.4.2. TBARS (TCA method) lipid peroxidation assay

The TBARS (TCA method) assay showed that SOL-DMSCs (n = 7) produced a significantly higher amount of MDA than NIL-DMSCs (n = 5, $p < 0.05$, **Figure 21**), thereby suggesting a higher potential of lipid peroxidation in SOL-DMSCs compared with NIL-DMSCs.

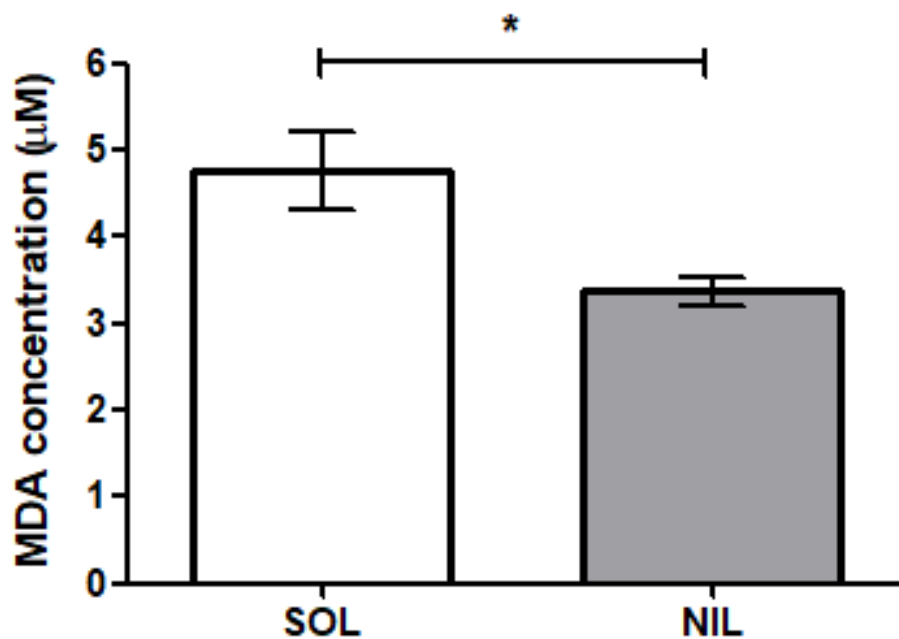


Figure 21. TBARS (TCA method) lipid peroxidation assay

SOL-DMSCs (n = 7) showed a statistically significant higher production of MDA, which is a measure of potential for lipid peroxidation, compared with NIL-DMSCs (n = 5, $p = 0.04$). The MDA concentration of SOL- and NIL-DMSCs was $4.764 \pm 0.458 \mu\text{M}$ and $3.372 \pm 0.158 \mu\text{M}$, respectively. Data were presented as mean $\pm$ SEM. P-value was calculated using Mann Whitney U-test. * $p < 0.05$.

4.3.5. Pro-inflammatory cytokine (IL-6 and IL-8) secretion

Prior to the assay, DMSCs were serum-starved to normalise the starting point of cytokine production (IL-6 and IL-8). Total removal of FCS from the medium (α -MEM complete) was not feasible since DMSCs cultured in this condition were detached, as shown through the decrease of cell indices in xCELLigence proliferation system (**Appendix IV - Supplementary figure 6**). DMSCs cultured in α -MEM medium supplemented with 1% FCS maintained the viability and the proliferation trend comparable to those cultured in the standard medium, which was supplemented with 10% FCS (**Appendix IV - Supplementary figure 6**). Thus, 1% FCS-supplemented medium was used as the serum-starved medium for DMSCs in this study. The ELISA revealed a statistically significant increase in IL-6 secreted by SOL-DMSCs compared with NIL-DMSCs ($p < 0.05$, **Figure 22**).

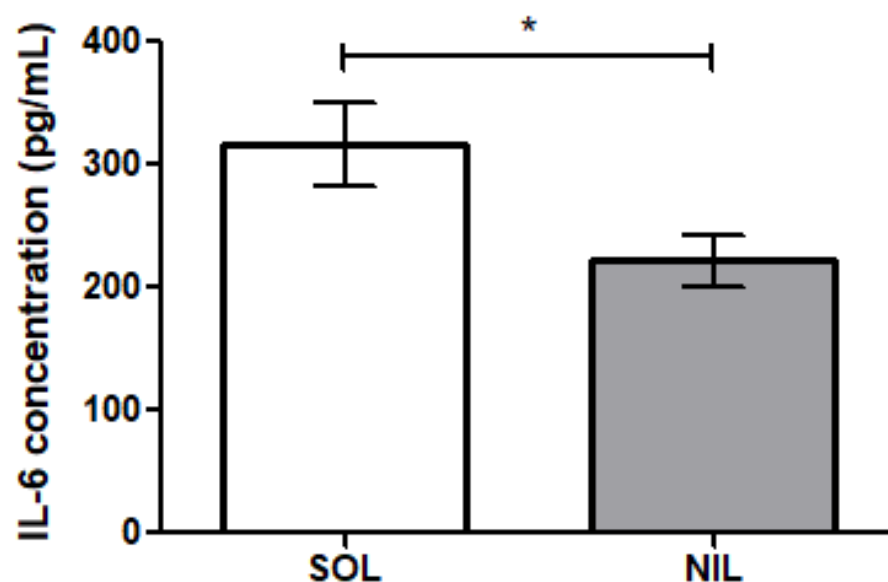


Figure 22. IL-6 secretion profile of DMSCs

SOL-DMSCs produced a significantly higher amount of IL-6 (316.90 ± 34.11 pg/mL) compared with NIL-DMSCs (221.30 ± 21.48 pg/mL, $p = 0.03$). IL-6 concentrations were normalized per 1000 μ g/mL cell lysate protein. Data were presented as mean $\pm$ SEM. P-value was calculated using Mann Whitney U-test. * $p < 0.05$.

The ELISA also revealed a higher concentration of IL-8 secreted by NIL-DMSCs (n = 7) compared with SOL-DMSCs (n = 8). However, the difference was not statistically significant ($p > 0.05$, **Figure 23**).

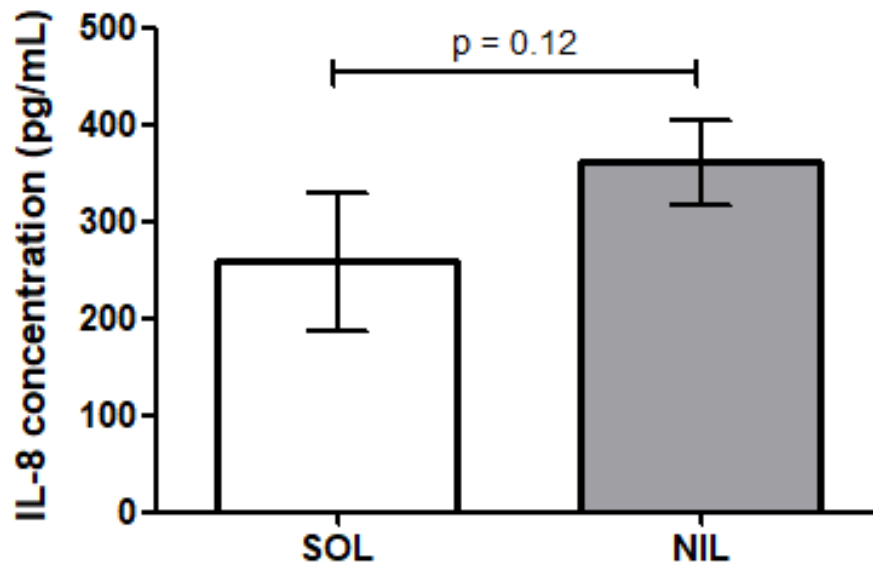


Figure 23. IL-8 secretion profile of DMSCs

SOL-DMSCs showed a decrease in IL-8 production (258.20 ± 72.09 pg/mL) compared with NIL-DMSCs (360.90 ± 44.06 pg/mL). Nevertheless, the differences were not statistically significant. IL-8 concentrations were normalized per 1000 μ g/mL cell lysate protein. Data were presented as mean $\pm$ SEM. P-value was calculated using Mann Whitney U-test.

4.4. Mass spectrometry-based lipid analyses

4.4.1. Identification of SOL- and NIL-DMSC lipidome

Using LipidSearch, a total of 570 unique lipid species were identified based on their MS/MS fragmentation patterns. These lipid species represented 5 lipid categories i.e., phospholipids (~74%), sphingolipids (~17%), neutral lipids (~9%), fatty acyls (0.2%), and other lipids (0.2%) with 17 lipid classes (**Table 2**).

Table 2. Summary of the number of identified lipids in SOL- and NIL-DMSCs

Lipid category/class		Count
Phospholipids (sub-total = 422)		
CL	Cardiolipin	31
LPC	Lysophosphatidylcholine	20
LPE	Lysophosphatidylethanolamine	9
LPI	Lysophosphatidylinositol	1
PC	Phosphatidylcholine	129
PE	Phosphatidylethanolamine	137
PG	Phosphatidylglycerol	34
PI	Phosphatidylinositol	46
PS	Phosphatidylserine	15
Sphingolipids (sub-total = 94)		
Cer	Ceramide	25
Hex1Cer	Hexosyl ceramide	6
Hex2Cer	Dihexosyl ceramide	2
Hex3Cer	Trihexosyl ceramide	4
SM	Sphingomyelin	57
Neutral lipids (sub-total = 52)		
TG	Triglyceride	52
Fatty acyl and other lipids (sub-total = 2)		
AcCA	Acyl Carnitine	1
Co	Coenzyme Q	1
Total		570

The highest numbers of lipid species identified were in class PE (~24%), followed by class PC (~23%) and SM (~10%). The remaining classes contained < 10% of the total identified lipid species. All lipid species were identified in both SOL- and NIL-DMSCs (n = 7 per group). The complete list of all lipid species identified in SOL- and NIL-DMSCs is shown in **Appendix V – Supplementary table 5 - 16**.

4.4.2. Quantification of SOL- and NIL-DMSC lipidome

In this section, the amount of each lipid species in SOL- and NIL-DMSCs was quantified as described in **Section 3.4.3** and compared. For practical reasons, in lipid classes containing more than 10 lipid species, only lipid species with differences in lipid amounts between SOL- and NIL-DMSCs where p-value less than 0.10 are shown. The remaining lipid species of those lipid classes with the individual p-value analyses are presented in the **Appendix V – supplementary 5-16**.

4.4.2.1. Phospholipids

In the phospholipid category, there were 9 lipid classes identified i.e., CL, LPC, LPE, LPI, PC, PE, PG, PI, and PS. In class CL, there were 31 lipid species identified. However, there were no statistically significant differences observed in the amounts between SOL- and NIL-DMSCs ($p > 0.05$, **Figure 24**). The descriptive statistics of all lipid species in Class CL are presented in **Appendix V – Supplementary table 5**.

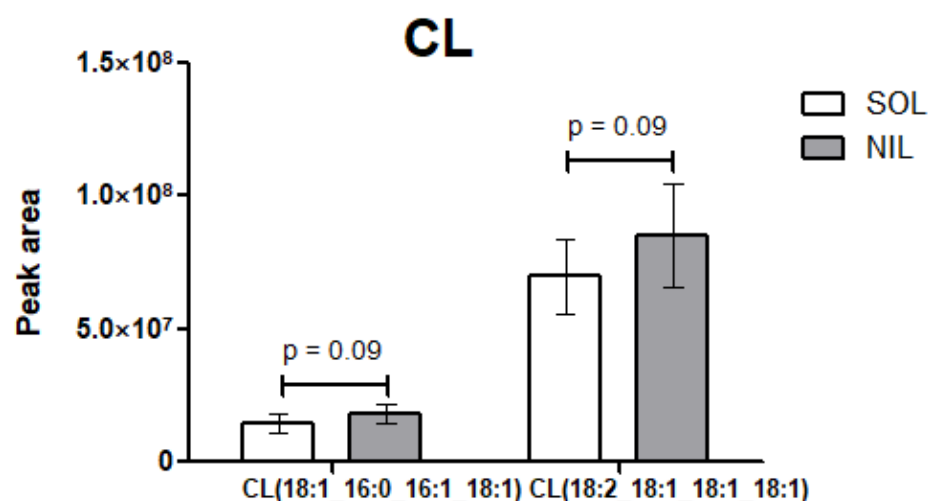


Figure 24. Relative quantification of class CL

Only lipid species with p-value < 0.10 between SOL-DMSCs and NIL-DMSCs are shown. The peak area was per 100 μ L sample. Data were presented as mean $\pm$ SEM. P-value was calculated using Mann-Whitney U-test.

In class LPC, LPE, and LPI, there were 20, 9, and 1 lipid species identified, respectively. However, there were no statistically significant differences observed in their amounts between SOL- and NIL-DMSCs ($p > 0.05$, **Figure 25**). The descriptive statistics of all lipid species in Class LPC, LPE, and LPI are presented in **Appendix V - Supplementary table 6**.

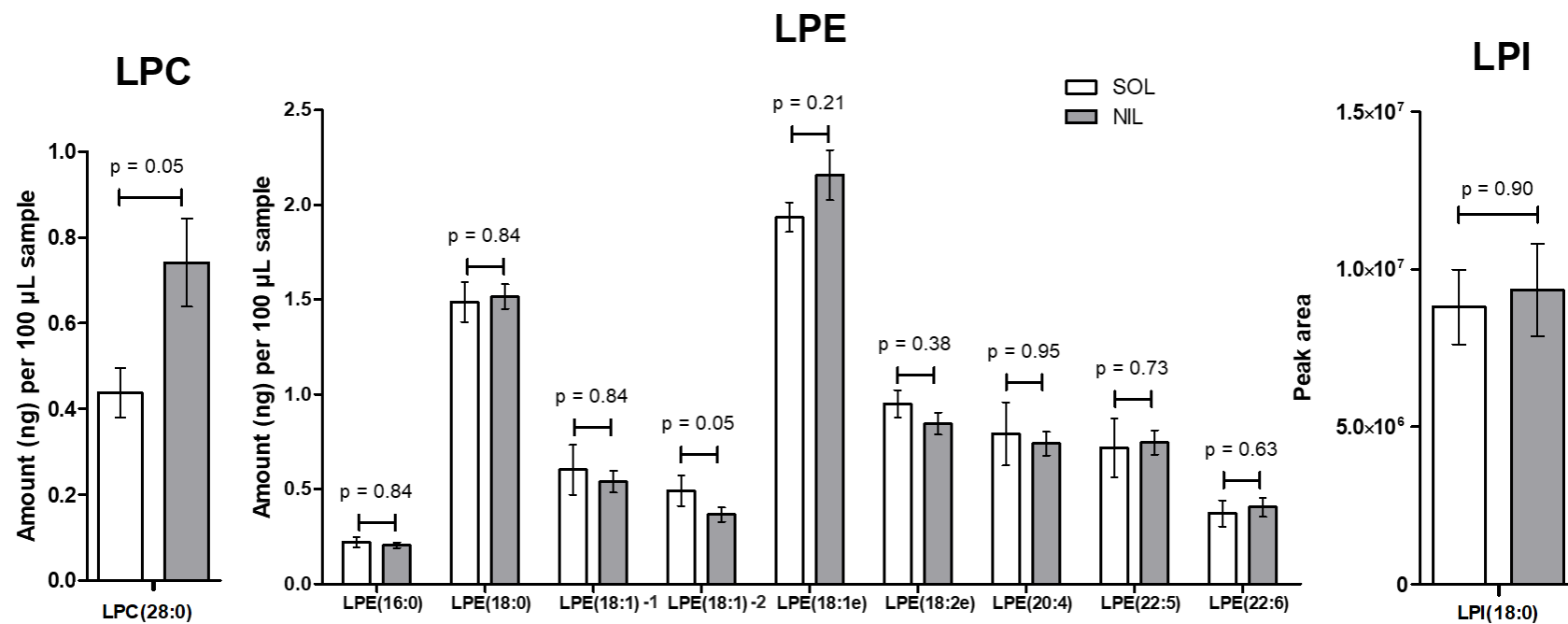


Figure 25. Quantification of Class LPC, LPE, and LPI

For class LPC, only lipid species with p -value < 0.10 between SOL-DMSCs and NIL-DMSCs is shown. For class LPI, the peak area was per 100 μ L sample. Data were presented as mean $\pm$ SEM. P -value was calculated using Mann-Whitney U-test.

In class PC, there were 129 lipid species identified. However, there were no statistically significant differences observed in their amounts between SOL- and NIL-DMSCs ($p > 0.05$, **Figure 26**). The descriptive statistics of all lipid species in Class PC are presented in **Appendix V - Supplementary table 7**.

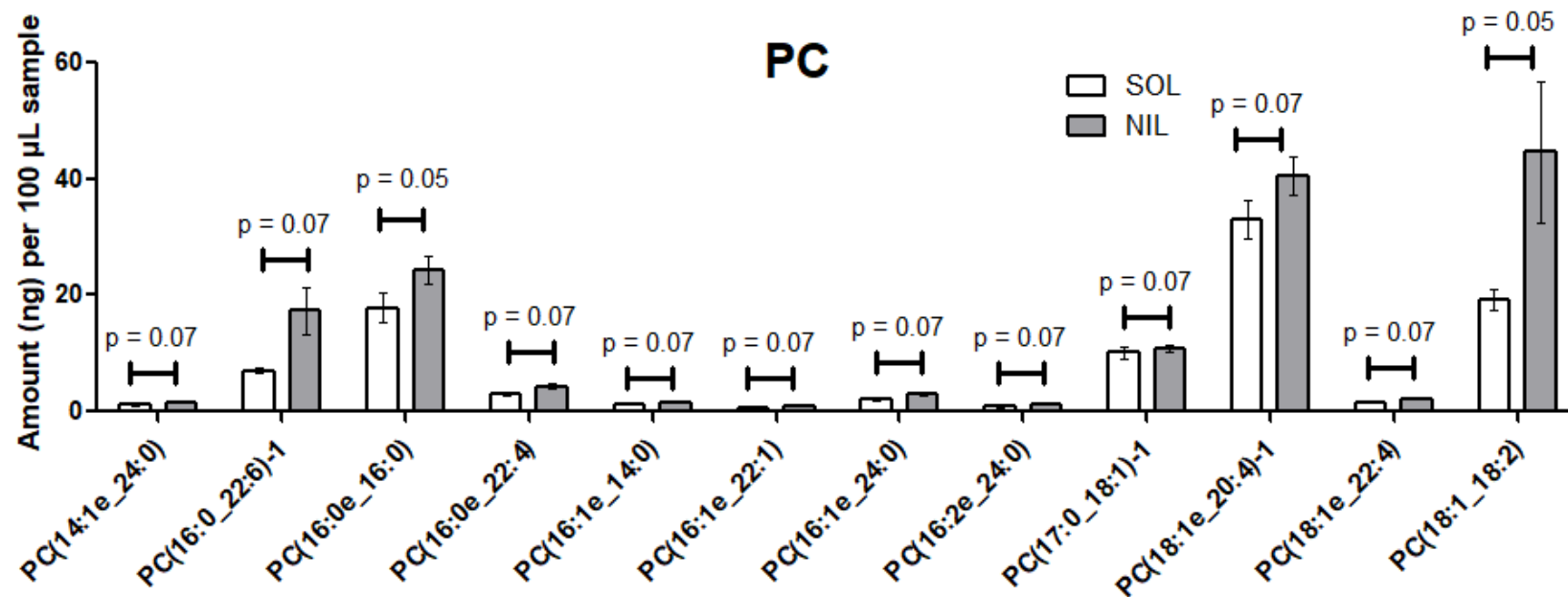


Figure 26. Absolute quantification of class PC

Only lipid species with p-value < 0.10 between SOL- and NIL-DMSCs are shown. Data were presented as mean $\pm$ SEM. P-value was calculated using Mann-Whitney U-test.

In class PE, 18 out of 137 lipid species identified showed statistically significant differences in the amounts of lipid species expressed between SOL- and NIL-DMSCs (Figure 27). These lipid species were PE(16:0p_22:5)*, PE(18:1p_22:4)*, PE(20:0p_22:4)*, PE(18:0p_22:4)*, PE(18:0_20:4)*, PE(18:1_20:4)-1*, PE(18:1_22:5)*, PE(18:1e_22:5)*, PE(18:2e_22:5)*, PE(18:3e_22:5)*, PE(20:1e_22:5)*, PE(20:1e_22:6)*, PE(32:1_20:4)*, PE(32:1_22:5)*, PE(16:0p_22:4)***, PE(18:2e_22:4)***, PE(20:1e_22:4)***, and PE(18:1_24:2)*** (*p < 0.05, **p < 0.01, ***p < 0.001). Overall, SOL-DMSCs had lower amounts of PE compared with NIL-DMSCs, except for PE(32:1_20:4) and PE(32:1_22:5), which showed the opposite trend.

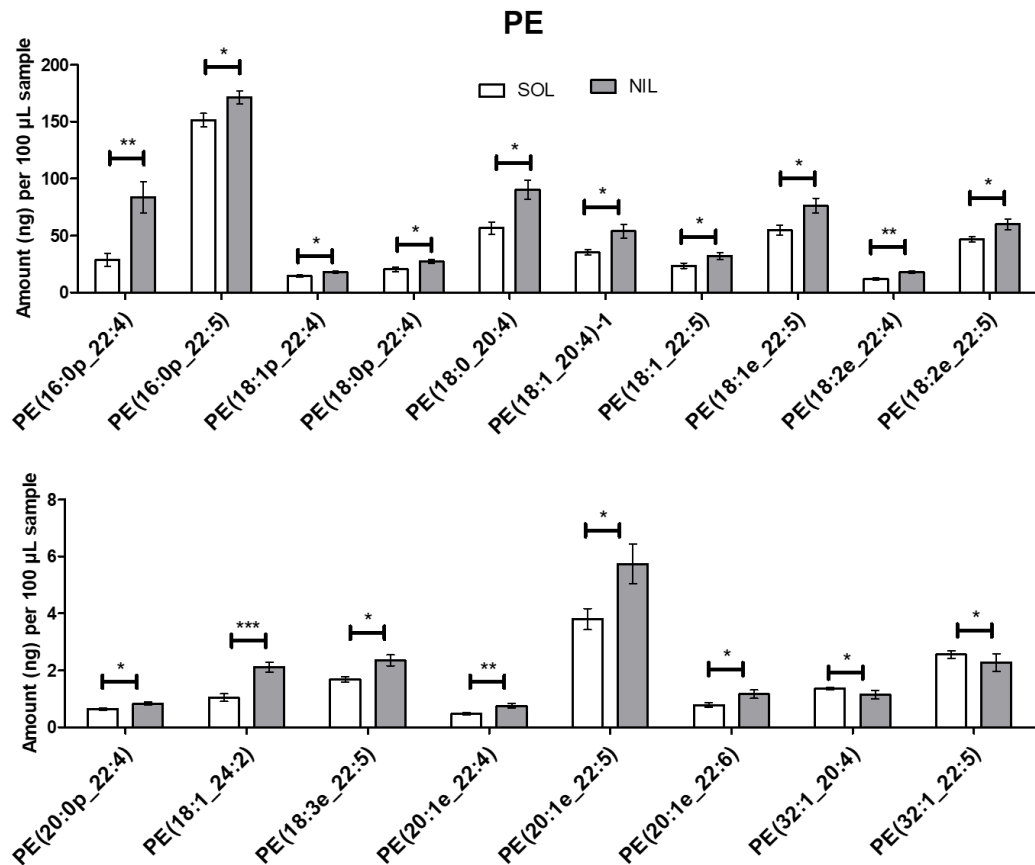


Figure 27. Absolute quantification of Class PE (p < 0.05)

Only lipid species with p-value < 0.05 between SOL- and NIL-DMSCs are shown. Data were presented as mean ± SEM. P-value was calculated using Mann-Whitney U-test. *p < 0.05, **p < 0.01, ***p < 0.001.

Other lipid species with the differences in lipid amounts between SOL- and NIL-DMSCs indicated $p < 0.10$ are shown in **Figure 28**. The descriptive statistics of all lipid species in Class PE are presented in **Appendix V - Supplementary table 8**.

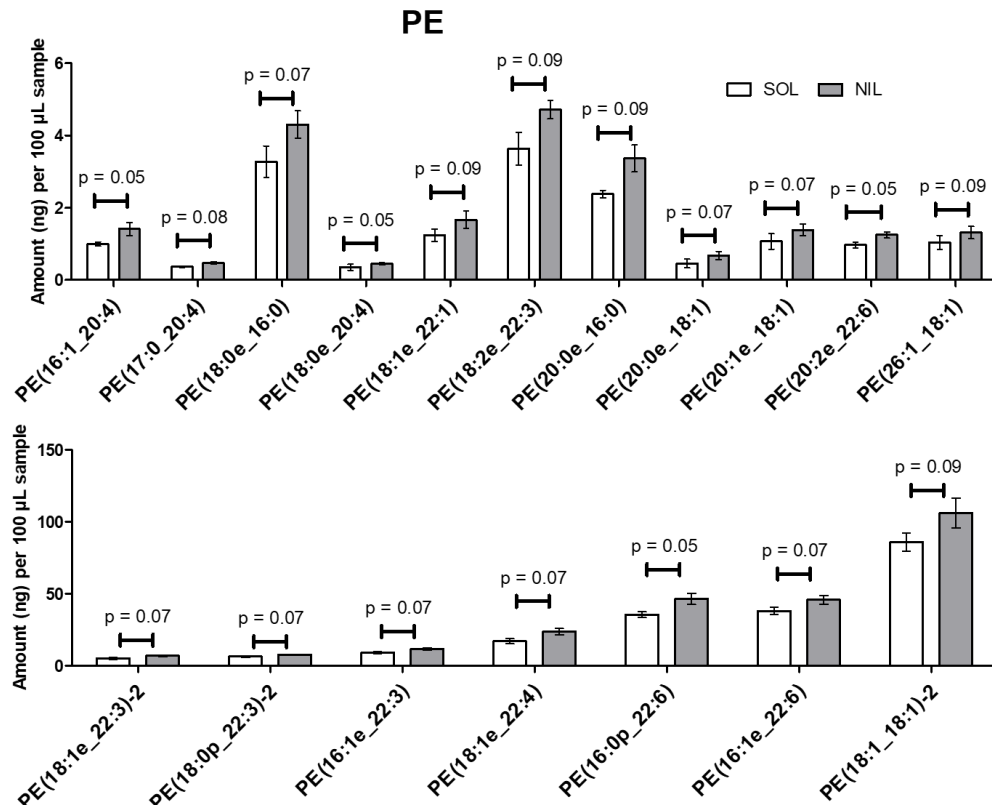


Figure 28. Absolute quantification of Class PE ($p < 0.10$)

Only lipid species with p -value < 0.10 between SOL- and NIL-DMSCs are shown. Data were presented as mean $\pm$ SEM. P -value was calculated using Mann-Whitney U-test.

In class PG, 6 out of 34 lipid species identified i.e., PG(16:0_20:4)*, PG(18:1_20:4)-1*, PG(18:0_18:1)***, PG(18:0_20:4)***, PG(18:1_20:4)-2**, and PG(20:3_22:5)-2*** showed statistically significant differences in the amounts of lipid species expressed between SOL- and NIL-DMSCs (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) (**Figure 29**). Overall, SOL-DMSCs had lower amounts of PG compared with NIL-DMSCs. The descriptive statistics of all lipid species in Class PG are presented in **Appendix V - Supplementary table 9**.

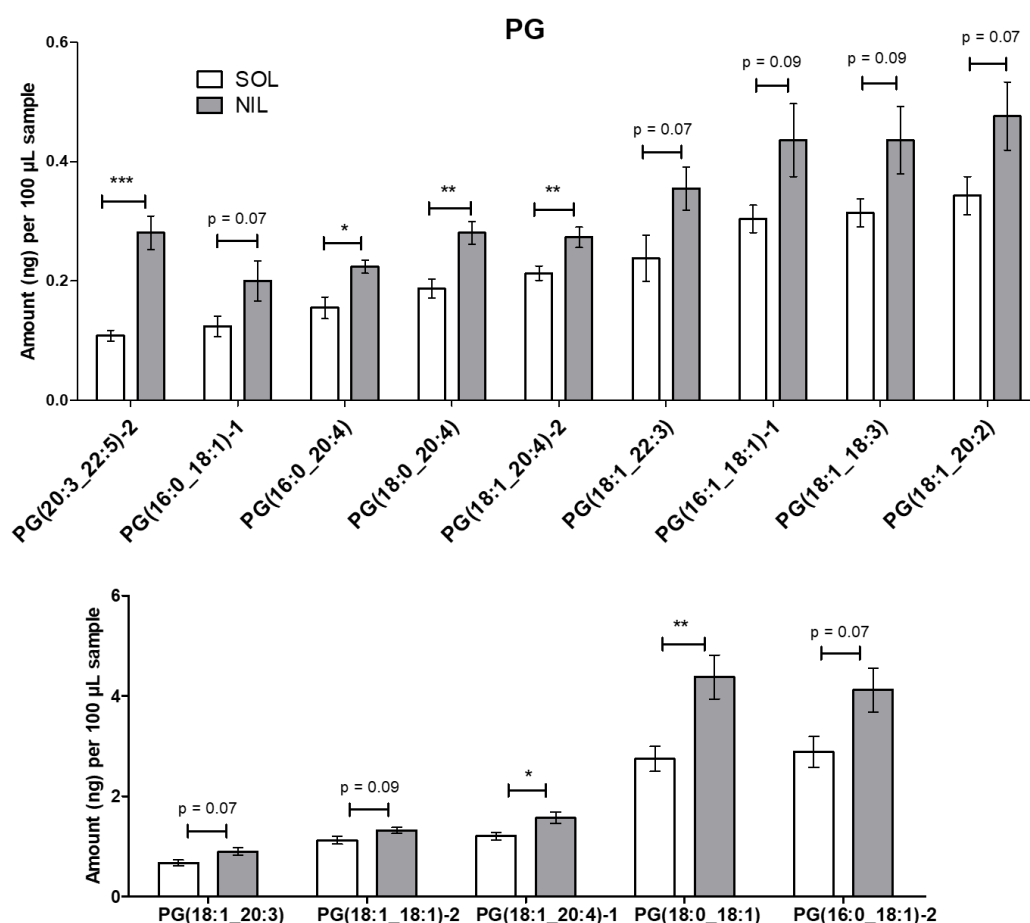


Figure 29. Absolute quantification of class PG

Only lipid species with p-value < 0.10 between SOL- and NIL-DMSCs are shown. Data were presented as mean $\pm$ SEM. P-value was calculated using Mann-Whitney U-test. *p < 0.05, **p < 0.01, ***p < 0.001.

In class PI, 10 out of 46 lipid species identified i.e., PI(16:0_20:4)*, PI(16:1_18:2)*, PI(17:0_20:4)-2*, PI(18:0_16:0)*, PI(18:0_17:0)*, PI(18:0e_22:4)*, PI(18:1_20:3)*, PI(18:1_22:4)*, PI(18:1e_22:4)-2*, and PI(18:1_14:0)** showed statistically significant differences in the amounts of lipid species expressed between SOL- and NIL-DMSCs (*p < 0.05, **p < 0.01) (**Figure 30**). Overall, SOL-DMSCs had lower amounts of PI compared with NIL-DMSCs. The descriptive statistics of all lipid species in Class PI are presented in **Appendix V - Supplementary table 10**.

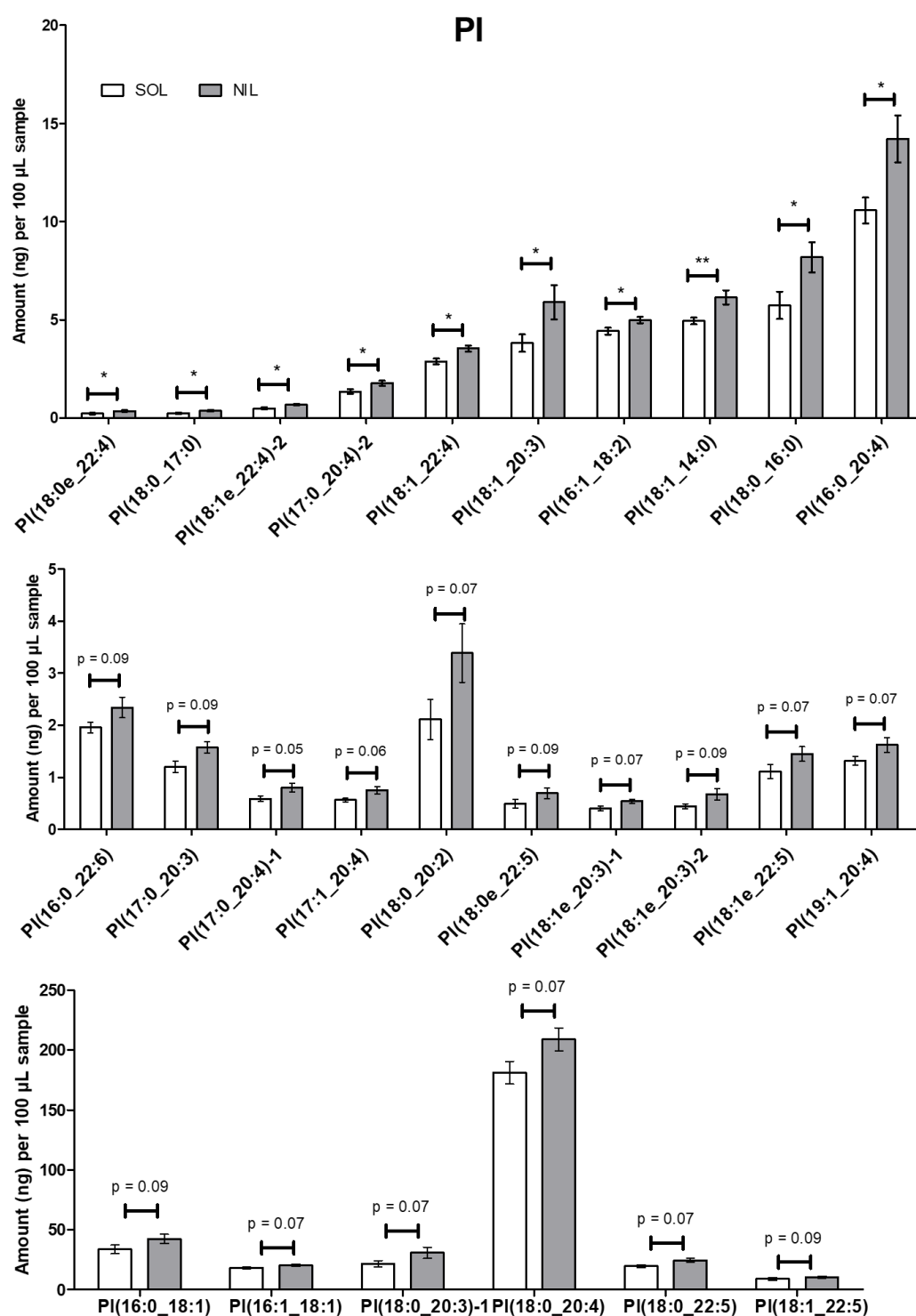


Figure 30. Absolute quantification of class PI

Only lipid species with p-value < 0.10 between SOL-DMSCs and NIL-DMSCs are shown. Data were presented as mean $\pm$ SEM. P-value was calculated using Mann-Whitney U-test. *p < 0.05, **p < 0.01.

In class PS, 5 out of 15 lipid species identified i.e. PS(18:0_20:3)*, PS(18:0_22:4)*, PS(18:0_20:2)**, PS(18:0_22:5)-2**, and PS(20:2e_18:1)** showed statistically significant differences in the amounts of lipid species expressed between SOL- and NIL-DMSCs (*p < 0.05, **p < 0.01) (**Figure 31**). Overall, SOL-DMSCs had lower amounts of PS compared with NIL-DMSCs. The descriptive statistics of all lipid species in Class PS are presented in **Appendix V - Supplementary table 11**.

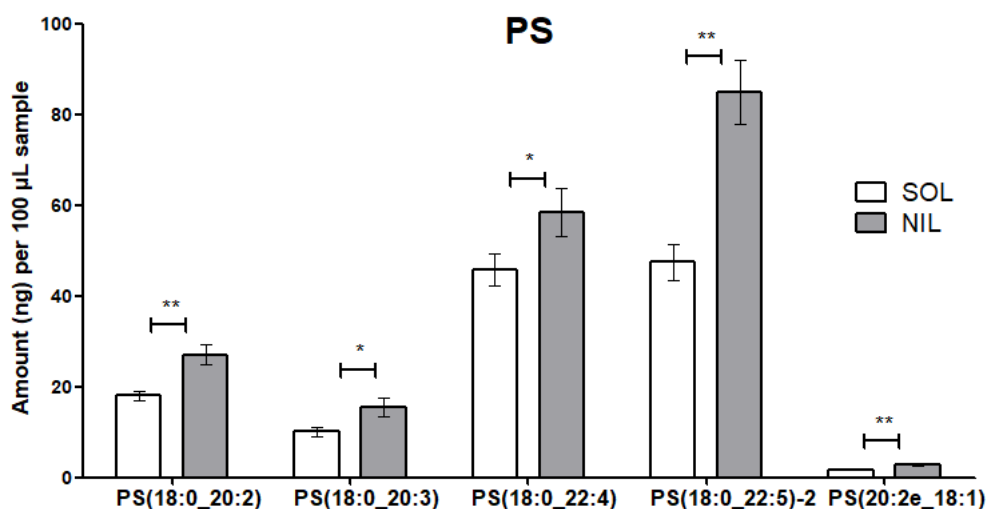


Figure 31. Absolute quantification of class PS

Only lipid species with p-value < 0.10 between SOL-DMSCs and NIL-DMSCs are shown. Data were presented as mean ± SEM. P-value was calculated using Mann-Whitney U-test. *p < 0.05, **p < 0.01.

4.4.2.2. Sphingolipids

In the sphingolipids category, there were 5 lipid classes identified i.e., Cer, Hex1Cer, Hex2Cer, Hex3Cer, and SM. In class Cer, 3 out of 25 lipid species identified i.e., Cer(d17:1_16:0)*, Cer(d18:1_14:0)*, and Cer(d18:1_16:0)** showed statistically significant differences in the amounts of lipid species expressed between SOL- and NIL-DMSCs (*p < 0.05, **p < 0.01) (**Figure 32**). Overall, SOL-DMSCs had lower amounts of Cer compared with NIL-DMSCs. The descriptive statistics of all lipid species in Class Cer are presented in **Appendix V - Supplementary table 12**.

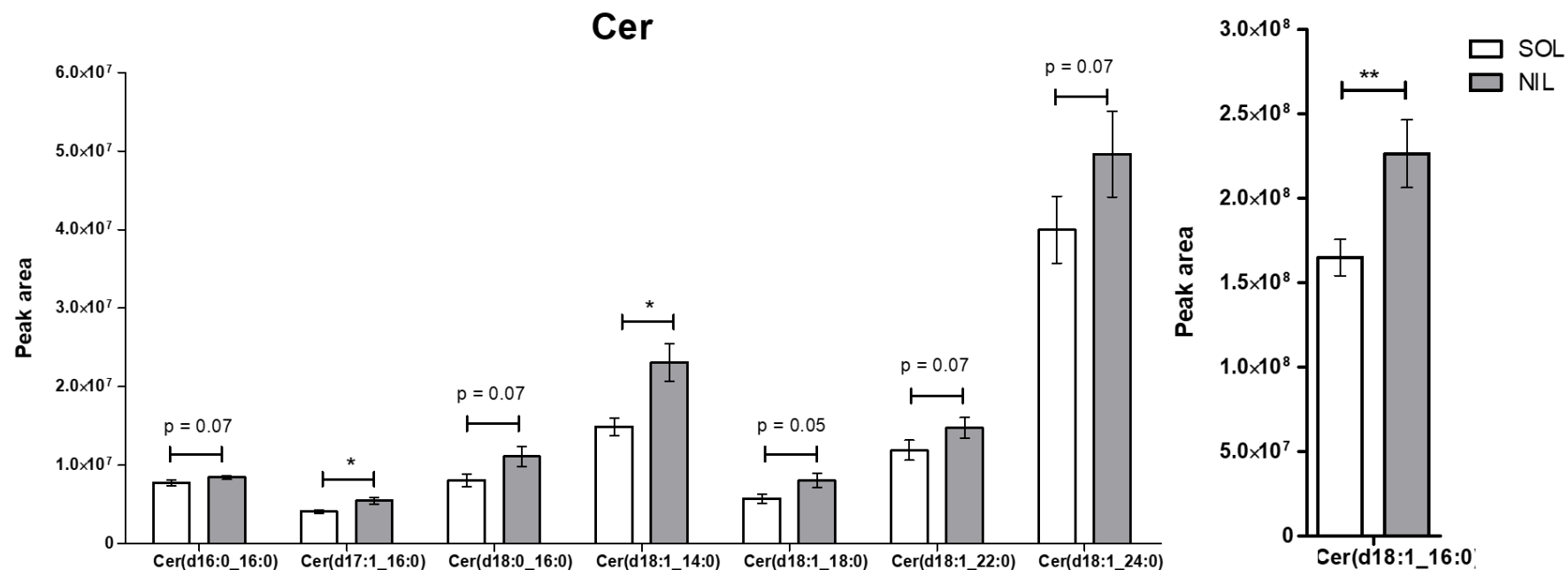


Figure 32. Relative quantification of class Cer

Only lipid species with p-value < 0.10 between SOL-DMSCs and NIL-DMSCs are shown. The peak area was per 100 μ L sample. Data were presented as mean $\pm$ SEM. P-value was calculated using Mann-Whitney U-test. *p < 0.05, **p < 0.01.

In class Hex1Cer, Hex2Cer, and Hex3Cer, there were 6, 2, and 4 lipid species identified, respectively. However, there were no statistically significant differences observed in their amounts between SOL- and NIL-DMSCs ($p > 0.05$, **Figure 33**). The descriptive statistics of all lipid species in Class Hex1Cer, Hex2Cer, and Hex3Cer are presented in **Appendix V - Supplementary table 13**.

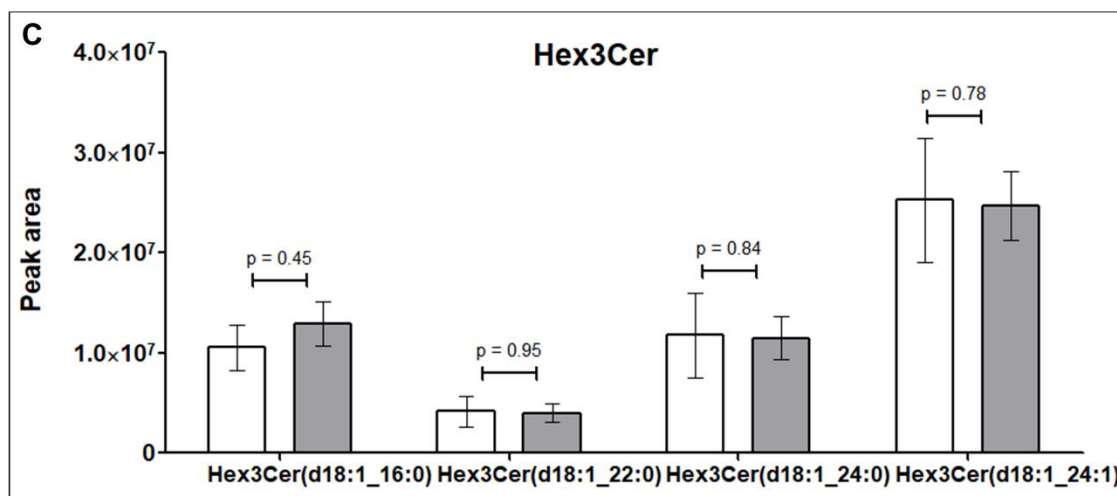
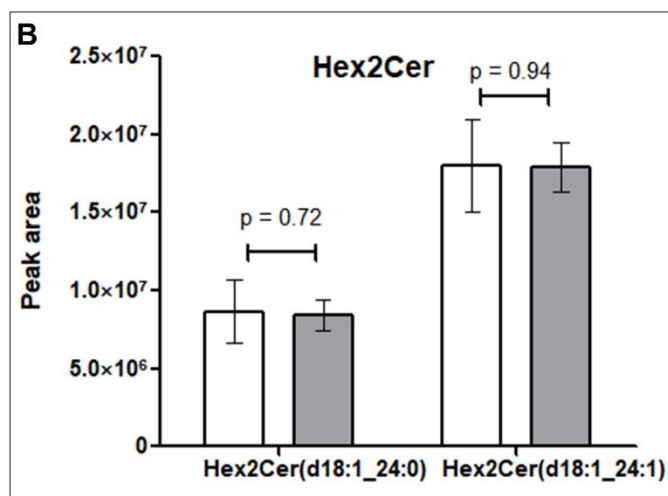
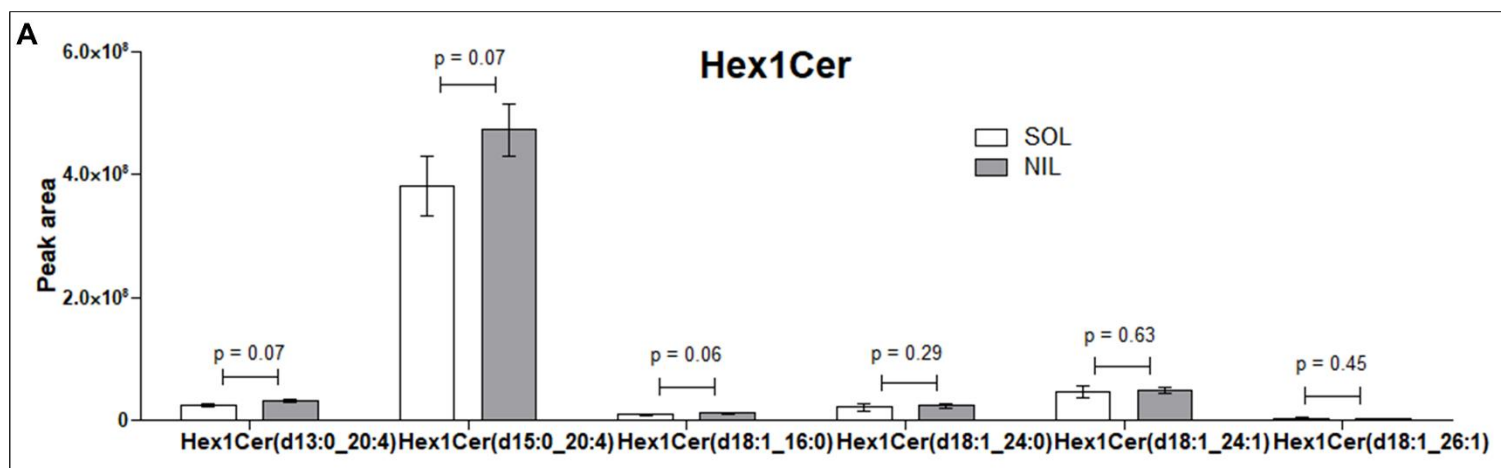


Figure 33. Relative quantification of class Hex1Cer, Hex2Cer, and Hex3Cer

The peak area was per 100 μ L sample. Data were presented as mean $\pm$ SEM. P-value was calculated using Mann-Whitney U-test.

In class SM, 2 out of 57 lipid species identified i.e., SM(d30:1) and SM(d32:1) showed statistically significant differences in the amounts of lipid species expressed between SOL- and NIL-DMSCs ($p < 0.05$) (**Figure 34**). Overall, SOL-DMSCs had lower amounts of SM compared with NIL-DMSCs. The descriptive statistics of all lipid species in Class SM are presented in **Appendix V - Supplementary table 14**.

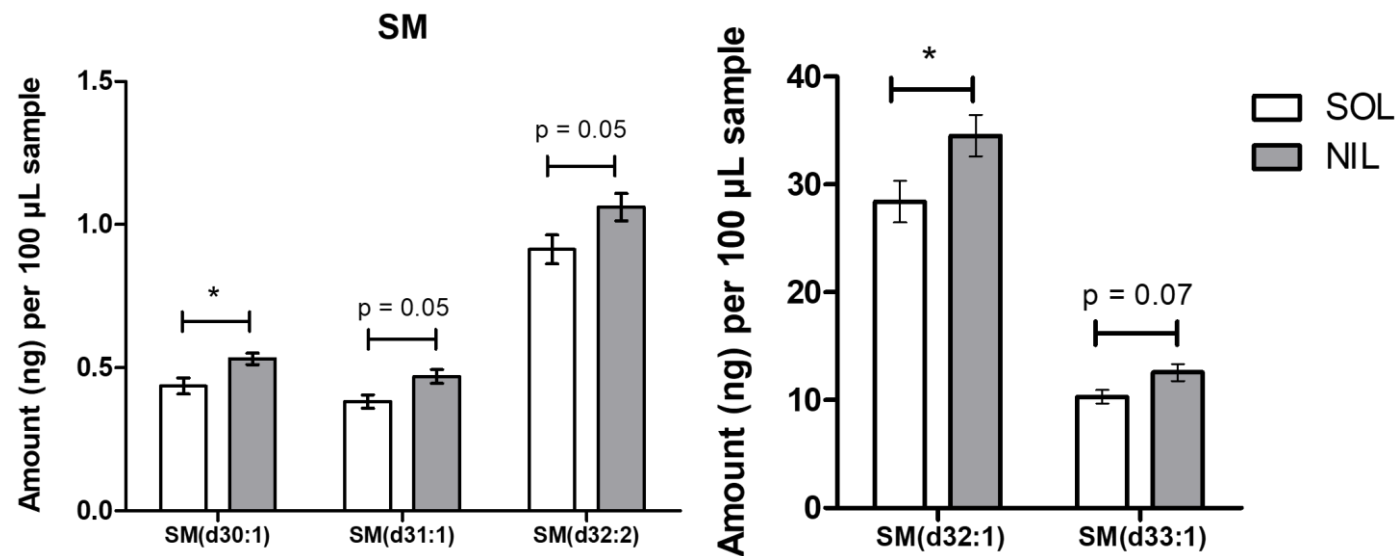


Figure 34. Absolute quantification of class SM

Only lipid species with p-value < 0.10 between SOL- and NIL-DMSCs are shown. Data were presented as mean $\pm$ SEM. P-value was calculated using Mann-Whitney U-test. *p < 0.05.

4.4.2.3. Neutral lipids

In the neutral lipid category, only one lipid class was identified; TG. In Class TG, 3 out of 52 lipid species i.e., TG(15:0_16:0_16:0)*, TG(16:0_14:0_16:0)**, and TG(16:0_16:0_16:0)* showed statistically significant differences in the amounts of lipid species expressed between SOL- and NIL-DMSCs (* $p < 0.05$, ** $p < 0.01$) (Figure 35). Overall, SOL-DMSCs had lower amounts of TG compared with NIL-DMSCs. The descriptive statistics of all lipid species in Class TG are presented in Appendix V - Supplementary table 15.

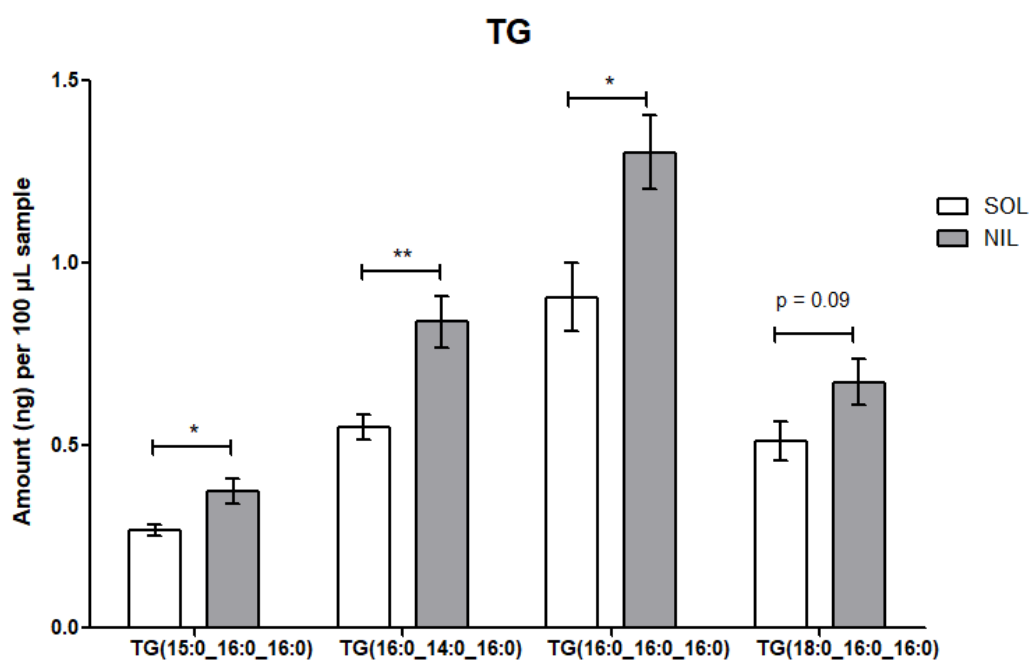


Figure 35. Absolute quantification of class TG

Only lipid species with p-value < 0.10 between SOL- and NIL-DMSCs are shown. Data were presented as mean $\pm$ SEM. P-value was calculated using Mann-Whitney U-test. * $p < 0.05$, ** $p < 0.01$.

4.4.2.4. Fatty acyl and other lipids

Only one fatty acyl lipid, AcCa(16:0), was identified. SOL-DMSCs had a significantly lower amount of AcCa(16:0) compared with NIL-DMSCs ($p < 0.01$,

Figure 36A). In addition, an unclassified lipid molecule, Co(Q10), was identified, but the difference in its amount between SOL- and NIL-DMSCs was not statistically significant ($p > 0.05$, **Figure 36B**). The descriptive statistics of AcCa(16:0) and Co(Q10) are presented in **Appendix V - Supplementary table 16**.

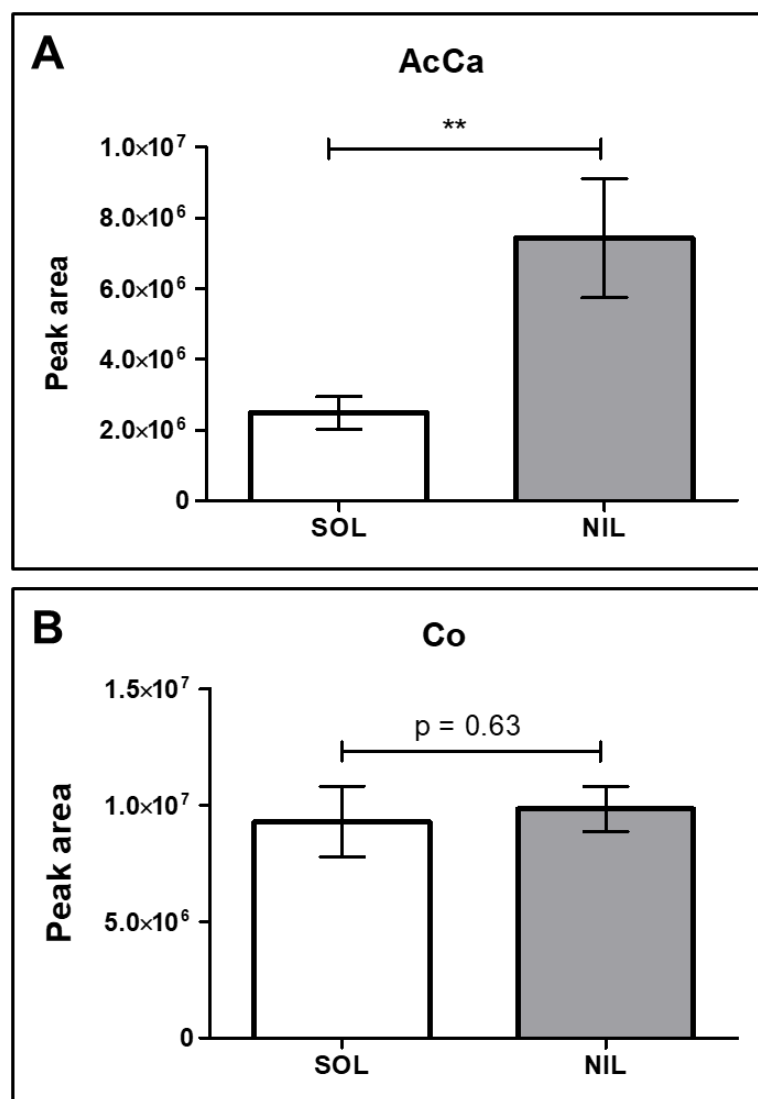


Figure 36. Relative quantification of Class AcCa and Co

Relative quantification of (A) AcCa(16:0) and (B) Co(Q10). The peak area was per 100 μ L sample. Data were presented as mean $\pm$ SEM. P-value was calculated using Mann-Whitney U-test. **p < 0.01.

5. DISCUSSION

5.1. Sample collection

5.1.1. Patient characteristics

Patients aged ≥ 35 years (advanced maternal age; AMA) with BMI ≥ 30 kg/m² (obese) were omitted because studies showed that AMA (Lean, Derricott, Jones, & Heazell, 2017; Lean, Heazell, Dilworth, Mills, & Jones, 2017) and obesity (Altmae et al., 2017; Ditchfield et al., 2015) adversely affect the function of placentae and placental stem cells (Alrefaei, Ayuob, Ali, & Al-Karim, 2015; Boyle et al., 2016). Several medical conditions as described in **Section 3.1** were also excluded because these conditions affected placental function and/or interfered with functions assessed in this study. Specifically, aspirin consumption was reported to enhance the attachment and reduce pro-inflammatory cytokine production of preeclampsia-DMSCs (Khanabdali et al., 2018) as well as inhibited MSC growth (Wang et al., 2006). PCOS (Al-Musawy, Al-Saimary, & Flaifil, 2018), hyperthyroidism (Antunes, Gagnon, Langille, & Sorisky, 2008), and gastric medicines (Biswas, Benedict, Lynch, & LeVine, 2012) were reported to affect IL-6 production, while IL-6 was reported to affect MSC proliferation, migration, and apoptosis (Pricola, Kuhn, Haleem-Smith, Song, & Tuan, 2009; Rattigan, Hsu, Mishra, Glod, & Banerjee, 2010). Breech birth (Kaya, 2000) and alcohol consumption (Kolesnikova et al., 2015) influence the level of lipid peroxidation.

5.1.2. Gestational age

Placentae were collected from full-term pregnancies because neonates delivered at this period are the healthiest and have fewest morbidities (The American College

of Obstetricians and Gynecologists Committee, 2013). The use of full-term placentae, therefore, minimised possible pathology-related confounding factors. Nevertheless, it is worth noting that samples from full-term NIL patients may be minutes/hours/days from showing the typical signs of spontaneous labour. Therefore, although these patients did not show any clinical signs of labour at the time of sample collection, labour-associated changes may have been taking place at the cellular or molecular level in the decidua since labour is a progressive event. Nonetheless, the changes in NIL patients were not prominent enough to stimulate labour phenotypes, hence were deemed appropriate for use as controls for SOL samples.

5.2. Isolation and characterisation of the primary cells

The isolation method used in this study has been used in numerous publications (Khanabdali et al., 2018; Kusuma et al., 2017; Kusuma et al., 2015; Qin et al., 2016; Shakouri-Motlagh, O'Connor, Kalionis, & Heath, 2019). Both in those publications and in this MPhil study, the effectiveness of this method to isolate DMSCs and remove possible contamination from other types of placental cells (e.g., trophoblast and endothelial cells) was validated through the characterisation of the isolated primary cells. The isolated primary cells were consistent with the ISCT criteria for defining MSCs i.e., adhered to the plastic cultureware (**Figure 8**), $\geq 95\%$ cells positively expressed MSC-specific antigens (e.g., CD90, CD166, CD73, CD44, and CD105) and $\leq 5\%$ cells positively expressed hematopoietic markers (e.g., CD45, HLA-DR, and CD19, **Figure 9**). The isolated primary cells were also able to differentiate into mesenchymal lineages [e.g., adipogenic (**Figure 10**) and

osteogenic (**Figure 11**)] and formed colonies when seeded at low density (**Figure 12**), which was a typical MSC characteristic showing MSC self-renewal capability. In addition, the cells were confirmed to be of maternal origin (**Figure 13**). Upon initial plating (P0), there were low numbers of other types of placental-derived cells present that survived the rigorous mechanical and enzymatic digestion. These cells remained in culture and contributed to the morphological diversity of the primary cells at P0. Nevertheless, after several washings and a medium change, the non-adherent cells were all washed out of the culture and after one passage (P1), the morphology of the adhered primary cells was uniform under the microscope. All primary cells (P1) showed the typical fibroblastic morphology of MSCs (**Figure 8**). The characterisation was performed on these morphologically uniform P1 primary cells. Specifically, for FISH analyses, since these analyses are not informative for the origin of the primary cells from placentae of female newborn, the assay was not performed on these cells. The maternal origin of primary cells from female newborn placentae are inferred based on the identical application of the isolation methods to all placental samples of both male and female newborns. In summary, since the primary cells were consistent with the criteria for MSCs, and were maternal in origin, they were subsequently referred to as decidual MSCs (DMSCs).

5.3. Labour-associated changes of DMSC functions

The following sections discuss several functional changes of SOL-DMSCs detected in this study within the framework of the association between ageing, parturition, and stem cells (subsequently referred as the “ageing-parturition-stem cell” axis). The general hypothesis of this section is presented in **Figure 37** below.

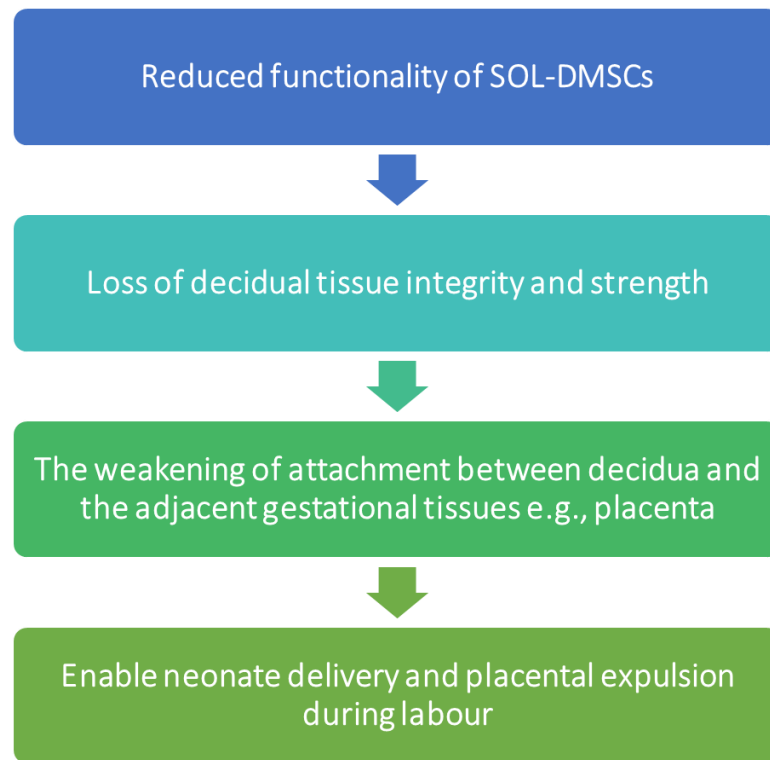


Figure 37. General hypotheses of functional changes in SOL-DMSCs at term

5.3.1. Proliferation capacity of SOL-DMSCs

Stem cell proliferation is essential to maintain the stem cell pool. In this study, SOL-DMSCs showed a reduced proliferation level, despite a slightly higher initial attachment level, compared with NIL-DMSCs. However, the differences for both cell attachment and proliferation (**Figure 14 - 15**) were not statistically significant ($p > 0.05$). In another study of the proliferation of SOL- and NIL-DMSCs from placentae of early-term patients (37-38 weeks of gestation, $n = 10$ per group) performed by Wijaya, Khanabdali, Georgiou, Kokkinos, Brennecke, & Kalionis (unpublished data, **Appendix VII – Figure 1**), this trend of reduced proliferation level of SOL-DMSCs was confirmed. A statistically significant lower level of proliferation was observed in early-term SOL-DMSCs compared with early-term NIL-DMSCs ($p < 0.05$). The attachment level was still not significantly different

between early-term SOL-DMSCs and early-term NIL-DMSCs ($p > 0.05$). The statistically significant differences of the proliferation levels were observed in early-term samples, but not in full-term samples. This may be because the early-term patients were further from the due date, when compared with the full-term patients. Consequently, the functional differences between early-term SOL- and NIL-DMSCs were more obvious compared with full-term SOL- and NIL-DMSCs. Higher sample size used in the early-term study may also contribute to the higher statistical power.

Numerous studies report the association between reduced proliferation level with MSC ageing (Choudhery, Badowski, Muise, Pierce, & Harris, 2014; Ksiazek, 2009; Maredziak, Marycz, Tomaszewski, Kornicka, & Henry, 2016; Mendes et al., 2002; Roobrouck, Ulloa-Montoya, & Verfaillie, 2008; Stenderup, Justesen, Clausen, & Kassem, 2003; Zhou et al., 2008). Nevertheless, thus far no study in the literature has directly associated this phenomenon with natural parturition. Studies of the functional decline of DMSCs in the pregnancy pathology of preeclampsia represent the only current evidence that associates stem cell exhaustion (i.e., ageing) with parturition. These studies report reduced DMSC proliferation in preeclampsia samples and this was attributed to the overexpression of miR-16 in preeclampsia-DMSCs (Wang et al., 2012). Accelerated placental ageing was also detected in women with preeclampsia (Cox & Redman, 2017; Manna, McCarthy, & McCarthy, 2019; Mayne et al., 2017), which suggests that functional changes in preeclampsia-DMSCs are associated with ageing. Since the same proliferation trend was observed in SOL-DMSCs, it is possible that this particular functional change of SOL-DMSCs also results from the ageing process to facilitate labour in the decidua at term.

5.3.2. *Migration capacity and pro-inflammatory secretion of SOL-DMSCs*

In this study, SOL-DMSCs showed a significant increase in migration compared with NIL-DMSCs ($p < 0.05$; **Figure 16 - 18**). Increased migration of SOL-DMSCs may be associated with the immune regulatory roles of MSCs during labour. Labour features many inflammatory aspects, particularly in the decidua, as the resident and infiltrating immune cells in this gestational tissue actively secrete a variety of pro-inflammatory cytokines at term (Gomez-Lopez et al., 2014; Norwitz et al., 2015). On the other hand, MSCs display pro-inflammatory phenotypes in highly inflammatory conditions (Le Blanc & Davies, 2015). In responses to inflammatory stimuli, MSCs migrate to the site of infection or injury and stimulate immune induction (Waterman, Tomchuck, Henkle, & Betancourt, 2010). Through the secretion of various pro-inflammatory cytokines, MSCs then increase the recruitment and functions (i.e., pro-inflammatory state activation and cell migration) of various immune cells, such as neutrophils and macrophages (Ulivi, Tasso, Cancedda, & Descalzi, 2014). In the case of tissue injury, this response is required to augment the initial inflammatory responses, which is the key element in activating wound healing responses (Ulivi et al., 2014). With regard to parturition, increased migration of leukocytes e.g., neutrophils and macrophages, to the labouring decidua was observed at term and this was suggested to contribute to the activation of decidua during the initiation of labour (Hamilton et al., 2012). Based on these data, it is possible that during labour, DMSCs also actively migrate to the inflammatory site in decidua to stimulate immune induction by secreting higher levels of pro-inflammatory cytokines. These cytokines then recruit more immune cells [e.g., neutrophils and macrophages] to amplify the initial inflammatory

responses required to initiate labour at term. This hypothesis is supported by the finding that SOL-DMSCs secreted a significantly higher level of IL-6 compared with NIL-DMSCs ($p < 0.05$; **Figure 22**). IL-6, together with IL-8, are principal pro-inflammatory cytokines reported in decidua during labour (Osman et al., 2003). Nevertheless, IL-8 production was not significantly changed in SOL-DMSCs compared with NIL-DMSCs ($p > 0.05$; **Figure 23**), which suggests that DMSCs may not be the major sources of decidual IL-8 during labour at term. Since increased secretion of pro-inflammatory cytokines e.g., IL-6 and IL-8, was also observed in MSCs from aged donors accompanied with the presence of MSC senescence (Gnani et al., 2019), it is possible that increased secretion of pro-inflammatory cytokines in SOL-DMSCs also resulted from ageing mechanisms, e.g., senescence and SASPs as described in **Section 1.5.2**.

5.3.3. *Cell death (necrosis and apoptosis) profile of SOL-DMSCs*

A decline in cell viability, either in the form of apoptosis or necrosis, is associated with MSC ageing (Asumda & Chase, 2011; Beane, Fonseca, Cooper, Koren, & Darling, 2014; Choudhery et al., 2014) and parturition in the decidua (Gu et al., 1994; Larsen, 1963). In this study, SOL-DMSCs had a significantly higher percentage of necrotic cells compared with NIL-DMSCs ($p < 0.05$, **Figure 19**). SOL-DMSCs also had a higher percentage of apoptotic cells (early, late, and total) and a lower percentage of healthy/viable cells compared with NIL-DMSCs. However, the differences were not statistically significant ($p > 0.05$, **Figure 19**). These data of the lower percentages of viable/healthy SOL-DMSCs are consistent with the findings of reduced SOL-DMSC survivability observed in the xCELLigence proliferation assay (**Figure 14**).

The overall decline of SOL-DMSC viability may result from the accumulation of cellular damages, resulting from higher exposure of SOL-DMSCs to stress stimuli (e.g., oxidative stress, inflammation) compared with NIL-DMSCs. Since these phenomena are commonly observed in aged organisms (Tower, 2015) and some types of aged stem cells (Koyuncu, Irmak, Saez, & Vilchez, 2015; Sethe, Scutt, & Stolzing, 2006), it can be inferred that these particular changes in SOL-DMSC viability may result from the ageing process during labour at term.

Furthermore, based on the findings in this study, it is likely that necrosis was the predominant type of cell death in SOL-DMSCs. This postulate is supported by a study on labouring fetal membranes (Menon, Mesiano, & Taylor, 2017). As parturition approaches, fetal membranes undergo senescence and secrete SASPs, which propagate the inflammatory signals for parturition by triggering the release of damage-associated molecular pattern molecules (DAMPs), e.g., high mobility group box (HMGB)-1, Hsp-70, uric acid, and fragments of cell-free DNA. The adjacent cells, e.g. decidual cells, recognise these DAMPs through pattern recognition receptors located in their plasma membranes or endosomes, which leads to higher inflammatory responses, the activation of complement system, as well as cell necrosis (Menon et al., 2017).

In addition, the inflammatory responses only increase during necrosis, not apoptosis (Wyllie, Kerr, & Currie, 1980). The necrosis of SOL-DMSCs, thereby, may contribute to the increase of pro-inflammatory responses during labour, as shown through a significant increase of IL-6 production in SOL-DMSCs ($p < 0.05$, **Figure 22**). A study on the age-dependent cell death of fibroblast cells also demonstrated reduced capacity of fibroblasts to induce apoptosis and an increasing shift from

apoptosis to necrosis with age, together with an increase in the level of necrotic inflammation in aged fibroblast cells (Miyoshi, Oubrahim, Chock, & Stadtman, 2006). These findings suggest a possible association between ageing, parturition, and cell death, particularly necrosis, of DMSCs at term.

5.3.4. *Oxidative stress profile of SOL-DMSCs*

As described in **Section 1.4** and **Section 1.5.2.**, increased levels of oxidative stress and decreased levels of antioxidant systems are associated with both ageing and parturition. To investigate these phenomena in the “ageing-parturition-stem cell” axis, this study assessed and compared the level of lipid peroxidation and the percentage of DMSCs expressing high levels of ALDH (so called ALDH^{br} DMSCs) between SOL- and NIL-DMSCs. Lipid peroxidation and ALDH expression were determined as the representative components of oxidative stress and antioxidant system in DMSCs based on a study of preeclampsia-DMSCs by Kusuma et al. (2017), which, as described in **Section 1.6.2.**, is indirect evidence of DMSC ageing during pregnancy. In preeclampsia-DMSCs, a significant increase in the level of lipid peroxidation and a significant decrease in the percentage of ALDH^{br} cells were reported (Kusuma et al., 2017).

In the current study, the lipid peroxidation level of SOL-DMSCs, as reflected through the level of MDA production, also significantly increased compared with NIL-DMSCs ($p < 0.05$, **Figure 21**). The percentage of ALDH^{br} SOL-DMSCs also decreased compared with the percentage of ALDH^{br} NIL-DMSCs. However, the differences were not statistically significant ($p > 0.05$, **Figure 20**). In support of these data, a study by Sugino et al. (2000) showed higher levels of lipid peroxidation, coupled with reduced levels of superoxide dismutase (SOD), in decidual tissue from

patients undergoing spontaneous abortion compared with those at the early stage of normal pregnancies. In the Sugino et al. (2000) study, these patterns were accompanied with a significant increase of PG-F_{2α} level, suggesting that in spontaneous abortion, parturition was accelerated due to early induction of PG-F_{2α} synthesis as the response to high accumulation of ROS and their end products, i.e. lipid peroxide in the decidua (Hemler & Lands, 1980). This mechanism may also occur in DMSCs during normal term parturition, but at a rate and a level that is sufficient to initiate parturition without evoking any pathological effects. With regard to the antioxidant system, since SOL-DMSCs did not show any significant differences in the percentage of ALDH^{br} cells compared with NIL-DMSCs ($p > 0.05$), ALDH may not be the major antioxidant enzymes used by DMSCs during labour at term. The ALDH family of enzymes detoxifies various aldehyde substrates by oxidizing them into their carboxylic acid forms (Vasiliou & Nebert, 2005). Other antioxidant enzymes, e.g., SOD, catalase, and peroxidase, which work by different mechanisms and target different types of ROS, may contribute more to the labour process in DMSCs. Nonetheless, further studies are required to fully understand the oxidative stress response of SOL-DMSCs.

5.4. Mass spectrometry-based lipid analyses of SOL- and NIL-DMSCs

In this study, SOL- and NIL-DMSCs expressed the same lipid species (a total of 570 lipid species). Most lipid species identified were phospholipids (~ 74%) and sphingolipids (~17%). The highest numbers of lipid species identified were in class PE (~24%), followed by class PC (~23%) and SM (~10%). The percentages were calculated per total count of lipid species identified. This was expected since

phospholipids and sphingolipids are the major lipid components of plasma membrane. Phospholipids are the building blocks of the lipid bilayer, while sphingolipids are abundantly found in the lipid rafts of the plasma membrane of animal cells, which accommodate the transportation and facilitate the function of various specific membrane proteins (Alberts et al., 2002). PC, PE, and PS are the types of phospholipids predominantly found in the plasma membrane. Together with SM, these lipids contribute to more than half the mass of lipid in most plasma membranes (Alberts et al., 2002).

Generally, the plasma membrane serves as the protective layer for the cells. The plasma membrane also plays an essential role in mediating various biological signals via several signalling pathways e.g., G-protein activators, second messengers, and nuclear receptor activators (Heldin, Lu, Evans, & Gutkind, 2016). Therefore, given that lipid are the major constituent of most animal cell membranes (~50% of the total mass), changes in lipid profile (i.e., quantities) may greatly affect numerous cellular signalling pathways. In this study, SOL- and NIL-DMSCs significantly differed in the lipid quantities of various specific lipid species, specifically in the category of phospholipids [class PE (~13%), PG (~18%), PI (22%), and PS (~33%)], sphingolipids [class Cer (~12%) and SM (~4%)], neutral lipids [class TG (~6%)], and fatty acyl (class AcCa). The percentages were calculated per total count of lipid species identified in particular lipid class. Compared with NIL-DMSCs, SOL-DMSCs had lower lipid quantities in almost all identified lipid species, except for PE(32:1_20:4) and PE(32:1_22:5), which showed the opposite trend. Based on these findings, it can, therefore, be inferred that changes

in lipid quantities of SOL-DMSCs may be associated with various molecular and signalling pathway changes in the decidual tissue in preparation for labour at term. Major changes in the lipid profile (i.e., increased or decreased quantities of various lipid species) are reported in various parturition-related studies. Increased lipolytic activity in maternal adipose tissue, which is derived by adipogenic differentiation of MSCs, was observed during parturition and found to contribute to increased maternal plasma lipid level (Herrera & Ortega-Senovilla, 2010). Increased levels of maternal plasma lipid is associated with preterm birth, hence there is a potential role for lipid profile alterations in the initiation of parturition (Moayeri et al., 2017; Vrijkotte et al., 2012). In cord blood, the total quantities of TG and cholesterol increase during pregnancy, decrease during labour, and increase again after delivery (Jo, Cheon, Ahn, Kim, & Lee, 2017).

A pilot study by Birchenall, Welsh, and López Bernal (2019) evaluated and compared the metabolite changes in the maternal and fetal plasma following SOL + vaginal delivery at term in humans (with NIL + elective C-section as the control group). There were significant changes in the metabolite quantities between SOL and NIL samples, with most changes were found in the lipid categories, particularly in class Cer and AcCa. Birchenall et al. (2019) also discussed the high similarity in metabolite changes between labour and strenuous exercise and suggested that the metabolism process of various macromolecules, such as lipids, may change in order to provide the energy required for these high energy consumption activities (i.e., labour and exercise). Birchenall et al. (2019) also suggested that the energy required for labour was very likely to originate from the maternal tissue (e.g., maternal skeletal muscle, which is derived from osteogenic differentiation of MSCs) since

the fetus/neonate is not thought to use its skeletal muscle during labour and delivery. Based on these observations, it is possible that the lipid components of SOL-DMSCs are catabolised through higher lipolytic activities during labour to provide at least in part, the energy source that fuels the labour process. Interestingly, similar to parturition, major changes in lipid quantities are reported in several studies of stem cell ageing (Ito & Suda, 2014) and MSC ageing (Kilpinen et al., 2013) with particular changes in the glycerophospholipids (PC, PS, PE, and PI) profile. These studies support the notion that changes in lipid quantities of SOL-DMSCs may also result from the ageing process during term labour. The general hypothesis for this association between lipid profile changes in SOL-DMSCs and labour is presented in **Figure 38** below.

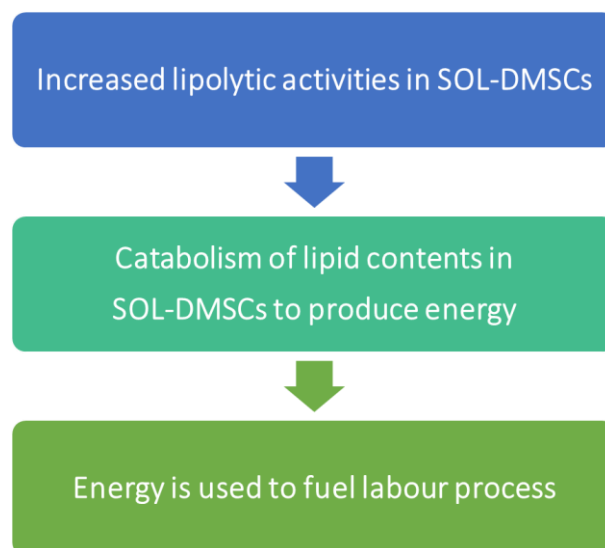


Figure 38. General hypothesis of the association between lipid profile changes in SOL-DMSCs and labour

5.5. Changes in DMSC function and lipid profile: a cause or consequence of labour?

In this study, there was evidence of significant ageing-related differences in various important functions (i.e., migration, IL-6 production, necrosis, lipid peroxidation)

and lipid profiles between SOL- and NIL-DMSCs, which suggests that active, spontaneous labour is associated with changes in the properties and behaviours of stem cells at full-term parturition. Some of these molecular and functional changes may be the consequence of labour itself. However, it is also possible that these changes trigger molecular pathways to cause SOL at term. The study design of this MPhil project was not sufficient to discriminate between cause or consequence with regard to the relationship between labour and the functional and molecular changes in SOL-DMSCs.

Additional group of samples could provide data to distinguish between cause or consequence. For examples, further studies could include a group of “emergency C-section shortly after SOL” patients as controls. In general, “emergency C-section shortly after SOL” patients are patients who have briefly experienced the labour process before undergoing C-section surgery due to various medical complications. In terms of the length of labour, the sequence of patient groups, from the shortest to the longest, should be patients undergoing (1) NIL + elective C-section, (2) emergency C-section shortly after SOL, and (3) SOL + vaginal delivery. If the functional and molecular profile of DMSCs from placentae of patients undergoing “emergency C-section shortly after SOL” (ECS-DMSCs) resemble those of NIL-DMSCs, it is likely that the changes detected in SOL-DMSCs were the consequence of labour. In other words, if the molecular and functional changes that are detected in SOL-DMSCs, and not in NIL-DMSCs, are not detected in ECS-DMSCs where the donors have undergone the initial phase of labour only, then it is likely that the changes in SOL-DMSCs are a consequence of the normal labour process. However, if the functional and molecular profile of ECS-DMSCs is similar to those in SOL-

DMSCs, then it is likely that these changes are the cause of labour since the changes are already occurring in the initial phase of the labour process (See **Figure 39** below).

Nevertheless, it is worth noting that the collection process of emergency C-section placentae for these analyses may be logistically challenging because of low numbers of samples with suitable medical conditions and the unpredictability of sample availability in normal working hours. Most emergency C-section deliveries are performed due to the fetal and/or maternal medical problem, therefore, the possibility of an underlying pathological condition must be taken into consideration when using these samples. To avoid confounding pathological factors, the ideal patient is one who plans to have an elective C-section, but in whom labour contractions start earlier than expected resulting in the “emergency C-section shortly after SOL” and an uncomplicated outcome for the mother or fetus.

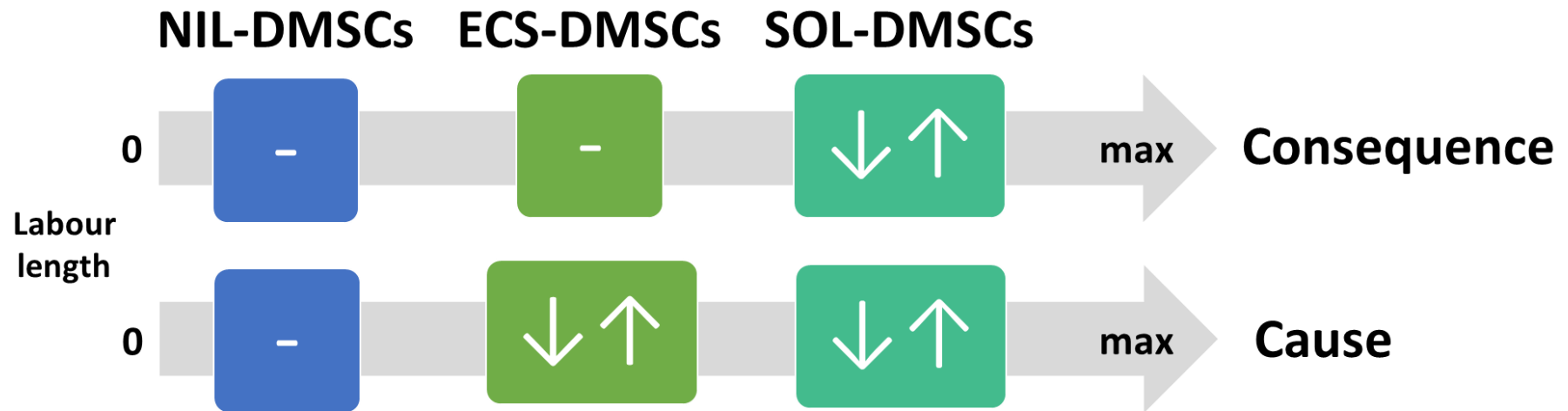


Figure 39. The rationale of the cause-consequence analyses

NIL-DMSCs: DMSCs from placentae of not-in-labour patients undergoing elective C-section delivery. ECS-DMSCs: DMSCs from placentae of patients who undergo a brief labour process before emergency C-section delivery is performed. SOL-DMSCs: DMSCs from placentae of patients undergoing spontaneous onset of labour + vaginal delivery. “-” indicates a control level of DMSC function and lipid profile, while “↓↑” indicates altered levels of DMSC function and lipid profile.

6. CONCLUSIONS, STUDY LIMITATIONS, AND FUTURE WORK

6.1. Conclusions

1. SOL-DMSCs showed evidence of ageing-related functional changes (i.e., reduced functionality and increased damage accumulation) compared with NIL-DMSCs, specifically:
 - a. SOL-DMSCs showed a significantly higher level of migration, necrosis, lipid peroxidation, and IL-6 production ($p < 0.05$)
 - b. No significant differences ($p > 0.05$) were found in cell attachment, proliferation, and survivability, apoptosis, ALDH expression, and IL-8 production. Nevertheless, there were trends towards increased apoptosis and reduced cell proliferation, cell survivability, and IL-8 production in SOL-DMSCs.
2. SOL- and NIL-DMSCs expressed the same lipid species (a total of 570 lipid species identified), however there was evidence of significant differences in lipid quantities of various specific lipid species, specifically in:
 - a. Phospholipid category, class PE (~13%), PG (~18%), PI (22%), and PS (~33%)
 - b. Sphingolipid category, class Cer (~12%) and SM (~4%)
 - c. Neutral lipid category, class TG (~6%)
 - d. Fatty acyl category, class AcCa

Overall, SOL-DMSCs had lower lipid quantities compared with NIL-DMSCs. The percentages were calculated per total count of lipid species

identified in particular lipid class. In contrast, SOL- and NIL-DMSCs did not show any statistically significant differences ($p > 0.05$) in the quantities of all lipid species identified in the following lipid classes:

- a. Phospholipid category, class LPC, LPE, LPI, and CL
- b. Sphingolipid category, class Hex1Cer, Hex2Cer, Hex3Cer
- c. Other lipid molecule, Coenzyme Q

These data suggest DMSCs may undergo changes in lipid metabolism during labour at term.

6.2. Study limitations and future work

1. The sample size used in this study ($n = 7 - 8$) was relatively small. However, a larger sample size was not feasible during the 1.5 year course of this study. There were significant time restraints due to the laborious nature of the isolation and characterisation of each DMSC preparation. Despite the small sample size, some functional assays in this study showed statistically significant results, whereas, for those assays that did not, some trends were observed. Thus, it would be worthwhile increasing the sample size to improve the statistical power in future studies.
2. As discussed in **Section 5.1.2**, labour-related changes may have occurred at the molecular or cellular level in the decidua of full-term NIL patients. To address this issue, future studies may utilise elective C-section samples from earlier gestational age e.g., early-term (37-38 weeks) or pre-term (36-37 weeks). Nevertheless, their possibly pathological nature must be taken into consideration during the analyses.

3. This study assessed limited numbers of DMSC functions (i.e., lipid peroxidation and ALDH expression for the oxidative stress function, only IL-6 and IL-8 for inflammation, and only mass spectrometry for lipid analyses). Nonetheless, significant differences were observed in some of these functions and more extensive analyses are warranted. For example, antibody arrays that cover more components of oxidative stress and inflammation, as well as mapping the signalling or molecular pathways for lipid analyses can be performed in future studies.
4. Other functional assays observing different DMSC functions can be performed on individual samples for more thorough assessment in the future. Some examples are senescence- or cell cycle-related assays, angiogenic activity analyses, miRNA or protein expression analyses, or extracellular vesicles characterisation.
5. The evidence obtained from this study is not sufficient to determine a causative or consequential relationship status between labour and the functional and molecular changes in SOL-DMSCs. One control group, which is the “emergency C-section shortly after SOL” patients who only briefly experienced labour before undergoing C-section, can be added to the study design of future studies to provide supporting evidence for a causal relationship, as discussed in **Section 5.5**.

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APPENDICES

Appendix I. Reagent components

Products	Components	Company
Alizarin Red	20 mg Alizarin Red 1 mL Milli Q water pH 4.1-4.3, adjusted with 0.5% ammonium hydroxide <i>Filtered (0.45 μm)</i>	Merck
AV-PI reagent mix	For each sample: 10 μ L 10 x binding buffer 10 μ L Propidium iodide 1 μ L Annexin V 79 μ L Milli Q water 100 μ L	Trevigen, Inc.
BCA working reagent	<i>Reagent A : B = 50 : 1</i> Reagent A: sodium carbonate, sodium bicarbonate, bicinchoninic acid, and sodium tartrate in 0.1M sodium hydroxide Reagent B: 4% cupric sulfate	Thermo Fisher Scientific
Crystal violet	0.5 g Crystal Violet 100 mL 20% methanol	LabChem TM Merck
Freezing medium	50% α -MEM 40% FCS 10% DMSO	Sigma Aldrich Gibco TM Chem-Supply
HBSS [-]	HBSS without Ca^{2+} and Mg^{2+} 1% P/S	Gibco TM Life Technologies
HBSS [+]	HBSS with Ca^{2+} and Mg^{2+} 1% P/S	Gibco TM Life Technologies
Oil Red O	1.8 mg Oil Red O 1 mL 60% isopropanol <i>Filtered (0.45 μm)</i>	Merck
P/S	100U/mlPen/100 μ g/mlStrep	Life Technologies
Red blood cell lysis buffer	0.25 M EDTA 1 M NaHCO_3 1 M NH_4Cl <i>Autoclaved</i>	Merck
α -MEM alone	α -MEM	Sigma Aldrich

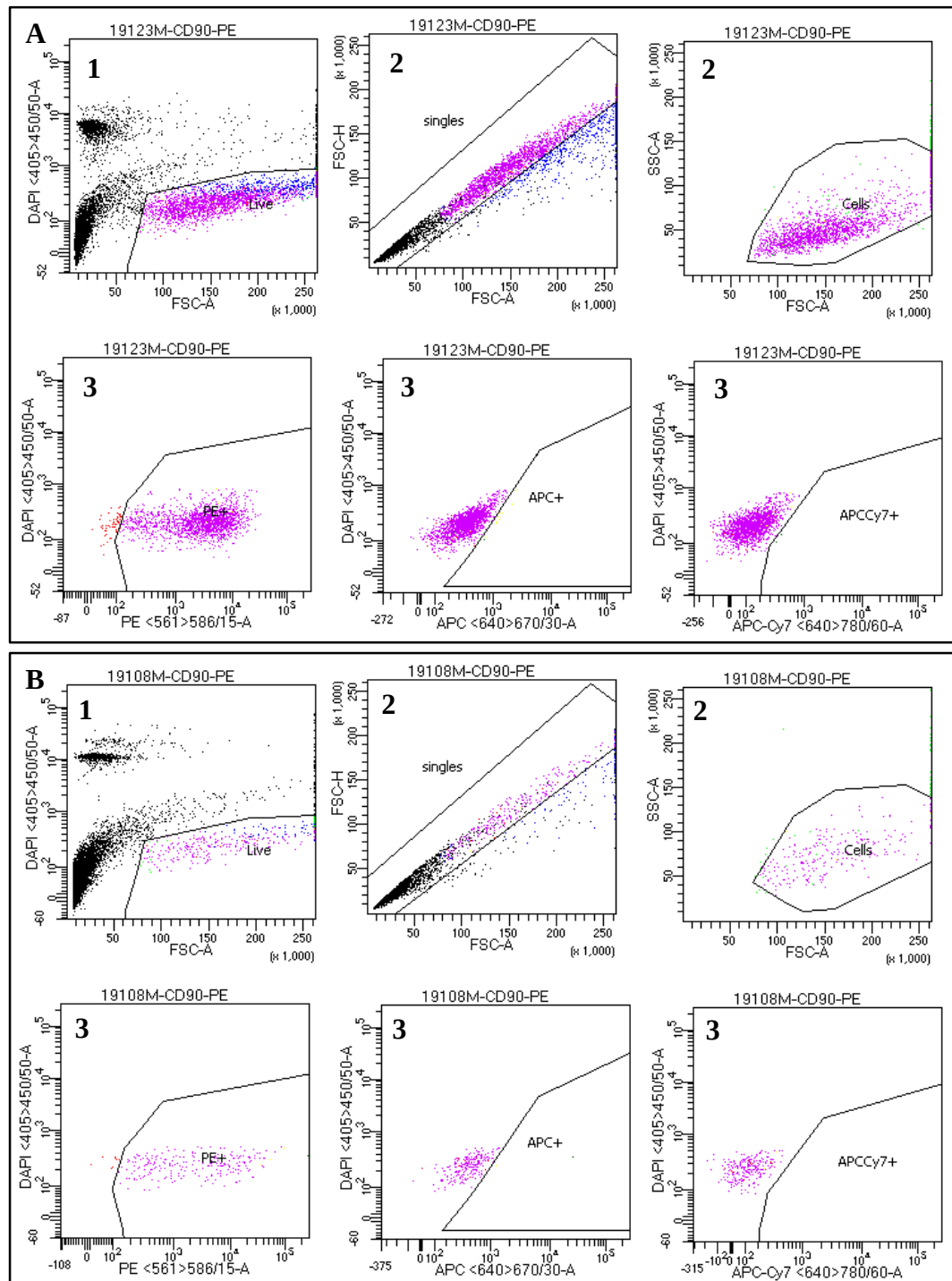
α -MEM complete medium	α -MEM 10% FCS 1% P/S 1% L-glutamine 2 mM		Sigma Aldrich Gibco™ Life Technologies Sigma Aldrich
Serum-starved medium	α -MEM 1% FCS 1% P/S 1% L-glutamine 2 mM		Sigma Aldrich Gibco™ Life Technologies Sigma Aldrich
SPLASH® LIPIDOMIX® Mass Spec Standard	Mixture Components	Concentration (µg/mL)	Avanti Polar Lipids, Inc.
	15:0-18:1(d7) PC	160	
	15:0-18:1(d7) PE	5	
	15:0-18:1(d7) PS	5	
	15:0-18:1(d7) PG	30	
	15:0-18:1(d7) PI	10	
	15:0-18:1(d7) PA	7	
	18:1(d7) LPC	25	
	18:1(d7) LPE	5	
	18:1(d7) Chol Ester	350	
	18:1(d7) MG	2	
	15:0-18:1(d7) DG	10	
	15:0-18:1(d7)-15:0 TG	55	
	18:1(d9) SM	30	
	Cholesterol (d7)	100	

Appendix II. Table of surface markers in the flow cytometry analyses

	Markers	Volume /100 μ L	Conjugate	Marker descriptions	Ref
MSC-specific	CD90	0.5 μ l	PE	- Glycosylphosphatidylinositol-linked protein (Thy1) - Involved in cell-cell and cell-matrix interactions	(Lin, Xin, Dai, & Lue, 2013)
	CD166	1.0 μ l	PE	- Activated leukocyte cell adhesion molecule (ALCAM) - Involved in active cell growth or migration - Mediating heterophilic and homophilic cell - cell interactions	(Halfon, Abramov, Grinblat, & Ginis, 2010)
	CD44	1.0 μ l	PE	- Homing cell adhesion molecule (HCAM) - Involved in adhesion, migration, homing, proliferation, apoptosis - Mediating stem cell - niche interactions	(Maleki, Ghanbarvand, Behvarz, Ejtemaei, & Ghadirkhomi, 2014)
	CD73	1.0 μ l	PE	- Ecto-5'-nucleotidase (5'-NT) - Converting extracellular AMP to adenosine	(Lin et al., 2013)
	CD105	0.5 μ l	APC	- A membrane glycoprotein type I (Endoglin) - An adjunct receptor for TGF-β ligands	(Lin et al., 2013)
Hematopoietic	CD45	1.0 μ l	APC-Cy7	Pan-leukocyte marker	(Dominici et al., 2006)
	HLA-DR	1.0 μ l	APC	- Human Leukocyte Antigen DR - Not normally expressed by MSCs unless stimulated	
	CD19	0.5 μ l	APC-Cy7	- B-lymphocyte antigen - Detecting B-cells that may adhere to MSCs	
Controls	Mouse IgG1	1.0 μ l	PE	- Isotype control - Excluding nonspecific antibody staining	(Maecker & Trotter, 2006)
	Mouse IgG2a	1.0 μ l	APC		
	Mouse IgG1	0.5 μ l	APC-Cy7		
	DAPI	1.0 μ l	N/A	4',6-diamidino-2-phenylindole - Blue-fluorescent nuclear stain	(Chazotte, 2011)

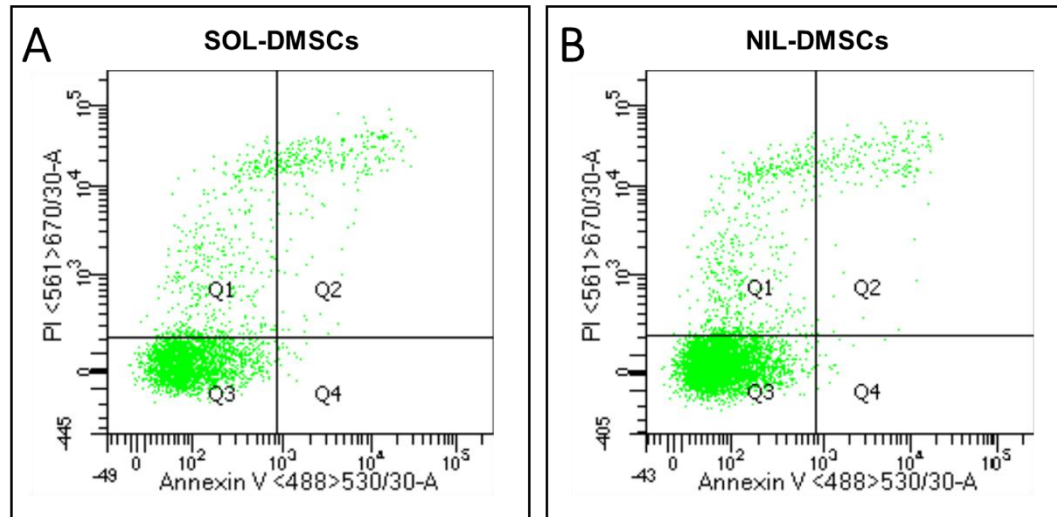
All antibodies corresponding to the markers listed above were supplied by BD Biosciences, except for CD105 (Life Technologies) and DAPI (Merck). All anti-human antibodies were mouse-derived. The suppliers did not specify the antibody concentration. PE: Phycoerythrin. APC: Allophycocyanin. APC-Cy7: Allophycocyanin-Cy7 (tandem conjugate).

Appendix III. List of gating adjustment for flow cytometry analyses



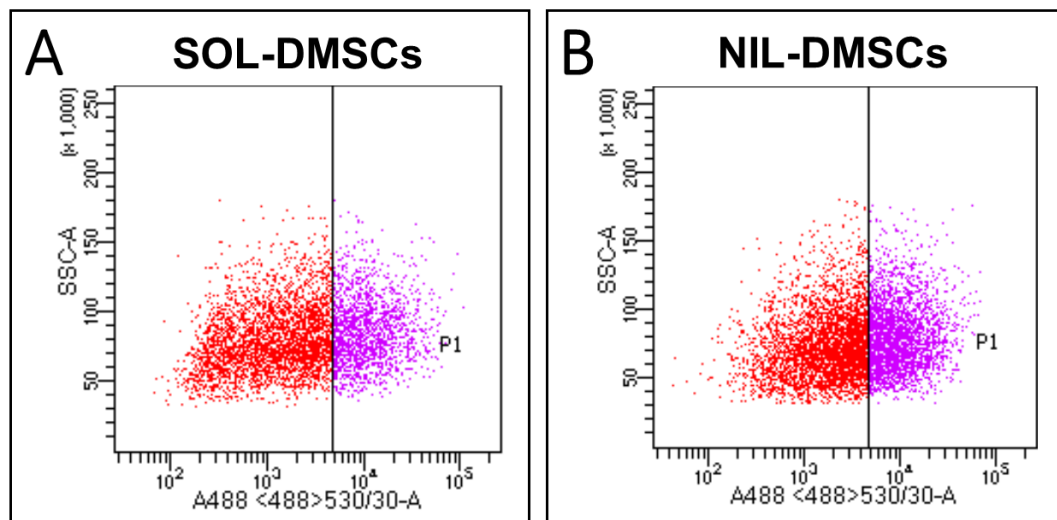
Supplementary figure 1. Representative gating adjustment for MSC surface antigen characterisation

(A) SOL-DMSCs and (B) NIL-DMSCs were analysed based on their (1) viability represented on their ability to exclude DAPI, (2) size and complexity represented on the forward and scatter light profile, then (3) surface marker expression reflected by fluorescence intensity of the corresponding antibody conjugates.



Supplementary figure 2. Representative gating adjustment for FITC Annexin V assay

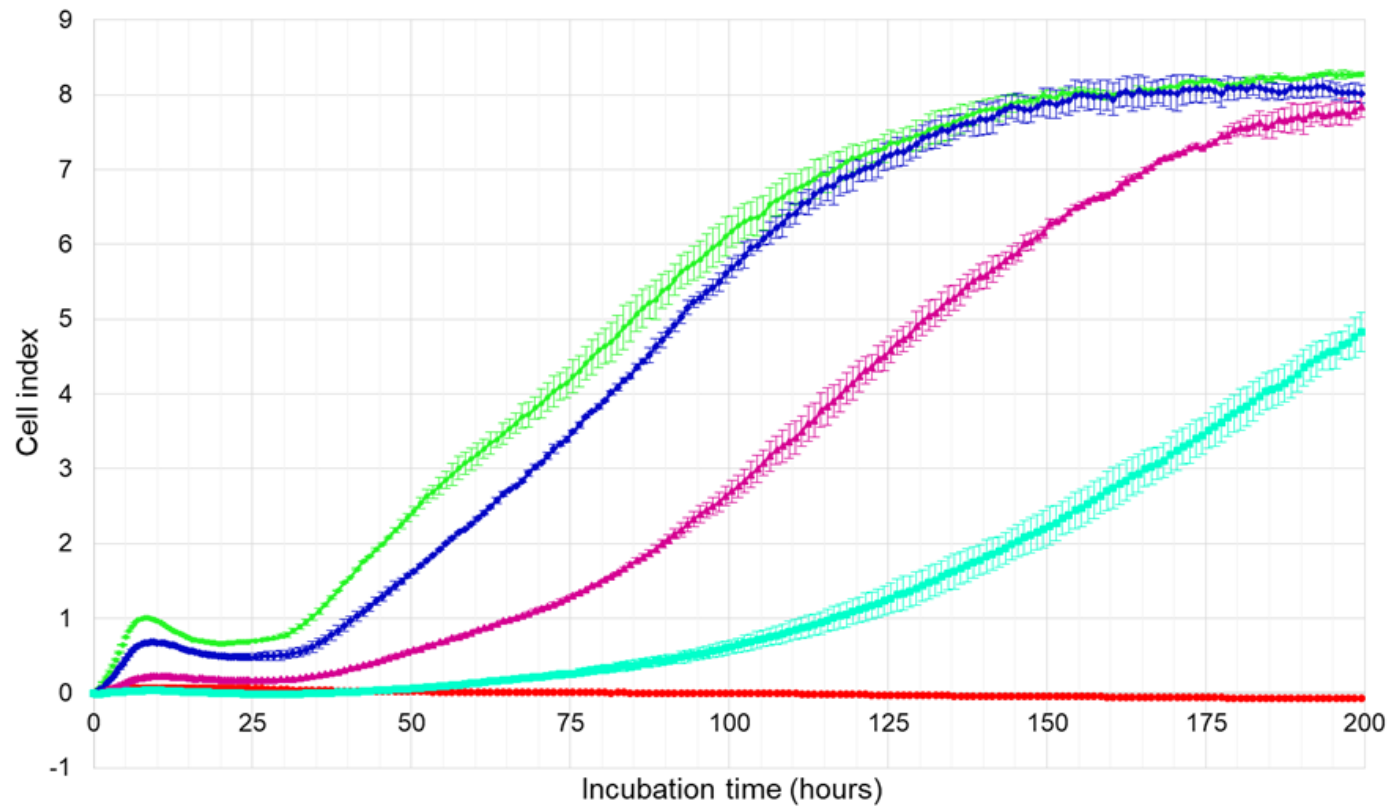
(A) SOL-DMSCs and **(B)** NIL-DMSCs. Q1(PI^+/AV^-): necrotic cells. Q2(PI^+/AV^+): late apoptotic cells. Q3(PI^-/AV^-): viable cells. Q4(PI^-/AV^+): early apoptotic cells. x-axis denotes the fluorescent intensity of AV, while y-axis denotes the fluorescent intensity of PI.



Supplementary figure 3. Representative gating adjustment for aldefluor assay

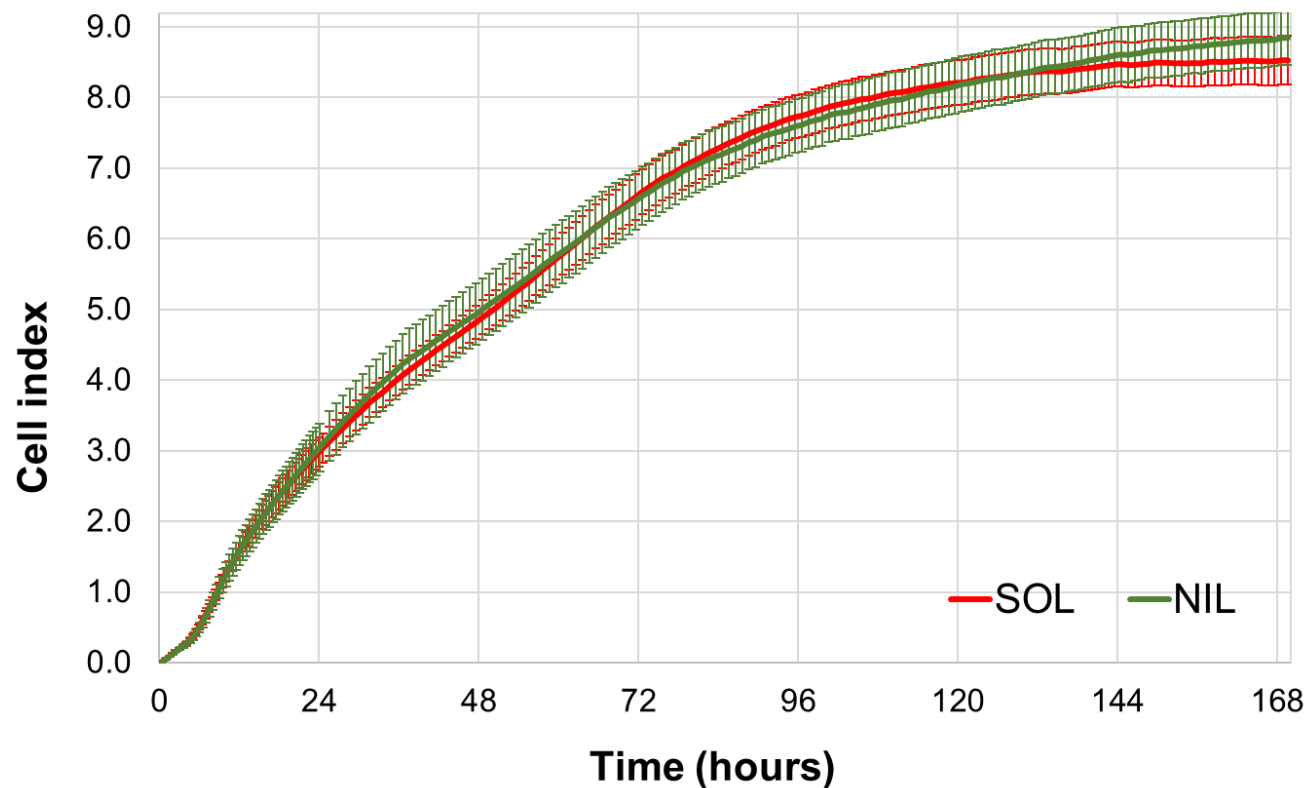
(A) SOL-DMSCs and **(B)** NIL-DMSCs. x-axis denotes the amount of intracellular fluorescence signals resulting from ALDH activity of DMSCs, while y-axis denotes the side scatter profile of DMSCs.

Appendix IV. Protocol optimisation results



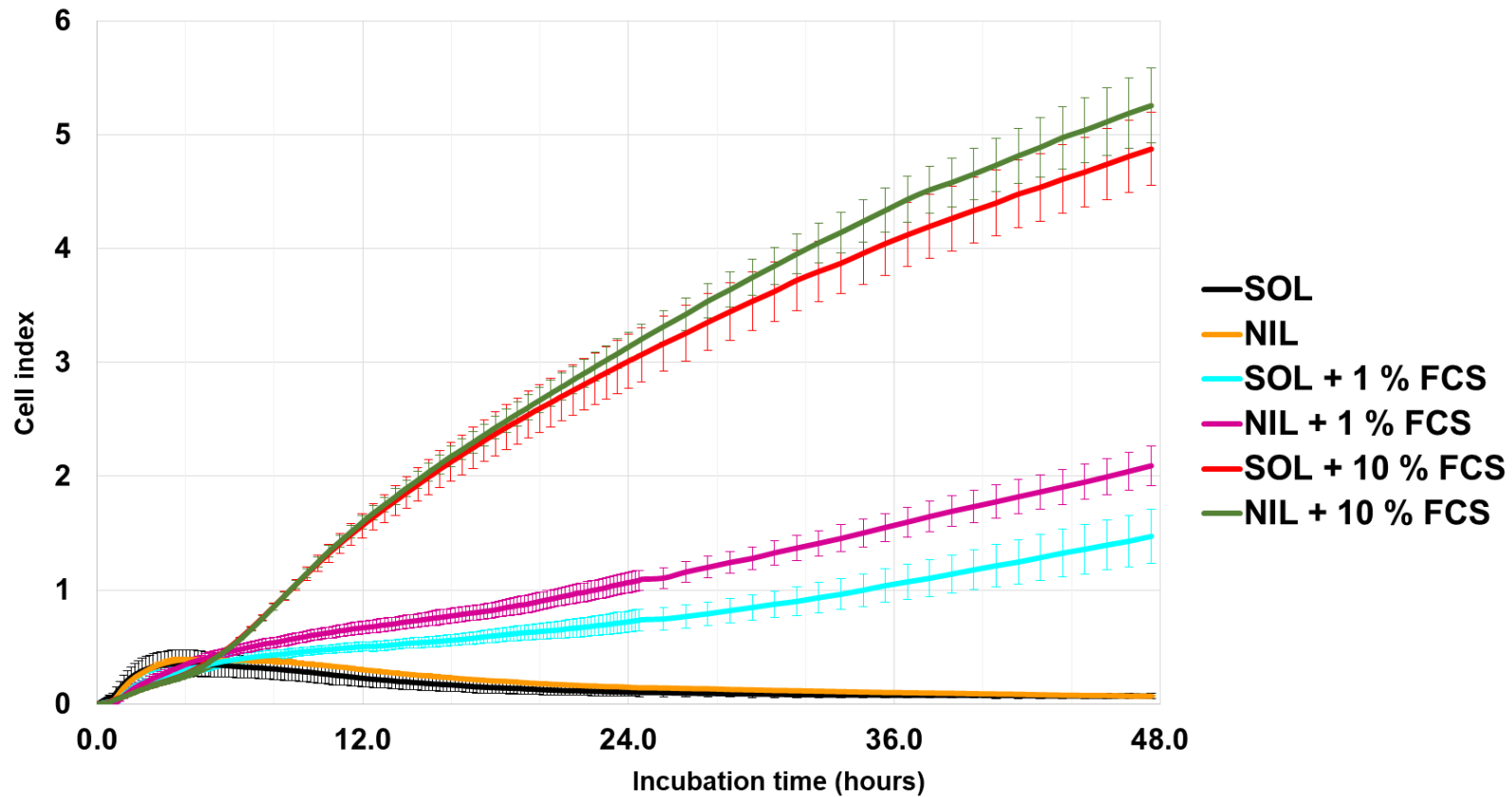
Supplementary figure 4. Optimisation of the seeding density of DMSCs

The lines represent the averaged cell indices of DMSCs ($n = 1$) seeded in duplicate at different seeding density/well: 1×10^4 cells (—), 7.5×10^3 cells (—), 5×10^3 cells (—), 2.5×10^3 cells (—), and medium only/no cells (—).



Supplementary figure 5. The complete growth profile of DMSCs

The lines represent the averaged cell indices of SOL- and NIL-DMSCs recorded at real time by the xCELLigence system. The error bars represent $\pm$ SEM of the cell indices. Arrow **(P)** and **(S)** point out the start of proliferation and survival phase, respectively. The attachment phase was designated as 0-5 hours, the proliferation phase was 5-80 hours, and the survival phase was from 80 hours and beyond.



Supplementary figure 6. Optimisation of serum concentration of the serum starvation medium

Real-time xCELLigence analysis of cell proliferation. The lines represent the cell indices of SOL- and NIL-DMSCs resuspended in growth medium supplemented with 0 (n = 3 per group), 1 (n = 3 per group), and 10% FCS (n = 8 for SOL-DMSCs and n = 7 for NIL-DMSCs). Data were presented as mean $\pm$ SEM. Cell indices were measured for 48 hours. Cells seeded in serum-starvation medium (1% FCS) showed a similar proliferation trend to those seeded in α -MEM complete medium (10% FCS), but at a reduced level of cell indices.

Appendix V. Descriptive statistics of the quantitative assays

Supplementary table 1. Clonogenic efficiency

Observed parameters	SOL-DMSCs (n = 8)	NIL-DMSCs (n = 7)
Clusters (%)	17.9 ± 2.03	21.29 ± 1.21
CFU-F (%)	8.25 ± 0.45	8.14 ± 0.94
Clusters + CFU-F (%)	26.00 ± 2.24	29.43 ± 1.89

Data were presented as mean ± SEM.

Supplementary table 2. xCELLigence real-time proliferation assay

Time point (hours)		Cell index	
		SOL-DMSCs (n = 8)	NIL-DMSCs (n = 7)
A	2	0.126 ± 0.011	0.124 ± 0.013
	3	0.189 ± 0.016	0.188 ± 0.020
	4	0.269 ± 0.021	0.264 ± 0.032
P	24	3.011 ± 0.237	3.136 ± 0.130
	48	4.874 ± 0.321	5.255 ± 0.327
	72	6.686 ± 0.463	7.225 ± 0.809
S	135	8.383 ± 0.295	8.600 ± 0.468
	145	8.493 ± 0.319	8.796 ± 0.529
	165	8.542 ± 0.369	9.014 ± 0.588

A: Attachment. P: Proliferation. S: Survival. Data were presented as mean ± SEM.

Supplementary table 3. CCK-8 end-point proliferation assay

Time point (hours)	OD 450 nm	
	SOL-DMSCs (n = 8)	NIL-DMSCs (n = 7)
24	0.93 ± 0.11	1.08 ± 0.17
48	2.02 ± 0.25	2.25 ± 0.27
72	2.89 ± 0.28	2.96 ± 0.25
165	3.25 ± 0.18	3.51 ± 0.18

Data were presented as mean ± SEM.

Supplementary table 4. FITC Annexin V assay

Quadrant	The percentages (%)	
	SOL-DMSCs (n = 8)	NIL-DMSCs (n = 7)
Necrosis (Q1)	16.75 ± 0.92	14.29 ± 0.57
Early apoptosis (Q4)	0.65 ± 0.09	0.63 ± 0.12
Late apoptosis (Q2)	6.15 ± 0.67	5.43 ± 0.40
Total apoptosis (Q2 + Q4)	6.84 ± 0.66	6.06 ± 0.45
Viable/healthy cells (Q3)	76.59 ± 1.23	79.63 ± 0.84

Data were presented as mean ± SEM.

Supplementary table 5. Lipid species identified in Class CL

Lipid Species	Peak area per 100 μ L sample				p-value	
	SOL-DMSCs (n = 7)		NIL-DMSCs (n = 7)			
	Mean	SEM	Mean	SEM		
CL(17:1_18:1_18:2_18:1)	8.35E+06	1.72E+06	1.00E+07	2.08E+06	0.2086	
CL(17:1_20:4_18:1_18:2)	4.14E+06	4.99E+05	5.21E+06	9.05E+05	0.3176	
CL(18:1_16:0_16:0_18:1)	7.21E+06	2.30E+06	9.40E+06	1.77E+06	0.1649	
CL(18:1_16:0_16:1_18:1)	1.45E+07	3.37E+06	1.81E+07	3.56E+06	0.0973	
CL(18:1_16:0_18:1_18:1)	1.41E+07	3.19E+06	1.80E+07	4.24E+06	0.1282	
CL(18:1_16:0_20:4_16:1)	8.44E+06	1.82E+06	9.94E+06	1.52E+06	0.1098	
CL(18:1_16:1_16:1_18:1)	2.63E+07	4.99E+06	2.97E+07	5.28E+06	0.5350	
CL(18:1_16:1_18:1_14:0)	4.42E+06	1.01E+06	4.96E+06	1.04E+06	0.4557	
CL(18:1_16:1_18:1_18:1)	6.08E+07	1.15E+07	7.05E+07	1.41E+07	0.3829	
CL(18:1_16:1_20:4_18:1)	3.36E+07	5.23E+06	3.75E+07	5.53E+06	0.2593	
CL(18:1_18:1_18:1_18:1)	3.52E+07	5.92E+06	4.11E+07	9.10E+06	0.5224	
CL(18:1_20:3_18:2_20:3)	7.70E+06	1.13E+06	8.81E+06	1.41E+06	0.4557	
CL(18:2_16:1_18:1_16:1)	1.35E+07	2.79E+06	1.53E+07	2.90E+06	0.5649	
CL(18:2_18:1_16:1_18:1)	5.96E+07	1.04E+07	6.94E+07	1.28E+07	0.3829	
CL(18:2_18:1_16:1_20:3)	1.35E+07	3.07E+06	1.75E+07	4.09E+06	0.2086	
CL(18:2_18:1_18:1_18:1)	6.98E+07	1.40E+07	8.53E+07	1.97E+07	0.0973	
CL(18:2_18:1_18:1_18:2)	4.74E+07	9.11E+06	6.02E+07	1.37E+07	0.3374	
CL(18:2_18:1_18:2_16:1)	1.92E+07	3.56E+06	2.29E+07	5.13E+06	0.6200	
CL(18:2_18:1_20:3_18:1)	1.26E+07	2.25E+06	1.47E+07	3.01E+06	0.6544	
CL(18:2_18:1_20:3_18:2)	1.68E+07	3.11E+06	2.07E+07	4.10E+06	0.4057	
CL(18:2_18:1_20:4_20:3)	1.14E+07	1.93E+06	1.34E+07	2.22E+06	0.3829	
CL(18:2_18:1_22:5_16:1)	3.17E+07	4.65E+06	3.91E+07	7.27E+06	0.4557	
CL(18:2_20:4_16:1_18:1)	3.52E+07	5.71E+06	4.06E+07	6.56E+06	0.3829	
CL(18:4_18:1_16:1_18:2)	7.82E+06	1.28E+06	9.06E+06	1.44E+06	0.4057	
CL(20:4_16:1_16:1_18:1)	1.59E+07	2.79E+06	1.74E+07	2.61E+06	0.6200	
CL(20:4_18:1_16:0_18:1)	7.97E+06	1.43E+06	9.80E+06	1.73E+06	0.1098	
CL(20:4_18:1_18:1_18:1)	2.13E+07	3.14E+06	2.38E+07	4.20E+06	0.7104	
CL(20:4_18:1_18:1_18:2)	4.19E+07	6.61E+06	4.92E+07	8.39E+06	0.2593	
CL(20:5_18:1_16:1_18:2)	1.13E+07	1.18E+06	1.42E+07	2.64E+06	0.4817	
CL(20:5_18:1_18:2_18:2)	1.20E+07	2.18E+06	1.45E+07	2.82E+06	0.4817	
CL(20:5_18:1_18:2_20:3)	8.28E+06	1.36E+06	1.02E+07	1.78E+06	0.3829	

P-value was calculated using Mann-Whitney U test.

Supplementary table 6. Lipid species identified in Class LPC, LPE, and LPI

Lipid species	Amount (ng) per 100 μ L sample				p-value	
	SOL-DMSCs (n = 6)		NIL-DMSCs (n = 7)			
	Mean	SEM	Mean	SEM		
LPC						
LPC(14:0)	0.063	0.004	0.068	0.007	0.7202	
LPC(15:0)-1	0.031	0.001	0.031	0.002	0.7157	
LPC(15:0)-2	0.326	0.014	0.319	0.009	0.8357	
LPC(16:0)-1	0.435	0.042	0.428	0.037	0.9452	
LPC(16:0)-2	1.388	0.105	1.711	0.219	0.3660	
LPC(16:1)-1	0.062	0.010	0.053	0.006	0.5672	
LPC(16:1)-2	0.202	0.016	0.180	0.013	0.3660	
LPC(17:0)	0.053	0.005	0.072	0.011	0.2949	
LPC(18:0)-1	0.142	0.011	0.186	0.028	0.2833	
LPC(18:0)-2	2.449	0.228	3.196	0.440	0.2949	
LPC(18:1)-1	0.555	0.055	0.498	0.045	0.5338	
LPC(18:1)-2	0.837	0.028	0.808	0.041	0.6678	
LPC(18:1)-3	0.106	0.009	0.132	0.018	0.3660	
LPC(18:1e)	0.237	0.020	0.235	0.012	0.8357	
LPC(18:2)	0.024	0.003	0.023	0.002	0.6625	
LPC(20:1)	0.057	0.004	0.060	0.005	0.7199	
LPC(20:4)	0.067	0.012	0.057	0.008	0.8357	
LPC(22:5)	0.036	0.009	0.033	0.003	0.6151	
LPC(26:0)	1.390	0.044	1.412	0.035	0.8357	
LPC(28:0)	0.438	0.058	0.741	0.103	0.0513	
LPE						
LPE(16:0)	0.221	0.027	0.205	0.014	0.8357	
LPE(18:0)	1.488	0.105	1.516	0.065	0.8357	
LPE(18:1)-1	0.602	0.131	0.540	0.057	0.8357	
LPE(18:1)-2	0.492 [■]	0.081	0.366	0.038	0.0513	
LPE(18:1e)	1.935 [■]	0.077	2.157	0.131	0.2086	
LPE(18:2e)	0.950	0.072	0.847	0.058	0.3829	
LPE(20:4)	0.792	0.166	0.741	0.063	0.9452	
LPE(22:5)	0.718	0.155	0.747	0.064	0.7308	
LPE(22:6)	0.373	0.070	0.405	0.049	0.6282	
LPI^a						
LPI(18:0)	8.80E+06 [■]	1.19E+06	9.35E+06	1.47E+06	0.9015	

P-value was calculated using Mann-Whitney U test. [■] n = 7. ^aLPI data were peak area per 100 μ L sample.

Supplementary table 7. Lipid species identified in Class PC

Lipid species	Amount (ng) per 100 µl sample				p-value	
	SOL-DMSCs (n = 6)		NIL-DMSCs (n = 7)			
	Mean	SEM	Mean	SEM		
PC(14:0_14:0)	5.482	0.521	6.605	0.456	0.1807	
PC(14:0_20:4)	8.044	0.551	9.429	0.630	0.2343	
PC(14:0e_22:5)	2.229	0.240	2.931	0.348	0.1000	
PC(14:0e_22:6)	1.323	0.139	1.320	0.084	0.8357	
PC(14:1e_18:1)	1.018	0.140	0.928	0.094	0.7308	
PC(14:1e_20:4)	1.671	0.136	1.542	0.110	0.5338	
PC(14:1e_22:5)	1.212	0.134	1.282	0.091	0.6282	
PC(14:1e_22:6)	0.690	0.060	0.660	0.060	0.6282	
PC(14:1e_24:0)	1.109	0.184	1.628	0.167	0.0734	
PC(15:0_14:0)-1	0.304	0.025	0.384	0.036	0.1014	
PC(15:0_14:0)-2	1.412	0.147	1.796	0.154	0.1526	
PC(15:0_16:0)	17.420	1.242	19.830	1.569	0.6282	
PC(15:0_16:1)	8.394	0.611	10.140	0.727	0.1807	
PC(15:0_18:1)	29.950	0.928	37.130	3.240	0.1375	
PC(15:0_18:3)	1.133	0.172	1.548	0.114	0.1014	
PC(15:0_22:3)	1.721	0.298	1.525	0.133	0.6282	
PC(15:0_22:4)	4.944	0.703	4.907	0.464	0.9452	
PC(15:0_22:5)	2.495	0.198	2.955	0.271	0.3660	
PC(15:0_22:6)	3.424	0.425	2.989	0.194	0.4452	
PC(16:0_14:0)	83.430	6.841	103.900	8.347	0.2949	
PC(16:0_16:0)	192.000	15.220	221.400	14.560	0.3660	
PC(16:0_16:1)	261.400	25.470	324.300	23.310	0.1807	
PC(16:0_17:0)-1	7.342	0.483	7.794	0.495	0.3660	
PC(16:0_17:0)-2	4.991	0.351	6.013	0.406	0.1375	
PC(16:0_18:1)	647.600	41.900	673.800	36.160	0.9452	
PC(16:0_18:2)	306.000	22.800	318.900	25.420	0.7308	
PC(16:0_18:3)	43.740	2.944	48.100	1.889	0.4452	
PC(16:0_20:3)	113.200	8.106	130.700	9.220	0.2343	
PC(16:0_20:4)	47.760	4.300	53.280	3.556	0.5338	
PC(16:0_20:5)	14.600	1.800	15.560	1.336	0.8357	
PC(16:0_22:6)-1	7.097	0.567	17.370	4.069	0.0734	
PC(16:0_22:6)-2	7.525	0.565	7.816	0.673	0.7308	
PC(16:0_24:1)	1.220	0.169	1.322	0.119	0.4452	
PC(16:0D3_14:1)	32.680	2.811	39.800	3.776	0.1807	
PC(16:0D3_18:2)	373.300	24.550	350.900	20.720	0.8357	
PC(16:0e_14:0)	4.111	0.415	5.476	0.551	0.2343	
PC(16:0e_15:0)	1.065	0.122	1.381	0.099	0.1014	
PC(16:0e_16:0)	17.890	2.464	24.360	2.394	0.0513	
PC(16:0e_20:4)	12.200	1.383	15.050	1.168	0.1014	
PC(16:0e_20:5)	7.886	0.641	8.393	0.542	0.5338	

PC(16:0e_22:3)-1	1.465	0.233	2.001	0.195	0.1375
PC(16:0e_22:3)-2	0.577	0.096	0.667	0.051	0.4744
PC(16:0e_22:4)	3.062	0.431	4.350	0.416	0.0734
PC(16:1_14:0)	22.850	3.224	25.460	3.238	0.6282
PC(16:1_14:1)	3.412	0.371	3.940	0.372	0.5338
PC(16:1_16:1)-1	17.090	1.281	19.210	1.947	0.6282
PC(16:1_16:1)-2	32.950	3.056	38.770	1.919	0.2949
PC(16:1_20:3)	9.837	0.954	12.120	0.895	0.1807
PC(16:1_20:4)	4.799	0.390	6.031	0.565	0.1375
PC(16:1e_14:0)	1.277	0.072	1.552	0.109	0.0734
PC(16:1e_16:0)	5.998	0.613	7.964	0.759	0.1014
PC(16:1e_22:1)	0.660	0.106	0.926	0.089	0.0734
PC(16:1e_24:0)	2.025	0.309	3.090	0.402	0.0734
PC(16:2e_18:0)	9.885	0.991	11.740	0.993	0.2343
PC(16:2e_24:0)	0.889	0.137	1.288	0.139	0.0734
PC(17:0_18:1)-1	10.100	0.953	10.980	0.588	0.0734
PC(17:0_18:1)-2	30.150	2.700	33.150	1.962	0.4452
PC(17:0_20:3)	3.022	0.357	3.375	0.321	0.4452
PC(17:0_20:4)	3.008	0.243	3.221	0.238	0.6282
PC(17:0_22:5)	2.628	0.261	3.123	0.236	0.2343
PC(17:1_16:1)	6.834	0.463	7.863	0.594	0.2949
PC(17:1_18:1)	27.060	1.704	29.480	1.708	0.3660
PC(17:1_18:2)	2.685	0.214	3.131	0.207	0.1807
PC(18:0_16:0)	44.260	3.425	48.880	2.588	0.3660
PC(18:0_18:0)	1.343	0.174	1.629	0.123	0.2343
PC(18:0_18:1)	307.500	30.840	323.800	17.890	0.5338
PC(18:0_18:2)	526.900	30.870	537.200	27.880	0.8357
PC(18:0_20:1)	3.306	0.413	3.556	0.272	0.5338
PC(18:0_20:3)-1	22.320	1.767	24.000	1.516	0.6282
PC(18:0_20:3)-2	18.540	2.025	20.990	1.943	0.4452
PC(18:0_20:3)-3	11.000	1.682	11.270	0.632	1.0000
PC(18:0_20:4)	78.000	5.458	80.550	5.093	0.8357
PC(18:0_20:5)	10.120	0.869	10.540	0.726	0.6282
PC(18:0_22:4)	7.279	0.820	8.115	0.541	0.3660
PC(18:0_22:5)	30.960	2.184	31.830	1.933	0.9452
PC(18:0e_16:0)	5.904	1.149	7.248	0.728	0.1807
PC(18:0e_20:4)	5.799	0.912	7.282	0.616	0.1375
PC(18:0e_22:5)	3.971	0.683	5.310	0.528	0.1014
PC(18:0e_22:6)	2.591	0.348	3.441	0.361	0.1807
PC(18:1_20:4)	125.600	10.990	147.200	12.500	0.2949
PC(18:1_21:0)	0.857	0.220	0.828	0.107	0.8357
PC(18:1_22:1)	2.301	0.333	2.427	0.213	0.3660
PC(18:1_22:2)-1	2.977	0.336	3.129	0.197	0.5197
PC(18:1_22:2)-2	5.478	0.717	5.649	0.384	0.9452
PC(18:1_22:4)-1	7.866	0.480	8.570	0.486	0.4452

PC(18:1_22:4)-2	2.760	0.191	2.666	0.148	0.6282
PC(18:1_22:5)	43.610	3.807	52.370	3.659	0.1375
PC(18:1_22:6)	8.200	0.568	8.398	0.512	0.7308
PC(18:1_24:1)	1.729	0.286	1.817	0.186	0.5338
PC(18:1_24:2)	1.420	0.191	1.416	0.111	0.7308
PC(18:1e_14:0)	12.750	1.366	15.400	1.149	0.2949
PC(18:1e_14:1)	3.503	0.225	3.938	0.302	0.2949
PC(18:1e_16:0)	51.100	6.903	65.640	5.773	0.2343
PC(18:1e_16:1)	7.343	0.655	9.039	0.736	0.1375
PC(18:1e_18:1)-1	11.730	1.629	11.890	1.103	0.8357
PC(18:1e_18:1)-2	1.438	0.226	1.725	0.170	0.2949
PC(18:1e_20:3)	1.083	0.099	1.115	0.093	0.9452
PC(18:1e_20:4)-1	33.040	3.301	40.560	3.161	0.0734
PC(18:1e_20:4)-2	3.113	0.292	3.809	0.334	0.1807
PC(18:1e_20:5)	5.633	0.667	6.317	0.444	0.5338
PC(18:1e_22:4)	1.581	0.179	2.257	0.208	0.0734
PC(18:1e_22:5)	9.991	0.810	12.370	1.217	0.1807
PC(18:1e_22:6)	6.614	0.660	8.005	0.738	0.1807
PC(18:2e_20:4)	12.170	0.954	14.650	1.441	0.3660
PC(18:3e_18:0)-1	2.234	0.224	2.411	0.203	0.6282
PC(18:3e_18:0)-2	4.093	0.435	5.072	0.501	0.2949
PC(19:0_18:1)	0.654	0.163	0.618	0.078	0.7308
PC(19:1_18:1)	7.249	0.806	8.008	0.483	0.3660
PC(20:0_22:6)	0.526	0.039	0.520	0.027	0.7206
PC(20:0e_16:0)	0.632	0.128	0.883	0.090	0.1014
PC(20:1_18:1)	26.730	2.981	29.180	1.843	0.7308
PC(20:1_22:5)	4.102	0.268	4.158	0.191	0.9452
PC(20:1e_16:0)	3.395	0.528	4.048	0.243	0.1807
PC(20:2_22:5)	3.530	0.310	3.875	0.295	0.4452
PC(20:4D8_18:1)	3.564	0.401	3.868	0.247	0.7308
PC(22:0_10:4)	1.088	0.075	1.200	0.092	0.6282
PC(22:0_20:4)	2.703	0.334	2.688	0.195	0.8357
PC(22:5_18:2)	2.697	0.206	3.459	0.326	0.1375
PC(22:5_20:4)	0.983	0.089	1.235	0.116	0.1375
PC(22:6_13:0)	2.574	0.279	2.406	0.213	0.2949
PC(24:0_20:4)	0.967	0.131	0.927	0.074	1.0000
PC(26:1_16:0)	0.780	0.136	0.825	0.093	0.6282
PC(26:1_18:1)	0.728	0.145	0.786	0.094	0.5338
PC(26:1_18:2)	0.605	0.097	0.626	0.061	0.7308
PC(18:1D7_14:0)	9.056 [■]	0.466	9.842	0.748	0.4452
PC(16:1_18:2)	18.900 [■]	3.270	25.160	4.464	0.1014
PC(18:1_18:1)	648.800 [■]	21.210	758.500	70.800	0.3660
PC(18:1_18:2)	19.260 [■]	1.727	44.700	12.160	0.0513
PC(18:1_20:3)	8.062 [■]	1.265	8.990	1.038	0.2343

P-value was calculated using Mann-Whitney U test. [■] n = 7.

Supplementary table 8. Lipid species identified in Class PE

Lipid species	Amount (ng) per 100 µl sample				p-value	
	SOL-DMSCs (n = 6)		NIL-DMSCs (n = 7)			
	Mean	SEM	Mean	SEM		
PE(16:0p_16:0)	2.608	0.203	2.916	0.124	0.1375	
PE(16:0p_18:1)	82.580	7.992	89.910	5.813	0.5338	
PE(16:0p_20:3)	5.140	0.754	4.505	0.474	0.2343	
PE(16:0p_20:4)	78.810	2.484	81.730	3.326	0.5338	
PE(16:0p_20:5)	11.040	1.066	11.880	0.790	0.5338	
PE(16:0p_22:3)	10.770	0.920	12.010	0.417	0.4452	
PE(16:0p_22:4)	28.930	5.467	83.920	13.630	**0.0012	
PE(16:0p_22:5)	151.500	5.999	171.400	5.896	*0.0350	
PE(16:0p_22:6)	35.660	2.060	46.420	3.914	0.0513	
PE(18:0_22:5)	87.110	4.520	89.290	2.771	0.7308	
PE(18:1p_18:1)	33.100	4.987	33.470	3.939	1.0000	
PE(18:1p_20:5)	8.337	1.827	7.431	1.465	0.8357	
PE(18:1p_22:3)	5.079	0.701	6.051	0.307	0.1807	
PE(18:1p_22:4)	14.570	0.924	18.050	0.859	*0.0350	
PE(18:1p_22:5)	55.800	4.466	62.710	4.085	0.3660	
PE(18:1p_22:6)	30.470	1.732	32.760	1.091	0.6282	
PE(20:0p_20:4)	3.773	0.452	4.712	0.378	0.1014	
PE(20:0p_22:4)	0.646	0.040	0.839	0.048	*0.0350	
PE(20:0p_22:5)	4.979	0.439	6.405	0.503	0.1014	
PE(18:0p_18:1)	41.660	5.778	48.100	4.695	0.4452	
PE(18:0p_20:4)	100.600	4.940	106.900	4.975	0.4452	
PE(18:0p_20:5)	20.690	4.328	14.010	1.849	0.2949	
PE(18:0p_22:3)-1	1.773	0.279	2.015	0.187	0.3660	
PE(18:0p_22:3)-2	6.439	0.611	7.807	0.297	0.0734	
PE(18:0p_22:4)	20.350	1.893	27.300	1.733	*0.0221	
PE(18:0p_22:5)	79.590	9.478	98.220	9.213	0.1375	
PE(18:0p_22:6)	42.460	3.302	48.810	3.374	0.3660	
PE(18:1_18:1)-1	77.220	3.817	74.990	2.352	0.6282	
PE(16:0_14:0)	0.722	0.088	0.798	0.053	0.5350	
PE(16:0_16:0)	1.912	0.209	2.290	0.230	0.2593	
PE(16:0_18:1)	42.820	4.022	48.710	5.570	0.3829	
PE(16:0_20:4)	4.645	0.320	5.481	0.470	0.2086	
PE(16:0_22:6)	9.089	0.987	9.314	0.913	0.7104	
PE(16:0_24:1)	0.242	0.038	0.278	0.021	0.2238	
PE(16:1_18:1)	5.288	0.440	5.445	0.388	0.8048	
	SOL-DMSCs (n = 7)		NIL-DMSCs (n = 7)		p-value	
PE(16:1_18:2)	2.867	0.254	2.868	0.209	1.0000	
PE(16:1_20:4)	0.999	0.057	1.412	0.185	0.0530	

PE(16:1_22:5)	4.184	0.246	5.436	0.671	0.1649
PE(16:1_22:6)	1.189	0.080	1.235	0.135	1.0000
PE(16:1e_16:0)	2.423	0.145	2.806	0.145	0.2086
PE(16:1e_18:1)	89.280	9.193	104.800	9.847	0.2086
PE(16:1e_18:2)	1.751	0.096	2.160	0.279	0.5350
PE(16:1e_20:3)	3.906	0.457	3.607	0.283	0.5350
PE(16:1e_20:4)	74.630	2.647	80.100	4.214	0.4557
PE(16:1e_20:5)	14.240	1.270	13.000	1.277	0.4557
PE(16:1e_22:3)	9.174	0.796	11.690	0.878	0.0728
PE(16:1e_22:4)	22.620	0.751	29.910	3.440	0.1282
PE(16:1e_22:6)	38.200	2.492	45.760	3.081	0.0728
PE(17:0_18:1)	1.625	0.096	1.859	0.204	0.8048
PE(17:0_20:4)	0.370	0.019	0.472	0.036	0.0842
PE(17:0_22:5)	0.737	0.075	0.914	0.058	0.1282
PE(17:1_18:1)	2.231	0.206	2.159	0.132	0.8048
PE(18:0_16:0)	3.786	0.385	4.398	0.280	0.2086
PE(18:0_18:0)	0.213	0.017	0.273	0.030	0.1649
PE(18:0_18:1)	167.700	17.550	194.400	16.010	0.1282
PE(18:0_20:2)	5.243	0.716	5.620	0.402	0.5350
PE(18:0_20:3)-1	20.670	2.182	22.390	2.086	0.8048
PE(18:0_20:3)-2	12.520	1.307	13.140	0.888	0.9015
PE(18:0_20:4)	56.700	5.327	90.190	8.427	*0.0111
PE(18:0_22:3)	6.000	0.826	6.607	0.512	0.3176
PE(18:0_22:4)	17.710	2.097	20.980	1.616	0.1282
PE(18:0_22:6)	11.240	1.211	12.320	1.209	0.7104
PE(18:0e_16:0)	3.274	0.439	4.309	0.380	0.0728
PE(18:0e_16:1)	0.600	0.104	0.724	0.063	0.1649
PE(18:0e_20:4)	0.355	0.087	0.454	0.035	0.0530
PE(18:0e_22:3)	0.165	0.024	0.220	0.025	0.1649
PE(18:1_14:0)	5.807	0.626	6.151	0.552	0.7104
PE(18:1_18:1)-2	85.910	6.250	106.100	10.370	0.0973
PE(18:1_18:2)-1	15.670	0.829	18.890	1.496	0.1649
PE(18:1_18:2)-2	15.680	0.830	18.900	1.495	0.1649
PE(18:1_20:3)-1	4.018	0.343	4.335	0.576	1.0000
PE(18:1_20:3)-2	7.103	0.447	8.496	0.625	0.1649
PE(18:1_20:4)-1	35.270	2.411	53.980	5.915	*0.0175
PE(18:1_20:4)-2	8.563	0.327	9.760	0.901	0.3829
PE(18:1_21:1)	0.645	0.091	0.727	0.059	0.4557
PE(18:1_22:0)	2.202	0.334	2.776	0.408	0.3829
PE(18:1_22:1)	5.725	0.836	7.046	0.618	0.2593
PE(18:1_22:3)	3.265	0.421	3.671	0.142	0.2086
PE(18:1_22:5)	23.700	2.452	32.240	3.033	*0.0262
PE(18:1_22:6)	13.420	1.682	12.210	0.792	0.8048
PE(18:1_23:0)	0.207	0.042	0.262	0.034	0.3176

PE(18:1_24:0)	3.055	0.550	3.924	0.530	0.2593
PE(18:1_24:1)	4.315	0.709	5.343	0.558	0.2086
PE(18:1_24:2)	1.046	0.139	2.121	0.170	***0.0006
PE(18:1e_16:0)	3.955	0.370	4.723	0.502	0.2593
PE(18:1e_18:1)	28.580	4.180	36.050	4.528	0.2086
PE(18:1e_20:1)	2.788	0.313	3.794	0.505	0.2593
PE(18:1e_20:2)	4.281	0.480	5.363	0.588	0.3829
PE(18:1e_20:3)	4.201	0.376	4.238	0.367	0.9015
PE(18:1e_20:4)	77.050	5.222	86.640	7.881	0.3176
PE(18:1e_20:5)	18.570	1.076	21.340	2.050	0.3176
PE(18:1e_22:1)	1.241	0.173	1.669	0.238	0.0973
PE(18:1e_22:3)-1	0.934	0.093	1.200	0.126	0.1282
PE(18:1e_22:3)-2	5.158	0.557	6.893	0.648	0.0728
PE(18:1e_22:4)	17.190	1.793	23.740	2.229	0.0728
PE(18:1e_22:5)	54.780	4.425	76.400	6.481	*0.0175
PE(18:1e_22:6)	32.270	2.708	39.490	3.952	0.4557
PE(18:2e_14:0)	2.093	0.221	2.262	0.165	0.9015
PE(18:2e_16:1)	3.987	0.175	4.285	0.312	0.7104
PE(18:2e_18:1)	26.860	2.743	30.130	3.042	0.5350
PE(18:2e_18:2)	0.959	0.054	1.133	0.080	0.1282
PE(18:2e_20:2)-1	1.870	0.140	2.167	0.230	0.3829
PE(18:2e_20:2)-2	1.671	0.153	2.054	0.165	0.1649
PE(18:2e_20:4)	145.900	6.999	169.800	10.340	0.1282
PE(18:2e_20:5)	8.428	0.755	8.028	0.667	0.4557
PE(18:2e_22:1)	1.711	0.207	2.323	0.298	0.1649
PE(18:2e_22:3)	3.638	0.455	4.715	0.255	0.0973
PE(18:2e_22:4)	11.780	0.894	17.930	1.243	**0.0041
PE(18:2e_22:5)	46.740	2.400	60.130	4.821	*0.0379
PE(18:2e_22:6)	22.730	1.302	28.630	3.546	0.3176
PE(18:3e_22:5)	1.689	0.085	2.354	0.193	*0.0111
PE(19:0_18:1)	0.585	0.059	0.683	0.044	0.1792
PE(19:1_18:1)	2.561	0.272	2.795	0.165	0.3176
PE(20:0_18:1)	3.879	0.470	4.545	0.470	0.5350
PE(20:0e_16:0)	2.380	0.105	3.367	0.370	0.0973
PE(20:0e_18:1)	0.458	0.121	0.681	0.111	0.0728
PE(20:1_18:1)	7.027	0.833	8.033	0.695	0.3176
PE(20:1e_16:0)-1	5.133	1.014	6.714	0.710	0.1282
PE(20:1e_16:0)-2	0.468	0.033	0.575	0.071	0.5350
PE(20:1e_18:1)	1.070	0.212	1.384	0.162	0.0728
PE(20:1e_20:4)	2.875	0.259	3.926	0.529	0.1282
PE(20:1e_22:4)	0.485	0.044	0.748	0.079	**0.0070
PE(20:1e_22:5)	3.805	0.373	5.735	0.696	*0.0262
PE(20:1e_22:6)	0.785	0.078	1.163	0.146	*0.0379
PE(20:2e_22:6)	0.974	0.081	1.250	0.087	0.0530

PE(22:0_20:4)	0.181	0.038	0.252	0.036	0.1649
PE(24:0_20:4)	0.123	0.029	0.163	0.026	0.3706
PE(26:1_18:1)	1.042	0.197	1.315	0.171	0.0973
PE(32:1_18:1)	1.225	0.115	1.098	0.149	0.4557
PE(32:1_20:4)	1.362	0.044	1.147	0.143	*0.0379
PE(32:1_22:5)	2.562	0.136	2.283	0.306	*0.0379
PE(32:1_22:6)	0.658	0.050	0.625	0.092	0.3829
PE(34:1_18:1)	1.264	0.120	1.470	0.268	0.8048
PE(34:1_22:4)	0.496	0.042	0.585	0.097	0.8048
PE(34:1_22:5)	2.910	0.217	3.023	0.465	0.3374
PE(34:1_22:6)	0.932	0.092	1.011	0.170	0.9490
PE(36:1_22:5)	0.827	0.081	0.999	0.188	0.9490

P-value was calculated using Mann-Whitney U test. *p < 0.05. **p < 0.01, ***p < 0.001.

Supplementary table 9. Lipid species identified in Class PG

Lipid species	Amount (ng) per 100 µl sample				p-value	
	SOL-DMSCs (n = 7)		NIL-DMSCs (n = 7)			
	Mean	SEM	Mean	SEM		
PG(16:0_18:1)-1	0.124	0.017	0.200	0.034	0.0728	
PG(16:0_18:1)-2	2.887	0.304	4.121	0.437	0.0728	
PG(16:0_20:4)	0.155	0.018	0.224	0.011	*0.0175	
PG(16:1_18:1)-1	0.304	0.023	0.436	0.061	0.0973	
PG(16:1_18:1)-2	0.448	0.029	0.487	0.039	0.5350	
PG(16:1_22:5)	0.992	0.052	1.238	0.140	0.1792	
PG(18:0_18:1)	2.746	0.248	4.378	0.439	**0.0070	
PG(18:0_20:4)	0.187	0.016	0.281	0.019	**0.0041	
PG(18:1_18:1)-1	2.402	0.177	3.238	0.396	0.1282	
PG(18:1_18:1)-2	1.122	0.079	1.317	0.058	0.0973	
PG(18:1_18:3)	0.314	0.024	0.436	0.056	0.0973	
PG(18:1_20:2)	0.343	0.032	0.476	0.057	0.0728	
PG(18:1_20:3)	0.669	0.058	0.894	0.074	0.0728	
PG(18:1_20:4)-1	1.204	0.077	1.568	0.114	*0.0379	
PG(18:1_20:4)-2	0.213	0.012	0.273	0.017	**0.0070	
PG(18:1_22:3)	0.238	0.039	0.355	0.036	0.0728	
PG(18:1_22:4)	0.991	0.110	0.950	0.074	0.8048	
PG(18:1_22:5)-1	2.252	0.124	2.593	0.190	0.3176	
PG(18:1_22:5)-2	0.916	0.053	1.041	0.060	0.2086	
PG(18:1_22:6)	5.215	0.220	5.307	0.270	0.9015	
PG(18:1D9_20:4)	3.232	0.611	3.491	0.536	0.5350	
PG(18:2_22:6)	1.124	0.034	1.248	0.091	0.3176	
PG(20:0_18:1)	0.047	0.006	0.071	0.011	0.1282	
PG(20:1e_24:1)	1.289	0.228	1.213	0.275	0.7104	
PG(20:2_22:5)	0.346	0.024	0.402	0.022	0.1792	
PG(20:3_22:5)-1	0.095	0.007	0.087	0.008	0.6089	
PG(20:3_22:5)-2	0.108	0.009	0.281	0.028	***0.0006	
PG(20:3_22:5)-3	0.809	0.055	0.856	0.056	0.7104	
PG(20:3_22:6)	1.647	0.076	1.768	0.194	1.0000	
PG(20:5_22:5)	0.183	0.013	0.216	0.022	0.2593	
PG(20:5_22:6)	0.442	0.028	0.438	0.041	0.9015	
PG(22:5_18:2)	0.368	0.080	0.502	0.072	0.1282	
PG(22:5_22:6)	2.330	0.096	2.150	0.203	0.4557	
PG(22:6_22:6)	3.216	0.151	2.691	0.287	0.1282	

P-value was calculated using Mann-Whitney U test. *p < 0.05. **p < 0.01, ***p < 0.001.

Supplementary table 10. Lipid species identified in Class PI

Lipid species	Amount (ng) per 100 µl sample				p-value	
	SOL-DMSCs (n = 7)		NIL-DMSCs (n = 7)			
	Mean	SEM	Mean	SEM		
PI(16:0_16:0)	2.187	0.324	2.842	0.493	0.1649	
PI(16:0_18:1)	33.600	3.662	42.400	3.727	0.0973	
PI(16:0_20:3)	3.392	0.261	3.951	0.363	0.3176	
PI(16:0_20:4)	10.570	0.656	14.210	1.202	*0.0175	
PI(16:0_22:6)	1.958	0.102	2.339	0.197	0.0973	
PI(16:0e_20:4)	0.242	0.039	0.341	0.040	0.1282	
PI(16:1_16:1)	0.971	0.049	1.075	0.087	0.3176	
PI(16:1_18:1)	18.070	0.722	20.370	0.956	0.0728	
PI(16:1_18:2)	4.426	0.178	4.985	0.167	*0.0262	
PI(16:1_20:4)	2.120	0.120	2.583	0.248	0.3176	
PI(16:1_22:5)	3.843	0.173	4.624	0.347	0.1282	
PI(17:0_20:3)	1.201	0.111	1.575	0.114	0.0973	
PI(17:0_20:4)-1	0.588	0.056	0.802	0.079	0.053	
PI(17:0_20:4)-2	1.337	0.116	1.773	0.124	*0.0262	
PI(17:1_16:0)	0.794	0.078	0.998	0.097	0.1282	
PI(17:1_18:1)	1.728	0.130	2.082	0.203	0.2086	
PI(17:1_20:4)	0.565	0.036	0.753	0.071	0.0636	
PI(18:0_16:0)	5.738	0.699	8.181	0.769	*0.0262	
PI(18:0_17:0)	0.241	0.031	0.370	0.041	*0.0175	
PI(18:0_18:1)	34.990	1.753	41.810	2.650	0.1282	
PI(18:0_20:2)	2.108	0.391	3.385	0.566	0.0728	
PI(18:0_20:3)-1	21.540	2.621	30.740	4.549	0.0728	
PI(18:0_20:3)-2	28.650	2.841	31.630	2.687	0.6200	
PI(18:0_20:4)	181.100	9.498	208.900	9.638	0.0728	
PI(18:0_22:3)	2.911	0.401	3.758	0.321	0.1649	
PI(18:0_22:4)	6.074	0.402	7.227	0.477	0.1282	
PI(18:0_22:5)	19.610	1.195	24.560	1.664	0.0728	
PI(18:0e_22:4)	0.221	0.037	0.353	0.042	*0.0379	
PI(18:0e_22:5)	0.490	0.081	0.697	0.103	0.0973	
PI(18:1_14:0)	4.946	0.175	6.144	0.356	**0.0070	
PI(18:1_18:1)	37.110	2.140	41.420	1.951	0.4557	
PI(18:1_18:2)	6.675	0.696	8.114	0.628	0.2593	
PI(18:1_20:3)	3.816	0.447	5.896	0.869	*0.0262	
PI(18:1_20:4)	63.650	3.226	69.270	4.358	0.3176	
PI(18:1_22:3)	1.308	0.176	1.704	0.127	0.1282	
PI(18:1_22:4)	2.877	0.145	3.539	0.156	*0.0111	
PI(18:1_22:5)	8.954	0.944	10.520	0.903	0.0973	
PI(18:1_22:6)	3.861	0.291	3.897	0.215	0.9015	
PI(18:1e_20:3)-1	0.401	0.048	0.539	0.041	0.0728	
PI(18:1e_20:3)-2	0.442	0.048	0.677	0.109	0.0973	

PI(18:1e_20:4)-1	2.129	0.176	2.349	0.255	0.8048
PI(18:1e_22:4)-2	0.488	0.051	0.678	0.041	*0.0297
PI(18:1e_22:5)	1.110	0.139	1.449	0.142	0.0728
PI(19:1_18:1)	1.114	0.087	1.328	0.129	0.2086
PI(19:1_20:4)	1.317	0.080	1.620	0.139	0.0728
PI(22:5_20:4)	0.270	0.021	0.293	0.016	0.4057

P-value was calculated using Mann-Whitney U test. *p < 0.05. **p < 0.01.

Supplementary table 11. Lipid species identified in Class PS

Lipid species	Amount (ng) per 100 µl sample				p-value	
	SOL-DMSCs (n = 7)		NIL-DMSCs (n = 7)			
	Mean	SEM	Mean	SEM		
PS(16:0_18:1)	44.260	3.209	52.310	4.437	0.1649	
PS(18:0_18:1)	41.620	3.858	42.750	2.967	0.7104	
PS(18:0_20:2)	18.130	1.079	27.120	2.245	**0.0041	
PS(18:0_20:3)	10.100	0.947	15.500	2.126	*0.0379	
PS(18:0_22:4)	45.840	3.521	58.450	5.354	*0.0379	
PS(18:0_22:5)-1	89.870	5.878	110.300	12.300	0.2086	
PS(18:0_22:5)-2	47.500	3.904	84.920	7.003	**0.0023	
PS(18:0e_22:5)	6.384	0.359	6.187	0.553	0.9015	
PS(18:1_18:1)	72.900	4.691	89.620	9.076	0.1282	
PS(18:1_22:0)	455.000	51.920	415.600	32.620	0.7104	
PS(18:1_22:1)	27.060	3.194	30.180	2.229	0.3176	
PS(18:1D7_17:0)	1.621	0.124	1.642	0.147	1.0000	
PS(18:1e_18:1)	5.015	0.377	6.492	0.847	0.1649	
PS(18:1e_22:5)	9.188	0.270	10.870	1.358	0.5350	
PS(20:2e_18:1)	1.798	0.104	2.968	0.315	**0.0023	
PS(18:1_24:1) ^a	3.38E+07	6.47E+06	3.51E+07	4.17E+06	0.7199	

P-value was calculated using Mann-Whitney U test. *p < 0.05. **p < 0.01. ^aSince PS(18:1_24:1) had different ion form with the PS internal standard, the amount of lipid (ng) cannot be quantified and thus, the data remained as the peak area per 100 µL sample.

Supplementary table 12. Lipid species identified in class Cer

Lipid species	Peak area per 100 μ L sample				p-value	
	SOL-DMSCs (n = 7)		NIL-DMSCs (n = 7)			
	Mean	SEM	Mean	SEM		
Cer(d16:0_16:0)	7.72E+06 [■]	3.38E+05	8.40E+06	2.42E+05	0.0734	
Cer(d17:1_16:0)	4.07E+06	2.09E+05	5.42E+06	4.28E+05	*0.0175	
Cer(d18:0_16:0)	8.04E+06	7.96E+05	1.11E+07	1.23E+06	0.0728	
Cer(d18:1_14:0)	1.49E+07	1.12E+06	2.31E+07	2.41E+06	*0.0105	
Cer(d18:1_16:0)	1.65E+08	1.09E+07	2.26E+08	2.01E+07	**0.0041	
Cer(d18:1_18:0)	5.72E+06	5.84E+05	8.02E+06	9.06E+05	0.0530	
Cer(d18:1_22:0)	1.19E+07	1.28E+06	1.47E+07	1.31E+06	0.0728	
Cer(d18:1_24:0)	4.00E+07	4.25E+06	4.96E+07	5.46E+06	0.0728	
Cer(d18:1_24:1)	9.00E+07	8.82E+06	1.05E+08	4.99E+06	0.1792	
Cer(d18:1_26:0)	1.78E+06	2.19E+05	2.19E+06	3.39E+05	0.3829	
Cer(d18:2_16:0)	2.57E+07	1.80E+06	3.41E+07	6.22E+06	0.4557	
Cer(d18:2_24:0)	2.86E+07	2.91E+06	3.45E+07	4.35E+06	0.3176	
Cer(d18:2_24:1)	4.55E+07	3.64E+06	4.54E+07	2.60E+06	0.9490	
Cer(d18:2_26:0+O)	2.13E+06 [■]	3.42E+05	2.73E+06	9.56E+05	0.8357	
Cer(d20:0_18:0)	5.70E+06 [■]	3.51E+06	2.90E+06	2.78E+05	0.2949	
Cer(d20:0_26:0)	7.87E+05 [■]	1.92E+05	9.72E+05	2.47E+05	0.8357	
Cer(d20:2_26:0+O)	1.23E+06 [■]	2.98E+05	1.59E+06	5.64E+05	0.9452	
Cer(d22:0_18:0)	8.97E+06 [■]	1.87E+06	1.15E+07	1.59E+06	0.1807	
Cer(d24:0_24:0)	6.78E+05 [■]	1.75E+05	9.14E+05	2.54E+05	0.6282	
Cer(m17:0_18:0+2O)	2.62E+08 [■]	8.55E+06	2.73E+08	9.80E+06	0.6282	
Cer(t18:0_24:0)	2.30E+06	2.93E+05	3.40E+06	8.82E+05	0.6200	
Cer(t42:0+O)	4.40E+06	4.68E+05	5.75E+06	1.22E+06	0.5350	
Cer(t20:0_24:0)	3.58E+06	6.58E+05	4.82E+06	8.95E+05	0.3176	
Cer(t20:0_24:0+O)	1.80E+06 [■]	3.73E+05	2.34E+06	6.52E+05	0.9452	
Cer(t20:0_26:0+O)	1.58E+06 [■]	3.30E+05	2.06E+06	6.21E+05	0.8357	

P-value was calculated using Mann-Whitney U test. *p < 0.05. **p < 0.01. [■] n = 6.

Supplementary table 13. Lipid species identified in class Hex1Cer, Hex2Cer, and Hex3Cer

Lipid species	Peak area per 100 μ L sample				p-value	
	SOL-DMSCs (n = 7)		NIL-DMSCs (n = 7)			
	Mean	SEM	Mean	SEM		
Hex1Cer						
Hex1Cer(d13:0_20:4)	2.42E+07	2.58E+06	3.15E+07	3.03E+06	0.0733	
Hex1Cer(d15:0_20:4)	3.81E+08	4.94E+07	4.72E+08	4.34E+07	0.0728	
Hex1Cer(d18:1_16:0)	8.62E+06 [■]	7.47E+05	1.11E+07	8.59E+05	0.0629	
Hex1Cer(d18:1_24:0)	2.13E+07 [■]	5.75E+06	2.31E+07	2.80E+06	0.2949	
Hex1Cer(d18:1_24:1)	4.68E+07 [■]	9.28E+06	4.87E+07	5.36E+06	0.6282	
Hex1Cer(d18:1_26:1)	3.11E+06 [■]	6.17E+05	3.26E+06	3.52E+05	0.4452	
Hex2Cer						
Hex2Cer(d18:1_24:0)	8.64E+06 [■]	2.05E+06	8.44E+06	9.60E+05	0.7195	
Hex2Cer(d18:1_24:1)	1.80E+07 [■]	2.97E+06	1.79E+07	1.60E+06	0.9430	
Hex3Cer						
Hex3Cer(d18:1_16:0)	1.06E+07 [■]	2.24E+06	1.29E+07	2.24E+06	0.4452	
Hex3Cer(d18:1_22:0)	4.19E+06 [■]	1.57E+06	4.01E+06	9.41E+05	0.9452	
Hex3Cer(d18:1_24:0)	1.18E+07 [■]	4.21E+06	1.15E+07	2.15E+06	0.8357	
Hex3Cer(d18:1_24:1)	2.53E+07 [■]	6.24E+06	2.47E+07	3.40E+06	0.7748	

P-value was calculated using Mann-Whitney U test. [■] n = 6.

Supplementary table 14. Lipid species identified in class SM

Lipid species	Amount (ng) per 100 µl sample				p-value	
	SOL-DMSCs (n = 6)		NIL-DMSCs (n = 7)			
	Mean	SEM	Mean	SEM		
SM(d30:1)	0.436	0.028	0.531	0.020	*0.0350	
SM(d31:1)	0.382	0.023	0.469	0.024	0.0513	
SM(d32:0)	3.666	0.264	4.353	0.229	0.2343	
SM(d32:1)	28.410	1.925	34.530	1.924	*0.0350	
SM(d32:2)	0.913	0.050	1.061	0.048	0.0535	
SM(d33:0)	0.356	0.028	0.428	0.037	0.1375	
SM(d33:1)	10.310	0.643	12.570	0.791	0.0734	
SM(d34:0)	37.000	2.133	40.660	2.376	0.2949	
SM(d34:1)	375.500	20.870	413.300	14.740	0.2949	
SM(d34:2)	25.250	3.318	32.160	2.102	0.1807	
SM(d34:4)	4.443	0.245	4.476	0.273	0.6282	
SM(d34:5)	0.267	0.016	0.305	0.022	0.2949	
SM(d35:0)	0.738	0.077	0.765	0.075	0.9452	
SM(d35:1)	8.496	0.513	9.251	0.690	0.3660	
SM(d35:2)	0.931	0.078	1.072	0.093	0.3660	
SM(d35:4)	1.293	0.077	1.377	0.049	0.2949	
SM(d18:0_18:1)	12.400	1.232	13.940	1.015	0.4452	
SM(d36:2)	4.042	0.261	4.451	0.218	0.4452	
SM(d36:4)	20.070	1.276	18.430	0.963	0.4452	
SM(d36:5)	2.932	0.227	3.392	0.297	0.2343	
SM(d38:1)	3.396	0.382	3.644	0.322	0.7308	
SM(d39:1)	0.618	0.087	0.689	0.059	0.6282	
SM(d39:3)	0.303	0.075	0.312	0.076	0.9452	
SM(d40:0)	1.030	0.202	0.917	0.091	1.0000	
SM(d40:1)	21.520	3.350	21.670	1.955	0.8357	
SM(d40:2)-1	7.842	0.770	8.251	0.368	0.7308	
SM(d40:2)-2	7.637	1.156	8.065	0.731	0.7308	
SM(d41:1)	5.971	0.909	5.698	0.431	0.9452	
SM(d41:2)-1	6.343	0.699	6.383	0.339	1.0000	
SM(d41:2)-2	2.453	0.374	2.714	0.281	0.6282	
SM(d42:1)	6.957	1.222	7.033	0.206	0.3660	
SM(d18:1_24:0)	43.790	7.854	39.570	3.575	0.9452	
SM(d42:2)	199.900	23.330	204.500	7.130	0.5338	
SM(d42:3)	54.900	1.992	58.270	2.052	0.2343	
SM(d18:1_24:2)	69.340	7.658	71.010	3.810	0.6282	
SM(d42:4)	9.596	0.664	9.341	0.365	0.7308	
SM(d18:1_24:3)	6.638	0.769	6.425	0.489	1.0000	
SM(d43:1)	1.013	0.144	0.878	0.063	0.5338	
SM(d43:2)	0.643	0.080	0.637	0.042	0.7308	
SM(d20:1_23:1)	3.014	0.442	2.320	0.268	0.2343	
SM(d43:3)	1.239	0.177	1.325	0.122	0.3660	

SM(d43:4)	2.738	0.313	2.680	0.168	0.9452
SM(d43:5)-1	1.482	0.080	1.416	0.048	0.6282
SM(d43:5)-2	0.730	0.054	0.742	0.051	0.7308
SM(d44:1)	0.555	0.110	0.512	0.047	1.0000
SM(d44:2)	5.111	0.840	4.584	0.306	0.8357
SM(d44:3)	3.285	0.539	3.188	0.196	0.8357
SM(d44:4)-1	1.690	0.222	1.516	0.136	1.0000
SM(d44:4)-2	20.400	2.814	18.610	1.300	0.9452
SM(d44:5)	56.210	4.496	51.080	2.463	0.3660
SM(d44:6)-1	7.718	0.393	6.978	0.289	0.2343
SM(d44:6)-2	7.766	0.396	7.018	0.290	0.2343
SM(t34:0)	0.420	0.048	0.371	0.031	0.3660
SM(t34:1)	2.644	0.226	2.505	0.162	0.2343
SM(t34:2)	0.532	0.044	0.490	0.026	0.2949
SM(t39:1)	0.281	0.020	0.285	0.012	0.4744
SM(t42:1)	0.932	0.106	0.878	0.043	0.8357

P-value was calculated using Mann-Whitney U test. *p < 0.05.

Supplementary table 15. Lipid species identified in class TG

Lipid species	Amount (ng) per per 100 μ L sample				p-value	
	SOL-DMSCs (n = 6)		NIL-DMSCs (n = 7)			
	Mean	SEM	Mean	SEM		
TG(15:0_15:0_15:0)	0.555	0.018	0.601	0.023	0.1979	
TG(15:0_16:0_16:0)	0.267	0.016	0.374	0.035	*0.0149	
TG(16:0_14:0_14:0)	0.252	0.032	0.331	0.015	0.1136	
TG(16:0_14:0_16:0)	0.550	0.034	0.839	0.071	**0.0080	
TG(16:0_14:0_16:1)	0.508	0.038	0.734	0.083	0.1807	
TG(16:0_14:0_18:1)	1.337	0.095	1.966	0.273	0.1807	
TG(16:0_16:0_16:0)	0.907	0.095	1.304	0.101	*0.0221	
TG(16:0_16:0_18:1)	2.215	0.340	3.411	0.485	0.1973	
TG(16:0_16:0_22:6)	0.247	0.025	0.339	0.064	0.3498	
TG(16:0_16:1_18:1)	3.267	0.256	4.850	0.661	0.1014	
TG(16:0_16:1_22:6)	0.802	0.071	1.079	0.240	0.5338	
TG(16:0_17:0_18:1)	0.268	0.040	0.440	0.061	0.1315	
TG(16:0_17:1_18:1)	0.497	0.055	0.707	0.108	0.3660	
TG(16:0_18:1_18:1)	3.937	0.341	5.313	0.839	0.2949	
TG(16:0_18:1_20:1)	1.600	0.179	2.266	0.445	0.3660	
TG(16:0_18:1_22:6)- 1	1.598	0.141	2.184	0.401	0.2833	
TG(16:0_18:1_22:6)- 2	0.853	0.054	1.109	0.194	0.2949	
TG(16:0_20:4_18:1)	2.563	0.306	3.801	0.690	0.1375	
TG(16:1_14:0_18:1)	0.905	0.074	1.306	0.185	0.1807	
TG(16:1_16:1_14:0)	0.248	0.022	0.360	0.048	0.1156	
TG(16:1_16:1_18:1)	1.627	0.107	2.394	0.371	0.2343	
TG(16:1_18:1_18:1)	3.365	0.229	4.599	0.732	0.1807	
TG(16:1_18:1_18:2)	1.878	0.111	2.761	0.468	0.2343	
TG(16:1_18:1_18:3)	1.072	0.076	1.637	0.295	0.1807	
TG(16:1_18:1_22:4)	3.725	0.224	5.184	1.021	0.2949	
TG(16:1_18:2_22:5)	0.912	0.061	1.183	0.219	0.2949	
TG(18:0_16:0_16:0)	0.513	0.053	0.674	0.064	0.0861	
TG(18:0_18:0_16:0)	0.225	0.015	0.281	0.035	0.2458	
TG(18:0_18:1_16:0)	0.988	0.124	1.473	0.223	0.1807	
TG(18:0_18:1_18:0)	0.228	0.027	0.304	0.059	0.1000	
TG(18:0_18:1_20:1)	0.208	0.028	0.283	0.052	0.3166	
TG(18:0_18:1_20:4)	1.022	0.066	1.524	0.287	0.2525	
TG(18:1_14:0_22:5)	1.962	0.113	2.729	0.487	0.1807	
TG(18:1_17:1_18:1)	0.433	0.066	0.584	0.093	0.1979	
TG(18:1_18:1_18:1)	3.622	0.239	4.663	0.868	0.2949	
TG(18:1_18:1_20:2)	1.908	0.147	2.574	0.433	0.1979	
TG(18:1_18:1_20:3)	1.475	0.128	1.987	0.333	0.2949	

TG(18:1_18:1_22:2)	0.473	0.046	0.607	0.103	0.3166
TG(18:1_18:1_22:3)	0.973	0.086	1.287	0.230	0.1807
TG(18:1_18:1_22:4)	1.442	0.095	1.929	0.379	0.4744
TG(18:1_18:1_22:5)	2.360	0.171	3.094	0.591	0.2240
TG(18:1_18:1_22:6)	1.513	0.106	2.050	0.394	0.2949
TG(18:1_18:2_18:1)	2.477	0.251	3.077	0.437	0.3660
TG(18:1_18:2_24:2)	0.272	0.029	0.340	0.057	0.2227
TG(18:1_20:2_22:3)	0.447	0.047	0.556	0.104	0.4308
TG(18:1_20:2_22:5)	0.947	0.088	1.256	0.248	0.1979
TG(18:1_20:3_22:5)	0.637	0.056	0.807	0.157	0.3660
TG(18:1_22:3_22:5)	0.332	0.044	0.423	0.086	0.2949
TG(18:1_22:4_18:1)	0.488	0.050	0.593	0.102	0.3907
TG(20:1_18:1_18:1)	0.808	0.072	1.126	0.190	0.2949
TG(20:1_18:1_22:5)	0.757	0.067	0.953	0.179	0.3660
TG(18:1_22:4_22:5)	409.800	24.720	592.400	99.400	0.2343

P-value was calculated using Mann-Whitney U test. *p < 0.05. **p < 0.01.

Supplementary table 16. Lipid species identified in Class AcCa and Co

Lipid species	Peak area per 100 μ L sample				p-value	
	SOL-DMSCs (n = 6)		NIL-DMSCs (n = 7)			
	Mean	SEM	Mean	SEM		
AcCa						
AcCa(16:0)	2.48E+06	0.46E+06	7.43E+06	1.69E+06	**0.0047	
Co						
Co(Q10)	9.29E+06	1.51E+06	9.84E+06	0.96E+06	0.6282	

P-value was calculated using Mann-Whitney U test. **p < 0.01.

Appendix VI. Review article manuscript

Ageing in Human Parturition: Impetus of the Gestation Clock in the Decidua

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Abstract

Despite sharing many common features, the relationship between ageing and parturition remains poorly understood. Human decidua is a specialised lining of endometrial tissue, which develops to prepare for pregnancy. The structure and location of the decidua supports its role as the physical scaffold of the growing embryo and placenta, and thus it is vital to sustain pregnancy. Approaching term, the physical support properties of the decidua are naturally weakened to permit parturition. In this review, we hypothesize that the natural weakening of decidual tissue at parturition is promoted by the ageing process of the decidual cells. We reviewed accumulating studies of the functional and molecular changes in decidua at parturition and classified those changes using the hallmarks of ageing proposed by López-Otín et al. (2013) as the framework. In primary ageing hallmark category, (1) increased DNA damage leading to apoptosis, (2) decreased telomere length leading to senescence, (3) reduced DNA methylation and histone acetylation as well as increased nuclear localization of the components of NFκB (p65), and (4) elevated level of Hsp-70 protein associated with decreased autophagy, were observed in the decidua as parturition approaches. In antagonistic ageing hallmark category, (1) increased mTORC1 signalling pathways, (2) accumulation of senescent cells and their secreted molecules known as Senescence Associated Secretory Phenotype (SASPs), and (3) increased ROS production and decreased antioxidant systems resulting in the disruption in mitochondrial biogenesis and autophagy (mitophagy), were observed in the decidua at parturition. In integrative ageing hallmark category, (1) less intimate cell-to-cell contact because of cell degeneration, and (2) upregulation of various pro-inflammatory signals (i.e., SASPs) released by the senescent cells, were observed in the decidua at parturition. Given that stem cell exhaustion is also one of the integrative ageing hallmarks, decidual mesenchymal stem/stromal cells (DMSCs), which are the predominant stem cells in the decidua, may also decline in numbers and functions to promote parturition. DMSCs may promote or facilitate parturition either directly through the decline of its regenerative functions or via paracrine secretion of extracellular vesicles (EVs) containing parturition-related miRNAs. All these findings support that the decidua age as the pregnancy progresses and this may promote and/or facilitate parturition, emphasizing a notion that ageing is the probable impetus of the gestational clocks in the decidua. This conceptual framework was created to provide a better understanding of the natural ageing process of decidua during parturition, hoping to raise awareness and encourage future studies of the importance of healthy ageing for healthy pregnancy outcomes.

Keywords: ageing hallmarks, parturition, decidua, gestational clocks, mesenchymal stem/stromal cells, extracellular vesicles, miRNA

Introduction

During the secretory phase of each menstrual cycle, the maternal endometrium develops a specialised lining of tissue, called the decidua, which is an important requirement for preparing, establishing and maintaining the pregnancy [1]. The main roles of the decidua are to facilitate proper embryo reception (i.e., recognition, selection, and acceptance), develop immune tolerance to protect the allogeneic embryo from possible attacks by the maternal immune cells, and to provide a physical scaffold as well as nutrients for the developing embryo prior to the development of the mature placenta [1, 2].

Recent studies show that the decidua progressively ages as pregnancy approaches term parturition [3], while premature and accelerated ageing of decidua are associated with pre-term parturition [4, 5]. Furthermore, ageing of the decidua may play an essential role in the initiation of labour, and thereby, be involved in determining the timing of parturition [6, 7]. Nevertheless, the current evidence that ageing, particularly of the decidua, is beneficial or even essential for natural parturition at term, is limited. Thus, there are ample opportunities for further investigation. Here, we review current findings and knowledge of the association between ageing, timing of parturition, and the decidua. Furthermore, the possible roles of important resident cells of the decidua, particularly mesenchymal stem/stromal cells (MSCs) and their secreted extracellular vesicles, will be reviewed in the framework of an “ageing-decidua-parturition axis”. Finally, we review the general advantages and disadvantages of models for studying the “ageing-decidua-parturition axis”.

General concept of biological ageing

Definition and evolutionary perspective of ageing

Human ageing is a complex process, which is difficult to define as it encompasses multiple processes operating at many levels from the single cell to the whole organism (e.g., molecular, cellular, physiological and psychosocial). Ageing can be defined as the accumulation of various physiological changes that lead to the decline of function over time [8, 9]. From an evolutionary perspective, ageing is considered to result from the accumulation of permanent damage as a consequence of the body's decision to limit energy and resources devoted to tissue repair at the level necessary for restoring the ability to reproduce [10, 11]. This strategy is important to ensure successful continuation of generations during a limited lifespan [12]. The perceptible outcomes of this survival strategy are loss of vigour and increased risk of diseases due to the overwhelming build-up of permanent damage over time [10]. Studies show that various common human chronic diseases including neurodegenerative diseases, cardiovascular disorders, cancer, and diabetes are strongly associated with advancing age [13, 14]. Therefore, although ageing occurs throughout life, it manifests most intensely at post-reproductive age, which is about the age of 50 and older in humans [11].

The multiple features of ageing: the impetus of the clock of life

Ageing is proposed to initiate, drive, and ultimately terminate a person's life by exerting different effects at various development stages (Figure 1). In contrast to the detrimental effects of advanced adult ageing (e.g., loss of vigour, increased morbidity and mortality risks) [15], recent evidence shows that ageing may play beneficial roles in early human development by facilitating pregnancy and parturition [16, 17]. For example, at the early stages of gestation, ageing of the syncytiotrophoblast layer is essential to maintain normal placental development and function required to sustain pregnancy [16]. At the later stages of gestation, the ageing of various gestational tissues, e.g., the fetal membranes [18, 19] and decidua [3, 4], may weaken various physical supports for the fetus [19] and promote the secretion of various cellular signalling molecules (e.g., pro-inflammatory cytokines) required for labour and parturition [3, 20]. After birth, the ageing features accumulate over time and begin to intensify post-adulthood as the dysfunctional burden of accumulated aged cells/tissues outpaces stem cell rejuvenation capacity. The stem cell population may also undergo age-related exhaustion (i.e., decrease in cell number and function) [21, 22]. This concept of stem cell exhaustion may also apply when ageing occurs to promote parturition in healthy term pregnancies, yet this area has not been fully investigated. In contrast to the extensive studies performed on the detrimental effects of ageing associated with advanced age, the beneficial effects of ageing at the early stages of life, particularly in association with parturition, are rarely explored despite many shared common features.

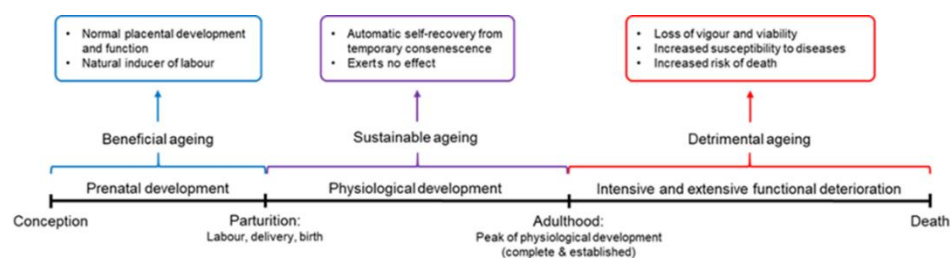


Figure 1. Various features of ageing throughout human's clock of life

Ageing can be beneficial, sustainable, or detrimental depending on the development stage of life. At the beginning of life, ageing is beneficial for sustaining pregnancy and promote parturition. During childhood and adulthood, ageing exerts no perceptible effect as the tissue repair system functions at its peak to support successful reproduction. Post-adulthood, the detrimental effects of ageing begin to take place with death being the endpoint.

The hallmarks of ageing

In 2013, Lopez-otin et al. proposed nine hallmarks of ageing, which are classified into three groups, i.e., primary, antagonistic, and integrative hallmarks, based on the similarity of their characteristics [23] (Figure 2). These hallmarks are commonly expressed in aged organisms, including mammals, and were determined based on the following criteria: manifested during normal ageing, cause acceleration of ageing if experimentally exacerbated, and hinder normal ageing process if experimentally alleviated [23].

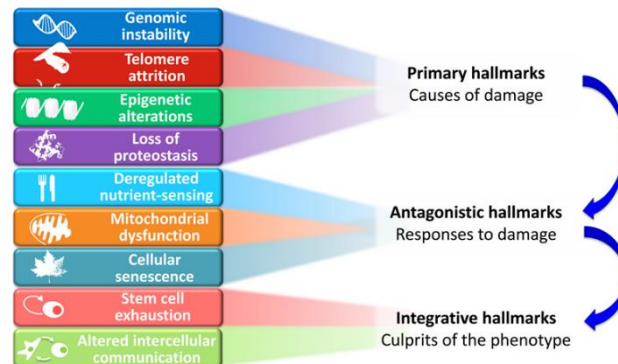


Figure 2. The hallmarks of ageing

Nine hallmarks of ageing were classified into three groups (primary, antagonistic, and integrative) to conceptualize the relationships between the main denominators of ageing. Further details on each hallmark can be obtained in [23]. Image is adapted from [23].

Since these hallmarks of ageing were proposed, many studies have employed them to describe the effect or process of ageing in a variety of study models or cases e.g., age-related diseases, either focusing on one hallmark [24-26], a combination of several hallmarks [27, 28], or all the hallmarks as a conceptual framework [29-32]. Later in this review, these hallmarks of ageing will also be used as the framework to systematically describe the ageing process of the decidua during gestation, and to propose the association of ageing with parturition.

General concept of human parturition

Human Parturition: Labour and Delivery

Parturition is the action or process of giving birth to offspring. This natural process comprises labour and delivery, and these terms are often used interchangeably with parturition. Labour is characterised by the regular and painful myometrial contractions that increase in duration, intensity, and frequency as the time of birth approaches [33]. Labour follows the withdrawal of inhibitory factors that maintain myometrial quiescence during pregnancy (e.g., progesterone, prostacyclin, relaxin, and nitric oxide) [34]. Once initiated, myometrial contractility is stimulated in response to the secretion of uterotropins (i.e., estrogen) and uterotonins (e.g., oxytocin) by endocrine glands [34], and by the production of pro-inflammatory factors e.g., prostaglandins (PGs), mainly PG-F_{2α} and PG-E₂, by the decidua and fetal membranes respectively [35-37], which act through the cyclooxygenase (COX)-2 pathway [38]. The main feature of myometrial re-activation and stimulation is the upregulation of specific contraction-associated myocytes and major gap components (i.e., connexin 43), which are required to transmit the contractile signals between myocytes [34, 39]. Besides the myometrial contractility, labour is also characterised with cervical ripening, which includes cervical dilatation and effacement, loss of the mucus plug, fetal membrane rupture, and leakage of amniotic fluid leading to the birth and delivery of the neonate, as well as placental detachment. These phenomena are clinically referred to as the three stages of labour [40]. Besides the mechanical stretching, endocrine regulation, and production of inflammatory factors [41] as described above, various studies associate labour with other molecular and biochemical pathways including apoptosis [42], oxidative stress [43], lipid metabolism [44], and remodelling of the uterine and cervical extracellular matrix (ECM) [45].

Timing of parturition: the key to pregnancy success and perinatal outcome

Human parturition normally occurs between 37–42 weeks of gestation, the period that is commonly called “term”. The American College of Obstetricians and Gynaecologists Committee (2013) re-defined “term” parturition into several specified periods i.e., early-term (37 weeks 0 days to 38 weeks 6 days), full-term (39 weeks 0 days to 40 weeks 6 days), late-term (41st week) and post-term (42 weeks and beyond) [46]. The re-definition of “term” parturition was necessary to address differences in the risk of

adverse perinatal outcomes throughout the “term” period (e.g., respiratory morbidity, stillbirth, and fetal growth restriction), with a higher risk observed in early and late/post-term [47, 48].

The timing of parturition varies between species and among individuals within the same species. The discrepancy between species indicates differences in the time to accommodate complete fetal growth before birth. Discrepancies within the same species (e.g., pre, early, full, late, and post-term period in human) indicates the existence of more than one regulatory mechanism, widely known as “gestational clocks”, that determine the end-point of human pregnancy. The gestational clocks work in diverse and redundant manners to compensate for the possibility that one or more regulatory pathway(s) may fail to occur at the appropriate time (as reviewed in [17, 49]).

Various type of gestational clocks are proposed and further classified based on the tissue compartments (i.e., fetal membrane, decidual, myometrial, placental, and fetal clocks) or the molecular pathways involved (i.e., endocrine, immune, proteomic, and epigenetic clocks) (Figure 3). In brief, the fetal membrane clock refers to the initiation of parturition by fetal membrane senescence [19]. The decidual clock signifies the initiation of parturition by the upregulation of inflammatory signals secreted by the decidua [20]. The myometrial clock refers to the initiation of parturition by increased inflammatory load on the gravid myometrium leading to progesterone withdrawal [17]. The placental and fetal clocks signify an increase in corticotrophin-releasing hormone (CRH) secretion by placental trophoblasts [50] and an upregulation of the fetal hypothalamic pituitary adrenal (HPA) axis to stimulate various hormonal cascades required for the maturation of fetal organs e.g., lungs [51] as pregnancy approaches term. The endocrine clock refers to the roles of fetal and placental hormones e.g., CRH and HPA axis during the onset of labour [17]. The immune clock is based on the chronology of immune cell behaviours in peripheral blood, their interconnected pathways and changes as pregnancy progresses [52]. The proteomic clock refers to the dynamic changes of the plasma proteome during pregnancy [53]. The epigenetic clock is based on the regulation of DNA methylation in association with labour [54, 55].

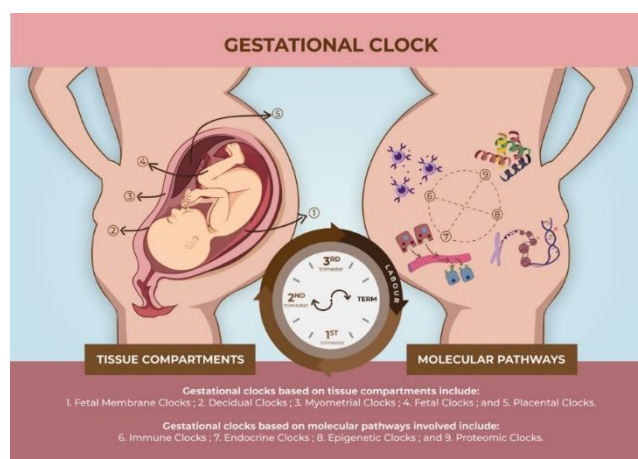


Figure 3. Various types of proposed gestational clocks

Despite abundant evidence of various gestational clocks, the mechanisms by which these gestational clocks sequentially or simultaneously work to initiate parturition remain unclear. The primary source of labour-inducer signals is also controversial with some studies favour the fetal membranes (as reviewed in [17]), while other studies favour the decidua (as reviewed in [20]). In this review, regardless of the origin, we propose ageing as the driving force behind the gestational clocks because many hallmarks of ageing are present in various gestational tissues during parturition and aberrant ageing of the gestation tissues e.g., placenta, fetal membranes, and decidua are strongly associated with pre-term parturition (as reviewed in [16]).

Building the link between parturition and ageing

Ageing and parturition share common features. First of all, increased sterile inflammatory responses are evident in both cases. The onset of labour involves the release of the endogenous sterile inflammatory stimuli (e.g., the nuclear protein high-mobility group box 1 HMGB1 [56], and cell-free fetal DNA [57]) by various gestational tissues. These stimuli activate Toll-like Receptor (TLR) pathways, which in turn trigger nuclear factor NF κ B to increase the transcription of many genes, particularly those encoding the inflammatory cytokines [58, 59], such as interleukin (IL)-1 β , IL-6, IL-8, IL-10, granulocyte macrophage colony-stimulating factor (GM-CSF), and tumour necrosis factor (TNF)- α [60, 61]. A significant

increase of IL-1 β , IL-6, and IL-8 is observed in the cervix and myometrium, interferon- γ (IFN- γ), IL-6 and IL-8 in the chorio-decidua, as well as IL-1 β and IL-8 in the amnion during labour at term [62, 63]. The elevation of pro-inflammatory cytokines in turn triggers the three stages of labour by stimulating the production of prostaglandins (PGs) and matrix metalloproteinases (MMPs). Cervical ripening involves the increase of MMP-1, MMP-3, MMP-8, MMP-9, cathepsin S, COX-2, and PG-E₂ [64, 65]. Membrane rupture involves the increase of MMP-9, collagenases, and PGs as well as the decrease of tissue inhibitor of metalloproteinase (TIMP)-2 [64, 65]. Myometrial contractility involves the increase of COX-2, PG-E₂, and oxytocin receptor that stimulate myocyte contraction [64, 65].

In relation to ageing, accumulating evidence shows the chronic and progressive increase of pro-inflammatory factors, known as “inflamm-ageing” [66], is one of the main features of ageing. With regard to the hallmarks of ageing, “inflamm-ageing” belongs to the integrative hallmarks, particularly in the altered intercellular communication category. Furthermore, most of the parturition-associated pro-inflammatory mediators mentioned above are also members of the senescence associated secretory phenotype (SASP) [67, 68]. SASP is a group of pro-inflammatory cytokines, chemokine, growth factors, and proteases commonly secreted by senescent cells. Senescent cells are characterised by the permanent growth arrest, morphological changes (e.g., overall increase in cell size and distorted nuclei), the presence of senescence-associated heterochromatin foci (SAHF), resistance to apoptosis, as well as increased nuclear factor NF κ B, which, as explained above, contribute to the parturition-related inflammation pathway [69]. Recent studies show that senescent cells accumulate in tissue with increasing age [70, 71]. Senescence is also implicated as a primary cause of age-related diseases [72] since experimental clearance of senescent cells in mouse models delays the appearance of ageing-related phenotypes and extends the health span of mice (as reviewed in [73]). In association with “inflamm-ageing”, it is proposed that chronic inflammation during the ageing process is promoted through the secretion of SASP by the senescent cells [68, 74]. Based on this understanding of ageing and parturition, it is likely that ageing of various gestational tissues contributes to increased production of pro-inflammatory factors with impending parturition, with cellular senescence as a possible underlying mechanism. Nevertheless, further investigation is still required on the type of aged cells or gestation tissues that contribute to the increase of the pro-inflammatory signalling during parturition. The threshold level of pro-inflammatory mediators required for normal parturition or their aberrant level associated with major pregnancy disorders may be important for the study.

Another common feature shared by ageing and parturition is weakening of physical support. This occurs in parturition, during labour, and indicates programmed tissue disintegration by the resident cells to promote the delivery of the fetus. In many studies, most notably those of muscle tissues, the loss of tissue integrity/strength is strongly associated with stem cell exhaustion during the ageing process [75-77]. Therefore, it is likely that as pregnancy approaches term, stem cell exhaustion is naturally scheduled to reduce gestation tissue integrity and allow parturition. There are various possible causes of the gestation tissue-derived stem cell exhaustion. Recent evidence shows that inflammation, through the prolonged secretion of SASP, may impair normal stem cell function and behaviour, and thus hamper tissue repair and regeneration in aged organisms [78-80]. As parturition is strongly related to inflammatory pathways [81], these findings provide indirect support to inflammation as the possible cause. Nonetheless, additional studies are required to confirm whether the exposure period and level of pro-inflammatory mediators circulated during pregnancy affect stem cell function leading to labour.

In addition to inflammation, stem cell exhaustion can also result from DNA damage and/or increased exposure to reactive oxygen species (ROS) [i.e., by exposure to oxidative stress], as commonly occurs in aged organisms [82]. Similarly, high exposure to oxidative stress is also observed at term pregnancy. The labouring gestational tissues are constantly exposed to high oxidative stress due to increased pro-inflammatory stimuli and prolonged exposure to circulating maternal factors. During delivery, various physiological changes in the uterine environment further elevate ROS production. These changes include alternating ischemia and reperfusion due to suppressed uteroplacental blood flow, a rapid transition from hypoxic intra-uterine to oxygen-rich extra-uterine environment, and higher oxygen consumption resulting in increased mitochondrial respiration [18, 83]. Higher lipid peroxidation and protein oxidation as well as lower expression of antioxidant enzymes e.g., glutathione peroxidase (GSHPx) are also detected in labour-myometrial samples, indicating an increased susceptibility of the labouring myometrium to oxidative damage [84]. With regard to stem cells, it is possible that gestation tissue-derived stem cells are also subjected to the similar oxidative stressors approaching parturition. Nevertheless, additional studies, particularly on the type of stem cell function affected, are required to obtain a better understanding of the role of stem cells in labour. In summary, the proposed association between ageing and parturition is shown on Figure 4 below.

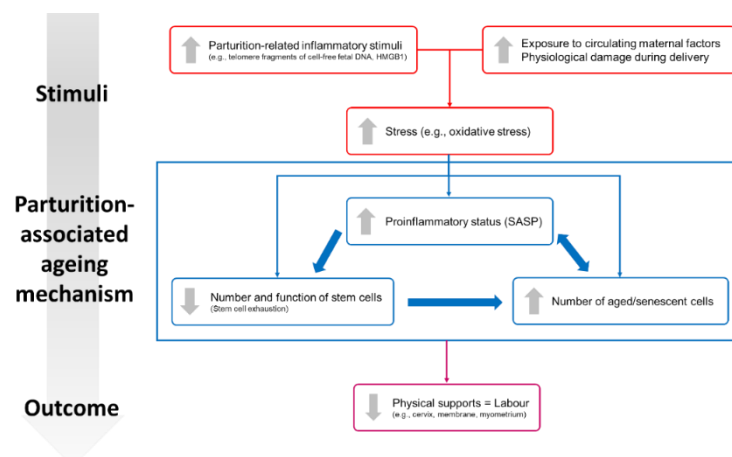


Figure 4. Proposed association between ageing and parturition

Approaching parturition, increased exposure to inflammatory stimuli, prolonged exposure to circulating maternal factors, and various physiological events during delivery induce oxidative stress within various gestation tissues. As a result, the production of pro-inflammatory factors, the number of senescent cells, and decreased number and function of stem cells within the tissue increase. These factors work in a cycle-like mechanisms with a similar outcome, which is the weakening of physical support (clinically referred as labour).

Decidua, decidual ageing, and parturition

General features of human decidua

The human endometrium routinely undergoes cyclical changes. The cycle begins with the **proliferative phase** where there is rapid proliferation of endometrial epithelium and stromal cells, resulting in a thickened endometrial lining. This is followed by the **secretory phase**, which is characterised by the differentiation of the glandular epithelium into its highly secretory state, the differentiation of the stromal cells from elongated, fibroblast-like mesenchymal cells into rounded, epithelioid-like cells, and vascular remodelling. The cycle ends with **menstruation**, which features the shedding of the thickened and differentiated endometrial lining, and **menstrual repair** where endometrial tissue and blood vessels are restored [85, 86].

The decidua is a specialised tissue lining resulting from the changes occurring during the secretory phase of the human endometrium. In pregnancy, the decidua is characterised by three regions: *decidua capsularis*, *decidua parietalis*, and *decidua basalis* [87]. The decidual region that surrounds the conceptus is the *decidua capsularis*. By week 22, *decidua capsularis* will fuse with *decidua parietalis*, which was the remaining decidual lining of the uterus, and attach to the fetal membranes, thus eliminating the uterine cavity [87]. *Decidua basalis* is the portion of the decidua that remains attach to the maternal side of the placenta during placental detachment [88]. The boundary area where the decidua is separated from the uterine wall at parturition is known as the **zone of separation** [89].

The formation of the decidua, called decidualisation, from the human endometrium is one of the essential events for a successful pregnancy. The process begins in response to elevated levels of estradiol (bioactive estrogen) and progesterone during the mid-secretory phase of the endometrial cycle, approximately 6 days after ovulation [1, 88]. Since the final structure of the decidua is highly receptive to the allogeneic embryo, decidualisation is essential for implantation and placenta formation. Impaired cyclic decidualisation, as shown with decreased expression of the decidual marker–prolactin, results in recurrent pregnancy loss [90]. Impaired decidualisation may encourage miscarriage in subsequent pregnancies by disrupting the natural process of embryo recognition and selection as well as the maternal responses to embryonic signals during implantation [90]. This evidence suggests that the decidua is essential as the physical scaffold during pregnancy and the loss of decidua or its physical support properties may result in the termination of pregnancy.

The properties of the decidua may be attributed to its major resident cells. One major resident cell type in the decidua is the decidualised stromal cells (DSCs) [91]. DSCs have large round nuclei, high numbers of nucleoli, accumulated glycogen and lipid droplets in the cytoplasm, dense membrane-bound secretory granules, and expanded rough endoplasmic reticulum and Golgi complex [85]. These cells are characterised by their expression of progesterone receptors and specific surface markers (e.g., Zn-dependent metallo-proteinases such as CD10, CD13, and CD21). DSCs also secrete a characteristic set of markers [e.g., prolactin, plasminogen activator inhibitor (PAI-1), insulin-like growth factor binding

protein 1 (IGFBP-1), and vascular endothelial growth factor (VEGF)] as well as various cytokines (e.g., IL-1, IL-11, and IL-15) [92]. Despite their well-defined characteristics, an understanding of the DSC functions, particularly during parturition, is still limited.

Decidual ageing and its potential role in parturition

The decidua is crucial for implantation of the blastocyst, supporting the early embryo, as well as anchoring and maintaining the developing placenta, and because of these important roles, the decidua remains the subject of intense research [1, 93]. In contrast, much less is known about the role of the decidua in the late stages of pregnancy, particularly during parturition where the physical support properties of the decidua may be weakened because of the ageing process. In this section, the general feature of decidual ageing and its potential role in parturition will be discussed in relation to the hallmarks of ageing proposed by Lopez-otin et al. (2013). Most evidence is derived from animal model studies, which provide insight into possible decidual ageing mechanisms in human. However, animal models of human decidua have significant limitations and where possible evidence from human studies is presented.

Physical features of decidual ageing

Studies of rabbit placentae by Larsen (1963) and Samuel et al. (1975) reported the physical phenotypes of decidual ageing. As gestational age progresses, the degeneration of decidual tissue (e.g., progressive decline in decidual thickness and strength of attachment to the uterine wall) was observed, and was proposed to assist the placental expulsion stage of labour [89, 94]. Ultrastructural observation revealed that at parturition, the degenerated DSCs and their fibrous debris accumulated at the “zone of separation”, and consequently, reducing the cell-to-cell interaction between the endometrium and placenta [94]. These deteriorating phenotypes occur to various degrees throughout the decidual tissue at term gestation (28–31 days) and peak at day 31 of gestation when most DSCs begin to break down and undergo fragmentation [89].

Genomic instability, decidual ageing, and parturition

A recent study of the early murine decidua at day 5 of gestation reported an increase in DNA damage (detected by DNA damage marker γ -H2AX) because of extensive proliferation of the epithelium and stromal cells during decidualisation. Increased DNA damage [detected by the upregulation of ribonucleotide reductase 2(RRM2)] also promotes decidualisation during early pregnancy in mice since administration of specific RRM2 inhibitors inhibits decidualisation [95]. While some progress has been made in understanding the role of DNA damage in the decidua in early pregnancy, its role in parturition at the late stage of gestation is poorly understood. A study of rodent deciduae suggested strong association between decidual tissue degeneration and apoptosis of DSCs, which is characterised by increased nucleosomal DNA fragmentation [96]. Increased DNA damage is strongly associated with the ageing process [23]. In both the anti-mesometrial (later forming the *decidua capsularis*) and mesometrial regions (later forming the *decidua basalis*) of the rodent decidua, the degree of DNA fragmentation increased with time resulting in the apoptosis of DSCs in both tissues [96]. Since *decidua capsularis* and *decidua basalis* are in direct contact with the fetal membranes, and with the maternal side of the placental disk respectively, it is likely that the progressive regression of these regions contribute to the detachment of the fetal membranes and placenta from the uterine wall at parturition.

Telomere attrition, decidual ageing, and parturition

Bonney et al. (2016) used a murine model of telomere attrition to show that the telomere length decreased as a function of gestational age in fetal membranes, placenta and decidua [97]. Telomere attrition is regarded as one of the primary hallmarks of ageing and considered as the common, causative factor of cellular senescence [98]. As term approaches, progressive activation of p38 mitogen-activated protein kinase (MAPK), which regulates telomere-dependent senescence, accompanies the progression of telomere attrition in decidua, placenta, and fetal membranes [97]. Based on the SA- β -Gal assay, which is a distinctive measure of cellular senescence, Bonney et al. (2016) reported telomere-dependent cellular senescence in the placenta and decidua, however, the extent of cellular senescence was lower than that in the fetal membranes. Since the effect of senescent cells on parturition was strongly associated with their secreted SASPs, Bonney et al. (2016) described several potential SASP genes that may be activated in the decidua in relation to ageing or senescence during parturition in the mouse. The potential role of decidual telomere attrition in parturition noted in this study was consistent with previous results in placental samples (as reviewed in [99]). Previous findings in the placenta indicate that the onset of labour is initiated by the shortening of telomeres, leading to apoptosis of placental trophoblast and chorion cells that, subsequently results in the release of fetal cell-free DNA, which

induces oxidative stress and the innate inflammatory responses (e.g., via TLR-9) required for labour [99]. This mechanism may also apply to the decidua, however, further studies are needed for verification.

Epigenetic alterations, decidual ageing, and parturition

Reduced DNA methylation associated with increased TLR-2 and TLR-9 gene expression was detected in decidual tissue, specifically in decidual leukocytes, from term–not in labour, term–labouring, and spontaneous preterm birth patients [100]. In general, DNA methylation affects gene expression by regulating gene transcription [101]. Ageing is proposed to be associated with both hypo- and hyper-methylation, which may contribute to functional decline by initiating chromosome instability through heightened gene responsiveness (hypo-methylation) or by highly suppressing gene expression (hyper-methylation) [102]. With regard to parturition, as described above, the activation of TLR pathway results in the upregulation of labour-associated proinflammatory cytokines. Decreased DNA methylation of TLR-2 and TLR-9 (i.e., hypo-methylation) is associated with increased expression of the receptor proteins and contributes to increased sensitivity of decidual tissue to inflammatory stimuli and leukocyte infiltration. The decline in DNA methylation of TLR-2 and TLR-9 observed in term–labouring decidual tissues is even greater in spontaneous preterm birth samples [100].

In regulating gene expression, DNA methylation operates concurrently with histone modifications [e.g., histone acetylation and deacetylation (catalysed by histone acetyltransferase (HAT) and histone deacetylase (HDAC) respectively)]. Histone acetylation is associated with increased transcriptional activity, whereas deacetylation produces the opposite effect [102]. With regard to parturition, studies of the human myometrium and murine models report a decline in the expression of progesterone receptors (PRs) coactivator containing HAT and the level of histone acetylation at term. Moreover, in a murine model, delayed parturition was observed after administration of a potent histone deacetylase inhibitor–trichostatin A–that increased the level of myometrial histone acetylation [103]. These findings suggest a mechanism that links changes in acetylation with decreased PR expression near term, which reduces tissue responsiveness to progesterone stimuli and leads to increased tissue sensitivity to the contractile stimuli required to initiate labour: a phenomenon called functional progesterone withdrawal. Consistent with this proposed mechanism, a significant decline in PR expression was observed in decidua at term labour. The decrease in decidual PRs may also result from exposure to PG-F_{2α}, which is typically secreted by the labouring decidua at term [104]. Studies of the expression of HAT and histone acetylation are limited to myometrial tissue and have not been carried out in decidual tissue. Nonetheless, this epigenetic mechanism may also apply to the decidua since the same outcome of reduced expression of PRs is detected in both tissues [103, 104]. Whether alterations in histone modification patterns as pregnancy approaches term–parturition are caused by the ageing process or not remain to be determined. Establishing the pattern of histone modifications in the ageing decidua is an important prerequisite.

Increased nuclear localization of p65, which is one of the subunit of transcription factors NFκB, has also been observed in the decidua at term and at preterm parturition. The increase in nuclear localization of p65 occurs with advancing gestation and thus, suggests a potential role for NFκB, or more specifically p65, in regulating various parturition-related genes in decidua [105]. As described earlier, NFκB regulates the activation of many labour-associated pro-inflammatory cytokines. Additionally, NFκB expression increases in tissues with age and age-related degenerative diseases e.g., Alzheimer's, diabetes, and osteoporosis, while its inhibition in mouse models delays the progress of age-related symptoms and pathologies [106]. These findings provide indirect support that parturition and ageing share similar biochemical pathways.

Loss of proteostasis, decidual ageing, and parturition

With advancing gestational age, the decidua undergoes changes in its protein homeostasis (proteostasis), particularly with regard to the expression of heat shock proteins (Hsp). During pregnancy, decreased expression of Hsp-90 and Hsp-70 were observed in human DSCs [107], whereas at parturition, elevated levels of Hsp-90 and Hsp-70 were found in the labouring decidua (specifically in the endometrial gland epithelial cells in both of spontaneous and glucocorticoid-induced labour samples) in a sheep model. Increased level of Hsp-70 and Hsp-90 are proposed to inhibit the uterine progesterone receptor leading to progesterone withdrawal and stimulation of estrogen receptor, which consequently initiate labour [108]. Nevertheless, increased levels of Hsp-70 above a certain threshold, which mimics the levels associated with systemic inflammation, oxidative stress, and hepatocellular injury, is associated with pregnancy complications e.g., preeclampsia (PE) [109].

Recent studies on the interaction between Hsp-70 and autophagy showed autophagy was inhibited by Hsp-70 induction (as reviewed in [110]). In contrast to Hsp-70 that is well-known for its chaperone function to repair and preserve proteins, autophagy works by eliminating the defective proteins, organelles, or cells to maintain proteostasis [110]. With regard to parturition, various findings indicate that decreased autophagy and the associated increased levels of Hsp-70 are important for initiating labour. A study by Signorelli et al. (2011) showed that autophagy was not detectable in placental sections (i.e. decidual tissue) of patients undergoing spontaneous labour followed by a vaginal delivery, but was observed in the placentae of patients who were not in labour and undergoing C-section [111]. A study of the murine ovary, where the crucial autophagy gene Beclin1 (Becn1) was conditionally knocked-out, also provided indirect evidence that lack of autophagy promoted the onset of labour, and if it occurred prematurely, preterm birth occurred [112].

The inverse relationship between Hsp-70 and autophagy may also involve the activation of mammalian target of rapamycin complex 1 (mTORC1) signalling pathways. Increased level of Hsp-70 promotes the activation of protein kinase B (Akt), which subsequently phosphorylates mammalian target of rapamycin (mTOR) protein. The phosphorylated mTOR protein combines with an adaptor protein called RAPTOR to form mTORC1. The mTORC1 subsequently inhibits autophagy and promotes phosphorylation and activation of heat shock factor 1 (HSF-1). HSF-1 attaches to HSP79-2 gene and promotes the production of Hsp-70 protein, resulting in the repetition of the cycle (as reviewed in [110]). Labour then become the consequence of increased levels of Hsp-70 and mTORC1.

With respect to ageing, Hsp-70 is known to be over-expressed when the amount of misfolded protein and its potential cause, ROS, is increased in tissues, which is a typical characteristic of tissue with ageing and age-related disease. The overexpression of Hsp-70 due to oxidative stress also promotes the secretion of various inflammatory cytokines and receptors e.g., TNF- α , IL-1 β , IL-6, and TLR-4, which are well-known for their roles in initiating labour [113]. Nevertheless, uncontrolled overexpression of Hsp-70-mediated inflammatory factors is also strongly associated with pelvic inflammation and endometriosis [114] indicating the importance of maintaining the level of ageing-related phenotypes only up to a beneficial threshold.

Cellular senescence - deregulated nutrient sensing, decidual ageing, and parturition

Recent studies using mice have reported a strong correlation between the senescence of DSCs and parturition [3-5, 7]. At term birth, DSCs naturally undergo progressive senescence as parturition approaches, while in preterm birth, this progression of decidual senescence is accelerated [3]. Senescent DSCs may cause parturition through their gradual accumulation within the tissue and their increased secretion of SASPs with gestational age (e.g., MMPs and various pro-inflammatory cytokines) [6]. In preterm birth, the accelerated rate of DSC senescence was reported in studies of p53^{d/d} mice, which harbour a uterine-specific deletion of Transformation-related protein 53 (*Trp53*) [3-5, 7]. *Trp53* encodes tumour suppressor protein 53 (p53), which is an extensively studied cancer-related protein [115]. In the process of parturition, the deletion of uterine *Trp53* causes the upregulation of mTORC1 signalling, which leads to premature DSC senescence that is characterised by increased decidual expression of Akt and cyclin-dependent kinase inhibitor (p21), and results in an increased incidence of preterm birth in p53^{d/d} mice [3, 4]. The upregulation of mTORC1 signalling pathway at these studies is consistent with parturition-related pathways of Hsp-70 and mTORC1 described in the previous section.

With regard to ageing, Akt and p21 are common factors involved in senescence. Early activation/upregulation of Akt and p21 leads to premature senescence by increasing cellular exposure and vulnerability towards oxidative stress [116] and by inhibiting progression of the cell cycle [117], respectively. On the other hand, mTORC1 protein complex is a sensor for nutrient/redox/energy-sensor pathways, particularly in the detection of a high concentration of amino acids [23]. Studies show that the upregulation of mTORC1 signalling promotes premature ageing, and its inhibition (e.g., through calorie restriction) has an anti-ageing effect (i.e., increased lifespan) [118, 119].

In murine model of parturition, inhibition of mTORC1 signalling by rapamycin normalised the progression of DSC senescence and consequently, averted preterm birth [3]. Thus, the dysregulation of mTORC1 pathways that lead to preterm birth show similarities with the pathways associated with premature ageing. Furthermore, the timing of parturition may be determined by the interaction of p53 with its transcriptional target, sestrin2 (SESN2). In murine studies, the interaction between p53 and SESN2 activated the 5' adenosine monophosphate-activated protein kinase (AMPK) signalling, which subsequently reduces the mTORC1 signalling and prevents preterm birth [7]. These findings are consistent in human studies [5, 7].

Unfortunately, investigations of senescence-associated parturition pathways mainly only focused on preterm birth [3-5, 7]. The pathways associated with decidual senescence leading to natural term birth, in the most part, can only be inferred. The postulate is that term parturition involves the same pathways that promote DSC senescence in preterm birth. However, the regulation of these pathways are different to those in preterm birth. For example, the timing of p53 activation and inhibition as well as the expression level or increasing rate of mTORC1 will differ to ensure the normal pace of decidual senescence ensues for term parturition to occur. Nonetheless, this hypothesis still requires further confirmation.

Mitochondrial dysfunction – oxidative stress, decidual ageing, and parturition

In parturition, the upregulation of SESN2 and AMPK leading to the downregulation of mTORC1 is known to delay the timing of parturition (as explained in previous section). In ageing, the same regulation generates anti-ageing effects in mammals. SESN is induced through various mechanisms i.e., p53, transcription factor Nrf-2, and JNK-AP-1 signalling axis by oxidative stress, which increases with age. Together with other sestrins (SESN1 and 3), SESN2 reduces the accumulation of ROS and stimulates antioxidant responses e.g., the upregulation of mitochondrial biogenesis and mitophagy (autophagy of abnormal mitochondria) by activating AMPK and inhibiting mTORC1 signalling pathway. This antioxidant regulatory pathway of sestrin plays an essential role in maintaining metabolic homeostasis and preventing age-related physiological deviations [120]. Nevertheless, the inverse conclusion of these findings, where decreased SESN2 and increased mTORC1 signalling may occur in DSCs and promote labour by increasing oxidative stress through the downregulation of mitochondrial biogenesis and mitophagy, requires further verification.

Pregnancy and parturition are processes associated with high oxidative damage in both maternal and neonatal tissues [83, 84]. With regard to the decidua, a recent study of endometrial stromal cells in the opossum and in humans, showed that decidualisation in humans and Eutherian mammals in general evolved from cellular stress responses to oxidative damage, which are a component of pregnancy-related signals [121]. As pregnancy approaches term, pro-inflammatory factors, which exist in the decidua at the later stages of gestation, are potent stimulators of ROS production. ROS, subsequently, recruit more pro-inflammatory factors required for labour and the cycle repeats [83].

Indirect evidence for a role of ROS in promoting term parturition in the decidua was provided in a recent study that compared the level of superoxide dismutase (SOD), lipid peroxide (LPO), and PG-F_{2α} in human decidual tissue from spontaneous abortion, and from early stages of uncomplicated pregnancies. Compared to decidual tissue from normal pregnancies, lower levels of SOD and higher levels of LPO and PG-F_{2α} were observed in the decidual samples from spontaneous abortion patients [122]. The accumulation of ROS, and their products, e.g., LPO, was found to accelerate the termination of pregnancy by stimulating the synthesis of PG-F_{2α} required for myometrial contraction [123]. Based on these findings, it can be assumed that at early stages of pregnancy, a high level of SOD is maintained to ensure the metabolism of LPO, which prevents the synthesis of PG-F_{2α} and maintains myometrial quiescence to sustain pregnancy [122]. Furthermore, although there is no study that has measured the level of decidual SOD and LPO approaching the end of pregnancy, it can be postulated that the level of decidual SOD may decline and LPO may increase to allow PG-F_{2α} synthesis for labour at term. In contrast, preterm birth, spontaneous abortion, or stillbirth may result from accelerated/premature decline of decidual SOD as pregnancy progresses and/or from the extremely low level of decidual SOD from the beginning of pregnancy. Further studies are required to confirm this hypothesis.

Since high oxidative stress is detected in both healthy and pathological parturition e.g., spontaneous preterm birth, abortion, IUGR, PE, placental ageing, and stillbirth [124], the antagonistic nature of oxidative stress where its effect, either beneficial or harmful for the organisms, depends on their level surge forward as a possible underlying rationale. With regard to ageing and ageing-related diseases, this antagonistic nature of oxidative stress, as expected, occurs in a similar fashion. Consequently, “*how high is too high for the oxidative stress*” becomes a fundamental question that needs to be investigated in both ageing and parturition studies to better predict the incidence of various associated disorders.

Altered intercellular communication, decidual ageing, and parturition

Both ageing and parturition involve changes in intercellular communication. Some examples of these altered intercellular interactions are chronic pro-inflammatory status, declining immune protection, and induced senescence by the adjacent senescent cells. In ageing studies, inflamm-ageing, immunosenescence, and bystander effects are some terms used to describe these phenomena, respectively. With regard to parturition, a recent study of decidual gene expression in spontaneous term labour reported gene changes related to inflammation signalling pathways. Notably, there is increased

gene expression of pro-inflammatory mediators and steroid receptors and decreased expression of anti-inflammatory mediators in the decidua at the onset of labour. These pro-inflammatory mediators and steroid receptors are IL-1 β , IL-8, estrogen receptor 1 (*ESR1*), homeobox gene A11 (*HOXA11*), progesterone receptor membrane component 2 (*PGRMC2*) and PG-E synthase (*PTGES*). The altered anti-inflammatory mediators include chemokine C-C motif ligand 2 and 5 (*CCL2* and *CCL5*), Lectin, Galactoside-Binding, Soluble 1 and 3 (*LGALS1* and *LGALS3*), and Progesterone Associated Endometrial Protein (*PAEP*) [125].

According to the “decidual clock” hypothesis, sterile inflammatory signals, which are associated with the onset of labour, are also significantly upregulated in the decidua through SASP production of the senescent DSCs. The inflammatory signals and other type of SASPs (e.g., MMPs) are then amplified and distributed from the decidua to other gestation tissues, such as the cervix (i.e., to stimulate its ripening), fetal membranes (i.e., to stimulate their rupture), and myometrium (i.e., to stimulate its contractile state), all of which lead to parturition [20]. Additionally, the factors that stimulate the decidua to undergo senescence and amplify the inflammatory signals are proposed to originate from senescent fetal membranes [17]. The claim is plausible given the proximal location of fetal membrane to the decidua [87] and the ability of senescent cells to induce adjacent cells to undergo senescence: the so-called “by-stander effect” [126]. One example of the proposed labour-inducer factors from fetal membranes is cell-free telomere fragments released by the senescent fetal membrane amnion cells, which are capable of triggering global oxidative stress leading to labour [18].

Stem cell exhaustion, decidual ageing, and parturition

To the best of our knowledge, no studies have investigated the potential role of stem cells, particularly decidual mesenchymal stem/stromal cells (DMSCs), in parturition despite some evidence of their possible involvement. DMSCs are the major stem cell types in the decidua. Like other type of stem cells, DMSCs have the ability to regenerate senescent cells, particularly senescent DSCs. During pregnancy, the regeneration of senescent DSCs are essential to sustain the receptive properties of the decidua towards the growing fetus. This hypothesis is supported by a recent study showing senescent DSCs undergo immunological clearance mediated by uNK to maintain decidual homeostasis in the cycling endometrium since endometrial MSCs cannot differentiate into new DSCs in the presence of senescent DSCs [127]. In contrast, the inability of MSCs to differentiate in the presence of senescent DSCs is important for parturition. DSCs should not be rejuvenated so that the physical support of the fetus and the placenta can be weakened during labour. As previously described, the deterioration of tissue physical support due to stem cell exhaustion has been reported in studies using muscle stem cells where their exhaustion was associated with the loss of tissue integrity and strength [75-77]. Nevertheless, further investigation are still required to confirm this hypothesis. Additional studies e.g., on why the senescent DSCs are not eliminated by the immune cells (i.e., uNK) as parturition approaches may also be conducted, with immunosenescence as the possible approach.

DMSCs are also known for their high resistance to oxidative stress and inflammatory stimuli because of their proximal location with the maternal blood circulation (i.e., located close to the endothelial cells that line the maternal blood vessels). As discussed earlier, high oxidative stress and pro-inflammatory stimuli are well-established components of parturition. Moreover, DMSCs are removed from the lining of the blood vessels during blood vessel remodeling as pregnancy progresses [128]. The removal of DMSCs from the blood vessel lining as parturition approaches indicates that parturition require DMSCs to stop functioning and/or be removed from the site of separation. With respect to ageing, the loss of cell numbers or functions of stem cells, termed stem cell exhaustion, is known to be one of the integrative hallmarks of ageing. Based on these findings, it can be inferred that DMSCs may also undergo ageing and/or removal to accommodate parturition. As there is no direct evidence to support this hypothesis, the potential role of other tissue-derived MSCs will be discussed in more detail on subsequent sections. To conclude, a summary of the features of decidual ageing and its role in parturition is shown in Table 1 and Figure 5 below.

Table 1. The general features of decidual ageing and their potential roles in pregnancy and parturition

	Hallmarks of ageing	Brief description in association with ageing process	Potential/Observed features on decidua	Potential roles in pregnancy and parturition	Ref
Primary	Genomic instability	The accumulation of permanent DNA lesions leading to disintegrated and dysfunctional genome.	Increased DNA damage through the upregulation of RRM2	Decidualisation	[95]
			Increased DNA fragmentation with time leading to apoptosis of DSCs	Fetal membrane rupture Placental expulsion	[96]
	Telomere attrition	Telomere is progressively shortened as the cells replicate due to DNA polymerase's "end replication problem". At certain level of telomere attrition, the cells stop dividing.	Decreased telomere length of DSCs with advancing gestational age, resulting in senescent DSCs	SASP-mediated labour process	[97]
	Epigenetic alterations	Stable and heritable modifications that regulate gene expression without directly altering the sequence of DNA base pairs.	Reduced DNA methylation in decidual tissue, leading to increased expression of TLRs near term	Increased sensitivity of decidual tissue to inflammatory stimuli	[100]
			Reduced histone acetylation in decidual tissue, leading to decreased expression of progesterone receptor (PR) near term	Progesterone withdrawal at the onset of labour	[103, 104]
			Increased nuclear localization of p65, one of the components of transcription factor NFkB, with advancing gestation	Regulation of parturition-related genes in decidua, e.g., that regulate synthesis of PGs and cytokines	[105]
Antagonistic	Loss of proteostasis	The loss of protein homeostasis (system required to maintain intact structure and function of protein molecules).	Elevated level of Hsp70 near term as the response to high oxidative stress and abundant misfolded protein resulting from the inhibition of autophagy through mTORC1 signalling pathway and by-products of senescent DSCs	Progesterone withdrawal and estrogen receptor function stimulation at the onset of labour	[108, 110]
	Deregulated nutrient-sensing	The deregulation of various nutrient-sensing pathways. Increased nutrient abundance and anabolism pathways are linked to ageing, while the contradicting pathways (nutrient shortage and catabolism) are linked to longevity.	Increased mTORC1 signalling pathways (participates in high concentration of amino acid-sensing), leading to natural DSC senescence	SASP-mediated labour process	[3, 7]
	Cellular senescence	The mitotic-competent cells stop dividing as the response towards stress	Increased number of senescent DSC (in normal pace)	SASP-mediated labour process	[3, 5, 6]
	Mitochondrial dysfunction	MtDNA undergoes mutations, due to the clonal expansion of mtDNA replication errors or the accumulation of oxidative stress generated as by-products of respiratory chain process in mitochondria, leading to abnormal mitochondrial functions.	High oxidative stress reflected through increased ROS production	ROS is required to promote proper cellular responses leading to decidualisation	[121]
Integrative	Altered intercellular communication	Ageing involves various changes in intercellular communication, such as in neuroendocrine systems, chronic pro-inflammatory status (inflammaging), immune protective system (immunosenescence), and senescence state of the adjacent cells (bystander effects).	Upregulation of various pro-inflammatory signals (SASPs) released by the senescent DSCs, probably is induced by "bystander effect" of the adjacent senescent fetal membrane cells	SASP-mediated labour process	[17, 20]
	Stem cell exhaustion	Progressive loss of stem cell functions and numbers	Decreased number and function of DMSCs, resulted in the failure to regenerate aged/ senescent DSCs	SASP-mediated labour process	-

Brief descriptions of the hallmarks of ageing are deducted from [23]. Abbrev. RRM2 stands for: ribonucleotide reductase 2; DSCs: decidual stromal cells; SASP: senescence-associated secretory phenotype; TLRs: toll-like receptors; PGs: prostaglandins; Hsp: heat shock protein; mTORC1: mammalian target of rapamycin complex 1; mtDNA: mitochondrial DNA; ROS: reactive oxygen species; DMSCs: decidual mesenchymal stem/stromal cells.

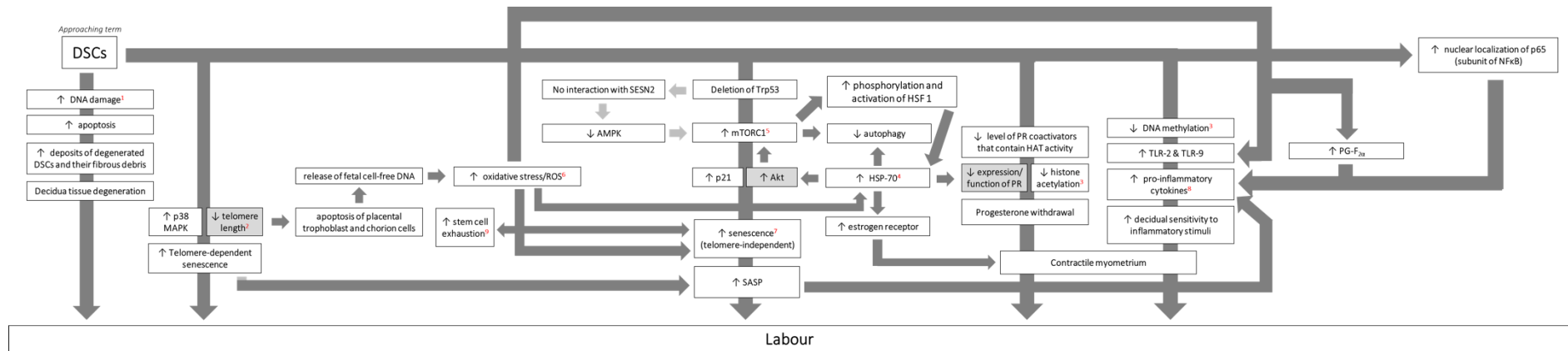


Figure 5. Labour-associated ageing pathway in decidua

Red-coloured numbers mark the hallmarks of ageing: 1: DNA damage; 2: telomere attrition; 3: epigenetic alterations; 4: loss of proteostasis; 5: deregulated nutrient sensing; 6: mitochondrial dysfunction; 7: cellular senescence; 8: altered intercellular communication; 9: stem cell exhaustion. Arrows indicate the sequence of the phenomena. Abbr. DSCs: decidual stromal cells, MAPK: mitogen-activated protein kinase, ROS: reactive oxygen species, SESN2: sestrin 2, AMPK: 5' AMP-activated protein kinase, mTORC1: mammalian target of rapamycin complex 1; p21: cyclin-dependent kinase inhibitor; Akt: protein kinase B, SASP: senescence-associated secretory phenotype, HSF: heat shock factor, HSP: heat shock protein, PR: progesterone receptor. HAT: histone acetylase, TLR: toll-like receptors, PG-F_{2α}: prostaglandin 2α, NFκB: nuclear factor κ-light-chain-enhancer of activated B cells.

Role of Mesenchymal Stem/Stromal Cells (MSCs) and their extracellular vesicles in human parturition

MSCs are multipotent stem cells that are able to differentiate into various mesenchymal cell lineages, such as adipocytes, osteoblasts, and chondrocytes. In addition to their differentiation capability, MSCs are characterised based on their expression of MSC-specific surface protein markers and adherence to plastic as described by the International Society for Cell & Gene Therapy (ISCT) [129]. In gestational tissues, MSCs are found in tissues of fetal-origin including cord blood, umbilical cord, amniotic fluid, and the fetal side of the placenta (i.e., amnion, chorion, and chorionic villi) and in tissues of maternal-origin including *decidua parietalis* and *decidua basalis* [130]. Based on current understanding of the origin of the placenta, MSCs are thought to be the precursor of all non-trophoblast cells of the villous core [131]. In the decidua, MSCs may also be responsible for giving rise to DSCs since the decidua is mostly composed of stromal cells and DSCs also express mesenchymal cell characteristics [132].

The abundant numbers of MSCs in various gestational tissues and their role in the formation and function of the placenta indicate the importance of MSCs during pregnancy. However, the role of MSCs in parturition remains poorly understood. Currently, studies of the association between MSCs and pregnancy mostly focus on the decline of MSC functions, properties, and differentiation capability in pregnancy-related disorders that are associated with placental ageing. For example, Rolfo et al. (2013) reported that chorionic villous-derived MSCs (CMSCs) from PE patients have reduced proliferation, increased senescence, and increased levels of various pro-inflammatory cytokines (e.g., TNF- α , IL-6, IL-8) and chemotactic factors (e.g., migration inhibitory factors/MIF, and soluble FMS-like tyrosine kinase-1/sFlt-1) compared with CMSCs from normotensive patients [133]. On the maternal side, Mayne et al. (2017) reported that PE-affected placentae show signs of premature senescence and ageing, including increased SASP, increased apoptosis and increased expression of ageing-related biomarkers [134]. Furthermore, Kusuma et al. (2017) showed that PE-DMSCs have decreased resistance to oxidative stress [135]. PE-DMSCs were also reported to have different microRNAs (miRNAs) expression (e.g., miR-16, miR-181a, miR-494), particularly those that regulate the immunosuppressive, proliferation, and angiogenesis-regulating potential of DMSCs, when compared with normotensive control [130]. miRNAs are short non-coding regulatory RNAs consisting of about 22 nucleotides that function in mRNA silencing (by cleaving the target mRNAs) and post-transcriptional regulation of gene expression (by suppressing gene translation) [136]. In MSCs, miRNAs are located within the cells or in the secretory, membrane-derived vesicles known as extracellular vesicles (EVs), which are known to facilitate the regenerative properties of MSCs via paracrine function [137, 138]. In pathological situations e.g., PE, the secretion of EVs containing aberrant miRNA expression by MSCs may contribute to the pathogenesis of PE [130]. In relation to term parturition, these findings may serve as indirect evidence that MSC functions may be altered at parturition, specifically in the decidua, since the decidua demonstrates strong evidence of ageing and senescence at parturition. Labour-associated senescence and ageing in the decidua may alter the properties of DMSCs, which subsequently act to facilitate labour, e.g., secreting EVs containing parturition-related miRNAs. This hypothesis requires verification.

Indirect evidence of a role of MSCs in parturition may also be obtained from studies that compare metabolic activities and function of mitochondria of human umbilical cord-derived MSCs (hUC-MSCs) of term not-in-labour and preterm parturition. A study by Pafolini et al. (2016) revealed that MSC-derived exosomes, one type of MSC-derived EVs, have the capacity to perform aerobic respiration independent of the mitochondria. However, only MSC-derived exosomes of neonates delivered at term not-in-labour were capable of generating ATP as the result of oxidative phosphorylation (OXPHOS) activity. MSC-derived exosomes of preterm neonates did not generate ATP after consuming the oxygen [139]. Additionally, Ravera et al. (2018) suggested that this metabolic difference may originate from different intracellular mitochondrial regulations. MSCs of preterm neonates have more scarcely organised mitochondrial reticulum and low expression of mitochondrial fusion-related proteins compared with MSCs of term not-in-labour neonates and these differences were reported to derive from different expression of CLUH, a cytosolic mRNA-binding protein that mainly governs intracellular biogenesis and distribution of mitochondria. In MSCs of preterm neonates, the expression of CLUH increased with gestational age, but was maintained in a low level. In MSCs of term-not in labour neonates, CLUH was also expressed in a similar pattern but at higher level compared with MSCs of preterm neonates. The silencing of CLUH in MSCs of term not-in-labour neonates resulted in the mitochondrial distribution and metabolic phenotypes similar with MSCs of preterm neonates. The resulting metabolic switch was detected from around the 34th week of gestational age [140].

These findings suggest that there is a metabolic switch in MSCs during gestation, indicating their possible role in promoting labour. The inference is that increased expression of CLUH leads to increased numbers of regularly organised mitochondria, resulting in higher aerobic respiratory activity, which is known to generate ROS as by-products. Consequently, the level of oxidative stress in MSCs and possibly, their microenvironment, increases, which is consistent with the oxidative stress features of decidual ageing associated with labour explained earlier. In preterm birth, expression level of CLUH is lower than normal, leading to incomplete aerobic respiration, which may cause higher ROS production than normal. Nevertheless, to investigate the direct association between this metabolic switch and labour, the expression of CLUH on MSCs of term in-labour neonates should be monitored as well.

Role of MSC-derived microRNAs in human parturition

MSCs exert their effects mostly by secreting miRNA-contained EVs. With regard to parturition, various miRNAs are associated with regulation of hormone receptiveness and main gene expression pathways in parturition (e.g., post-transcriptional regulation of progesterone-progesterone receptor signalling in relation to myometrial contractility during labour) [141]. To see possible associations between miRNAs, MSCs, and parturition, here we present a list of miRNAs that are reported to have roles in parturition and their expression in MSCs (Table 2).

Table 2. MSC-derived miRNAs and their possible role in parturition

Tissue source	miRNAs	Trend with gestational age	Principal findings/roles in parturition	Ref	Reported in MSCs [Ref]
Placenta	let-7 family	Upregulated	Involved in: - Regulation of innate/adaptive immune responses - Induction of cell differentiation - Function as tumour suppressors May contribute to inflammation and senescence aspects of parturition	[142]	[143]
	miR-34				
	miR-29a				
	miR-195				
	miR-181c				[144]
Chorio-amniotic	miR-338	Downregulated	Increases gene and protein expression of phospholipase PLA2G4B in decidual cells. PLA2G4B plays important role in PG biosynthesis at parturition	[145]	[146]
	Dicer*		Key miRNA-processing enzyme, may contribute to changes in miRNAs processing during labour at term		[147]
Reflected amnion*	miR-143	Downregulated	- The expression is higher in amnion mesenchymal cells than amnion epithelial cells - Increases Prostaglandin-Endoperoxidase Synthase 2 (PTGS2) synthesis. PTGS2, also known as COX-2, is enzyme involved in PG production at labour	[148]	[149]
	miR-145				[150]
Myometrium	miR-199a-3p	Downregulated	- Increases expression of COX-2, key enzyme in PG synthesis - Activated by progesterone, and inhibited by estrogen, mediating opposite effects of these hormones during labour	[151]	miR199a [152]
	miR-214				–
	miR-146b-3p	Upregulated	- Upregulated both in spontaneous and oxytocin-induced labour - Potential gene target in myometrium: IL-8, NFκB, TLR-2, TLR-4, BCL2-related protein A1, regulating the inflammatory process of labour	[153]	miR-146b-5p [152]
	miR-200 family	Upregulated	- Reduces expression of the transcription factors zinc finger E-box binding homeobox proteins ZEB1 and ZEB2 - ZEB1 and ZEB2 reduce expression of contraction associated genes: oxytocin receptor and connexin-43, and inhibit oxytocin-induced	[154]	miR200b [152]

			contractility in human myometrial cells Modulating the uterine quiescence and contractility during labour		
Cervix	miR-223	Upregulated	- Regulation of genes involved in cervical remodelling during labour, e.g., TLR3, claudin 8, and Calmodulin-like 3 (downregulated after labour) - TLR is involved in inflammatory signalling of labour - Claudin 8 is the key component of tight junction in cervix epithelium - Calmodulin suppresses synthesis of matrix metalloproteinases and collagenases	[155]	[156]
	miR-34b				–
	miR-34c				–

Notes: *amnion that encloses the fetus but is not attached to the placental disk and umbilical cord. “–” means it has not been reported.

Although these miRNAs may be expressed in MSCs, in most cases, their roles in parturition was observed in other type of cells, and therefore, require confirmation in MSCs. To the best of our knowledge, no studies have investigated the expression of MSC-derived miRNAs in association with parturition.

Studying decidual ageing in human parturition: the current and potential study models

Decidual ageing plays a significant role in parturition and further studies are required to confirm various aspects of this role. However, most studies of decidual ageing in parturition have been performed in animal models, e.g., rabbit and rodents (i.e., mice and rats) [4, 89, 157]. The advantages of using animal models in ageing and parturition studies of decidua are numerous. Since human is also classified as a deciduata species, the decidua of human and deciduata animals have similar structure (e.g., both human and mice have pre-DSCs that are joined to adjacent cells by gap junctions [158]). In comparison with human-based studies, animal-based studies also have fewer ethical constraints (e.g., where tissue biopsy of the decidua throughout different gestational age can be performed) and allow regular, tightly controlled and monitored, and precise time-point experimental procedures with least possible medical intervention and sample variations. Nevertheless, human parturition is a complex and unique process and thus, most animals are the inadequate model of human parturition. The findings in animals cannot be readily extrapolated to humans because of the many environmental and functional differences between humans and animal models, in terms of parturition and ageing (as reviewed in [159] and [160] respectively). For example, human decidualisation can occur in response to the hormonal changes released by the corpus luteum at the luteal phase with/without pregnancy. In contrast, mice do not have a luteal phase in their estrous cycle and decidualisation can only occur after mating [158]. Since the effect of those differences on the outcomes of the studies is unknown, animal studies, e.g., ageing-parturition studies of mice decidua [3], still require confirmation on human-derived tissues [161].

The human placenta is ideally suited to study ageing-associated parturition features as it contains both of the maternal (*decidua*) and fetal (*chorionic villi*) components of the gestational tissues at parturition [162]. There are many advantages for using human placenta to study ageing-associated parturition features in the decidua. Firstly, it allows studies directly on human decidual tissue that is attached to the maternal side of the placenta. Therefore, changes or specific functions can be directly measured in the tissue, making it more reflective to the *in vivo* condition. Human placentae are abundantly available and their collection does not require invasive procedures or raise any significant ethical constraints (compared with tissue biopsy of human decidua) as it is naturally expelled at the end of pregnancy [163]. Moreover, human placentae also serve as an abundant source of DMSCs [128, 144], which as explained above, may play significant roles in initiating parturition.

However, the use of human placentae in ageing-parturition studies has some limitations. The use of human placenta only allows for a narrow range of time-point observation as it is impractical and unethical to obtain the samples from non-pathological pregnancies before the delivery of the neonate. Placentae from spontaneous preterm delivery can be used for this purpose, but their potential pathological properties should be taken into consideration as possible confounding factors in assessing spontaneous labour mechanisms. Moreover, patient-to-patient variations as well as high numbers of

medical interventions performed to ensure the safety of the mother and neonate during parturition are inevitable and difficult to control. Nonetheless, ageing-parturition studies on human-derived decidua should be encouraged to validate the findings from animal-based studies.

Conclusion

Ageing propels the gestational clocks to parturition. In decidua, the ageing process of the decidual stromal cells as well as their corresponding stem cell population (i.e., DMSCs) may contribute to the initiation and/or facilitate various stages of labour (e.g., cervical ripening, delivery of neonate, and placental detachment). However, further investigation on various features of decidual ageing that are highly expressed at parturition, particularly those based on human-derived tissue studies, are still required to verify the findings from animal-based studies.

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Appendix VII. Research article manuscript

Functional Changes in Decidual Mesenchymal Stem/Stromal Cells are Associated with Spontaneous Onset of Labour

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ABSTRACT

Ageing and parturition share many common pathways, but their relationship remains poorly understood. This study focuses on the decidua, where there is evidence that decidual cells undergo ageing as parturition approaches term, and these ageing-related changes of the decidua may trigger labour. Mesenchymal stem/stromal cells (MSCs) are a predominant type of stem cell that resides in the decidua. Given that stem cell exhaustion is a hallmark of ageing, ageing of decidual MSCs (DMSCs) may contribute to the functional changes in decidual tissue required for spontaneous labour at term. This study aims to determine whether DMSCs from patients undergoing spontaneous onset of labour (SOL-DMSCs) show evidence of ageing-related functional changes compared with those from patients, not in labour, undergoing Caesarean section (NIL-DMSCs).

Placentae were collected from term (37 – 40 week's gestation), SOL (n = 18) and NIL (n = 17) healthy patients. DMSCs were isolated from decidua basalis that remained attached to the placenta after delivery. Primary cells were characterised to confirm their stem-cell properties and maternal origin. Important cell functions i.e., proliferation, migration, necrosis, apoptosis, lipid peroxidation, aldehyde dehydrogenase expression, and pro-inflammatory cytokine secretion were assessed and compared between SOL- and NIL-DMSCs. Mass-spectrometry-based lipid analyses were also performed to identify and quantify lipid species in both DMSCs.

SOL-DMSCs show reduced proliferation and increased lipid peroxidation, migration, necrosis, mitochondrial apoptosis, and IL-6 production compared with NIL-DMSCs ($p < 0.05$). Significant differences in lipid quantities between SOL- and NIL-DMSCs ($p < 0.05$) were found in various phospholipids [phosphatidylethanolamine (13%), phosphatidylglycerol (18%), phosphatidylinositol (22%), and phosphatidylserine (33%)], sphingolipids [ceramide (12%) and sphingomyelin (4%)], triglycerides (6%), and acyl carnitine. SOL-DMSCs had lower lipid quantities compared with NIL-DMSCs.

In conclusion, SOL-DMSCs showed evidence of ageing-related reduced functionality, accumulation of cellular damages, and changes in lipid quantities compared with NIL-DMSCs. These changes may play significant role in promoting spontaneous labour at term.

Keywords: *ageing, decidua, term parturition, spontaneous labour, decidual mesenchymal stem cells, functional changes, lipid analyses*

INTRODUCTION

As pregnancy approaches term, the decidua undergoes various changes to prepare for labour (Cox and Redman, 2017, Menon et al. , 2016). These changes are postulated to originate from

the cellular level e.g., from decidual stromal cells (DSCs) and mesenchymal stem cells (MSCs), which are some predominant resident cell types in the decidua (Abomaray et al. , 2016, Dunn et al. , 2003). DSCs are round, epithelioid-like cells that arise following differentiation of the elongated, fibroblast-like endometrial mesenchymal cells during the secretory phase of menstrual cycle (Okada et al. , 2018, Salamonsen et al. , 2009). As parturition approaches, DSCs undergo various changes similar to those seen in ageing processes, e.g., senescence (Cha et al. , 2013, Deng et al. , 2016, Hirota et al. , 2011, Hirota, 2010) and inflammation (El-Azzamy et al. , 2017). Studies in human and animal models show that senescent DSCs are dominant in labouring term decidua, while hindered progression of DSC senescence delays the timing of parturition (Hirota et al., 2011). Gradual accumulation of senescent DSCs stimulates labour possibly by weakening the physical strength of the decidual tissue, or by secreting senescence associated secretory phenotype molecules (SASPs), which mostly comprise pro-inflammatory cytokines required for stimulating the contractile state of the myometrium during labour (Cha and Aronoff, 2017, Norwitz et al. , 2015).

MSCs are multipotent stem cells capable of self-regeneration and differentiation into various mesenchymal cell lineages, including adipocytes, osteoblasts, and chondrocytes. In culture, MSCs are characterised by their adherence to plastic surface, and expression of a MSC-specific combination of surface markers, as defined by the International Society for Cellular Therapy (ISCT) (Dominici et al. , 2006). In the decidua, MSCs play an important role in rejuvenating the DSC population. Since labour is associated with ageing of DSCs, we propose that decidual MSCs (DMSCs) may also undergo ageing process, resulting in their reduced functionality at term. The decline in numbers or the loss of function of stem cells is called stem cell exhaustion and is a hallmark of ageing (Lopez-Otin et al. , 2013). Reduced functionality of DMSCs would limit their ability to replenish aged DSCs, and thus may contribute to the accumulation of senescent DSCs in the labouring decidua. Consequently, the attachment between the decidual tissue and its adjacent gestational tissues, e.g., placenta, may be weakened, thereby facilitated the delivery of the neonate and the placenta during labour at term. Indirect evidence that stem cells play an important role in maintaining tissue strength and integrity comes from studies of muscle cells, where stem cell exhaustion leads to loss of tissue strength during the ageing process (Goldspink, 2007, 2012, Mendelsohn and Larrick, 2016).

Therefore, this study aims to determine whether DMSCs from term (37-40 weeks of gestation) placentae of patients undergoing spontaneous onset of labour (SOL-DMSCs) show evidence of ageing-related functional changes compared with DMSCs from term placentae of patients, not in labour, undergoing Caesarean section (NIL-DMSCs).

MATERIALS AND METHODS

Tissue collection

Term placentae (37-40 weeks gestation) from patients undergoing SOL + vaginal delivery (n=18) and NIL + elective C-section delivery (n=17) were collected by research midwives at the Royal Women's Hospital, Parkville, Australia. Labour was defined as spontaneous if the contractions occurred naturally without induction and completed without pharmacological augmentation. Further exclusion criteria related to factors that affect placental function or interfere with particular stem cell function. Placental samples were not collected from donors > 35 years of age (advanced maternal age) and with a body mass index (BMI) > 30 kg/m² (obese range). Patients with multiple gestations, miscarriage, polycystic ovary syndrome, hyperthyroidism, gastric medicines and aspirin consumption, as well as major fetal and placental abnormalities were omitted from this study. Patients with breech birth and alcohol consumption were excluded from the lipid peroxidation assay. The project was approved by the Royal Women's Hospital Research and Ethics Committee, VIC, Australia (Projects 10/49 and 12/42). All patients have provided written consent prior to donating their samples.

Isolation, characterisation, and culture maintenance of DMSCs

Isolation, characterisation [i.e., flow cytometry analyses, in vitro adipogenic and osteogenic differentiation assays, and fluorescence in situ hybridisation (FISH) analyses], and culture maintenance of DMSCs were performed according to the published methods (Kusuma et al. , 2018). Primary cells of maternal origin that were consistent with the ISCT criteria for MSCs (Dominici et al., 2006) were referred as DMSCs. The standard culture medium used was α -MEM complete, which consists of α -MEM (Sigma Aldrich, USA) supplemented with 10% FCS (Gibco, USA), 1% L-glutamine (Life Technologies, USA), and 1% pen/strep (Sigma Aldrich, USA). Most experiments were performed at passage 3-5. DMSCs were used up to passage 5 since subsequent generations do not reliably maintain their stem cell-like properties (Portmann-Lanz et al. , 2006, Soncini et al. , 2007).

Functional assays

xCELLigence real-time proliferation assay

xCELLigence system (ACEA Biosciences, Inc., USA) recorded real-time changes in the electrical impedance resulting from cellular events detected by the microelectrode of the xCELLigence E-plate. These changes were reported as arbitrary cell indices. The growth profile of DMSCs was established using the xCELLigence system as previously described (Abumaree et al. , 2017, Alshabibi et al. , 2017, Alshabibi et al. , 2018) with following modifications. DMSCs were plated into a 96-well E-plate in triplicate (1×10^4 cells/100 μ L α -MEM complete/well), then incubated at 37°C, 5% CO₂ for 400 hours without a medium change. Cell index was measured in real-time every 5 min for the first 24 hours, and subsequently, every 1 hour using RTCA Software 1.2.1. Cell index of SOL- and NIL-DMSCs was compared at various time points in attachment and proliferation phase.

Cell viability measurement

DMSCs were prepared, plated, and incubated analogously to the xCELLigence real-time proliferation assay, but in a standard 96-well plate. At 250 hours (during survivability phase), cells were assessed for their viability using Trypan blue exclusion test (Strober, 1997). The numbers of viable SOL- and NIL-DMSCs were compared.

xCELLigence real-time migration assay

For the migration assay, the xCELLigence system (ACEA Biosciences, Inc., USA) utilises 16-well CIM plate consisting of two chambers (upper and lower) separated by a polyethylene terephthalate membrane with porous size of 8 μ m. Microelectrodes were embedded to the membrane at the lower chamber side. The migration of DMSCs was evaluated using the xCELLigence system as previously described (Abumaree et al., 2017, Alshabibi et al., 2017, Alshabibi et al., 2018) with following modifications. The α -MEM complete medium (165 μ L/well) and α -MEM alone (50 μ L/well) were added to the lower and upper chamber of the CIM-plate respectively. DMSCs were plated into the upper chamber of CIM-plate (4×10^4 cells/100 μ L α -MEM alone/well), then incubated at 37°C, 5% CO₂ for 24 hours. Cell index was measured and recorded real time every 15 min using RTCA Software 1.2.1. Cell index of 0.1 was determined as the migration starting point as per manufacturer's recommendation.

Mitochondrial apoptosis assay

This assay was performed as described by the manufacturer (Biotium, USA) with following modifications. DMSCs (1.5×10^5 cells) subjected to the JC-1 cationic dye to determine apoptotic cells. Cells were incubated at 37°C for 15 min, washed, and resuspended in 500 μ L PBS. The expression of JC-1 dye in DMSCs was measured using a BD LSRII flow cytometer and analysed using FACS Diva software (BD). Gating was set as in **Figure 3A-B**. The percentages of mitochondrial apoptotic SOL- and NIL-DMSCs were compared.

FITC Annexin V assay

The assay was performed using FITC Annexin V kit (Trevigen, Inc., USA) as previously described (Kusuma et al., 2017). Gating was set as in **Figure 4A-B**. Cells were classified into four quadrants: Q1: necrosis (Annexin V-/Propidium Iodide+), Q2: late apoptosis (Annexin V+/Propidium Iodide+), Q3: viable/healthy (Annexin V-/Propidium Iodide-), or Q4: early apoptosis (Annexin V+/Propidium Iodide-). The percentage of SOL- and NIL-DMSCs in each quadrant was compared.

Oxidative stress assays

ALDEFLUOR aldehyde dehydrogenase (ALDH) detection assay

The assay was performed using ALDEFLUOR Kit (STEMCELL Technologies, Canada) as previously described (Kusuma et al., 2017). Gating was set as in **Figure 5A-B**. The percentages of SOL- and NIL-DMSCs that positively expressed ALDH (ALDH^{br} cells) were compared.

TBARS (TCA method) lipid peroxidation assay

The assay was performed according to the manufacturer's instructions (Cayman Chemical, USA) to quantify the lipid peroxidation marker, malondialdehyde (MDA), produced by SOL- and NIL-DMSCs. MDA reacted with thiobarbituric acid (TBA) generated thiobarbituric acid reactive substances (TBARS), which the absorbance was measurable at 535 nm. This assay was performed on 2x10⁶ DMSCs homogenised in 50 µL PBS (Gibco, USA). The absorbance of each sample was measured using a SpectraMax Plus 384 microplate reader and processed using SoftMax Pro 5 software. Samples were tested in duplicate. The lipid peroxidation level of SOL and NIL samples proportional to the absorbance value, was compared.

Enzyme-Linked Immunosorbent Assays (ELISAs) for IL-6 and IL-8 detection

Serum-starvation and cell lysate collection

DMSCs (70% confluent) were serum-starved for 48 hours in α -MEM supplemented with 1% FCS and the serum-starved medium volume reduced to 75% from the standard volume. After 48 hours, the medium was collected, then centrifuged at 500 g for 5 min and 2,000 g for 10 min to remove cell debris. DMSCs were collected after dissociation with TrypLE (Life Technologies, USA). Cells were lysed in radioimmunoprecipitation assay buffer (RIPA buffer, Life Technologies, USA) supplemented with 0.002% AEBSF (MP Biomedicals, USA), then centrifuged at 14,000 g, 4°C for 15 min. Supernatants (cell lysates) were collected. The protein concentration of the cell lysates was then measured using Pierce BCA method using the kit from Sigma Aldrich, USA.

ELISAs

ELISA kits (Crux Biolab, Australia) were utilised according to the manufacturer's instruction to quantify the concentration of IL-6 and IL-8 secreted by SOL- and NIL-DMSCs. IL-6 and IL-8 concentrations were normalised to the protein concentration before being compared.

Mass-spectrometry based lipid analyses

Sample preparation

DMSCs at P5 were serum starved for 48 hours. Prior to cell lysis, cells were resuspended in 400 µL PBS (Gibco, USA) and only 50 µL of this cell suspension was lysed and homogenised in RIPA buffer (Life Technologies, USA). Cell lysates were used to measure the protein concentration of the samples using Pierce BCA method (Sigma Aldrich, USA). Cells resuspended in PBS was aliquoted to microtubes at a volume that would give the equivalent of 12 µg protein/sample. Each sample was then spiked in with 5 µL SPLASH® LIPIDOMIX® Mass Spec Standard (Avanti Polar Lipids, Inc., USA) Lipid components of these mixtures were extracted using Folch's lipid extraction protocol (Folch et al., 1957).

Mass spectrometry analyses

Sample analyses were performed in positive and negative mode on a Thermo Fisher Orbitrap Lumos (Thermo Fisher, Scoresby, Australia) combined with a Thermo Fisher U3000 nanoHPLC (Thermo Fisher, Scoresby, Australia). The resulting raw mass spectrometry data files were searched, aligned into their corresponding group (SOL- and NIL-DMSCs), and filtered using LipidSearch software v.4.2.20 SP1 (Thermo Scientific, USA) with parameters as described (Wang et al. , 2018). The ID and quantities of each lipid species identified from SOL- and NIL-DMSCs were compared. For the lipid species belonged to the lipid class that had the representative standard included in the SPLASH® LIPIDOMIX® mixture (Avanti Polar Lipids, Inc., USA), the absolute amount of each lipid species (µg) per 100 µL sample was determined using the formula:

$$\frac{\text{Peak area of the lipid species}}{\text{Peak area of the lipid class standard}} \times \text{the amount of the standard spiked into the samples (}\mu\text{g)}$$

For the lipid species belonged to the lipid class that was not included in the SPLASH® LIPIDOMIX® mixture (Avanti Polar Lipids, Inc., USA), the relative quantification was determined directly using the peak area of the lipid species, with the assumption of total lipid analysed was equal for all samples as it had been normalised based on the protein concentration of the samples.

Statistical analyses

Data distribution was estimated using the graphical assessment of skewness. Parametric data were analysed using unpaired student t-test (two-tailed), while non-parametric data were analysed using Mann-Whitney U test. Statistical analyses were performed using GraphPad Prism 5.01 software. For xCELLigence migration, Annexin V, ALDH, TBARS lipid peroxidation, and ELISAs, the data were generated from the combination of two different batches of patient samples. One sample was tested in both batches as a replication control. Prior to data merger, data were normalised using the average value of each group. The statistical significance was set at *p<0.05, **p<0.01, and ***p<0.001.

RESULTS

Patient characteristics

Clinical characteristics of placental donors are summarised in **Supplementary table 1**. The clinical characteristics of patients undergoing SOL and NIL were not significantly different although some patient-to-patient variation is unavoidable.

Isolation and characterisation of DMSCs

Upon isolation (passage 0), the primary cells isolated from SOL and NIL patients were morphologically diverse due to the presence of small amount of other types of placental cells that survived the rigorous mechanical and enzymatic digestion. Nevertheless, after several washings and a medium change, the non-adherent cells were washed out of the culture and after one passage (P1), the morphology of the adhered primary cells was uniform under the microscope. All primary cells (P1) showed the typical fibroblastic morphology of MSCs (**Supplementary figure 1**). Flow cytometry analyses showed that ≥95% cells from both patients positively expressed MSC-specific surface markers i.e., CD90, CD 166, CD 73, CD 44, and CD 105 and ≤5% of the cells were positive for hematopoietic markers i.e., CD 45, CD 19, and HLA-DR (**Supplementary figure 2**). Under appropriate stimulation, primary cells from both SOL and NIL patients underwent adipogenic and osteogenic differentiation, as shown through the formation of lipid droplets (**Supplementary figure 3**) and calcium deposits

(**Supplementary figure 4**), respectively. These cells also successfully formed colonies when seeded at low density, as expected of MSCs (**Supplementary figure 5**). Based on FISH analyses of the primary cells prepared from placentae of male newborn, primary cells from both patients were of maternal origin, as shown by the presence of two X chromosomes signals and the absence of Y chromosome signals (**Supplementary figure 6**). FISH analyses are not informative on cells from placentae of female newborn. Nevertheless, the cell preparations from the placentae of both female and male newborns were identically carried out with the same methods, thereby suggesting that the origin of all isolated primary cells were consistent. In conclusion, since primary cells isolated from term placentae of patients undergoing SOL and NIL were consistent with the minimum criteria set by ISCT for MSCs (Parolini et al. , 2008) and were of maternal origin, they were subsequently referred to SOL-DMSCs and NIL-DMSCs, respectively.

Functional assays of SOL- and NIL-DMSCs

Proliferation capacity

Proliferation capacity of SOL- and NIL-DMSCs (n = 10 per group) was assessed using xCELLigence real-time proliferation assay. Complete profile of cell attachment, proliferation and survival is shown in **Figure 1A**. There were no statistically significant differences observed in the attachment phase ($p > 0.05$, **Figure 1B**). SOL-DMSCs had a significantly reduced proliferation compared with NIL-DMSCs ($p < 0.05$, **Figure 1C**). Proliferation was examined by measuring cell indices at the 120 hour, and then normalised these to the indices at 15 hours, which was designated as the start of proliferation phase. SOL-DMSCs had fewer surviving cells compared with NIL-DMSCs ($p < 0.05$, **Figure 1D**). Cell survival was assessed based on the cell viability after 250 hours, which was designated as the start of survival phase of DMSCs in the xCELLigence real-time proliferation assay.

Migration capacity

Migration of SOL-DMSCs (n = 8) and NIL-DMSCs (n = 7) was determined using the xCELLigence real-time migration assay. SOL-DMSCs and NIL-DMSCs both migrated toward a higher concentration of FCS in the growth medium. SOL-DMSCs presented higher migration capacity compared with NIL-DMSCs throughout the time course (**Figure 2A**) and the differences were statistically significant ($p < 0.05$) at 16 hours (**Figure 2B**).

Cell death profile of DMSCs

Mitochondrial apoptotic DMSCs

SOL-DMSCs had a significantly higher percentage of mitochondrial apoptotic cells compared with NIL-DMSCs (**Figure 3C**, n = 10 per group, $p < 0.05$), based on the loss of mitochondrial membrane potential.

FITC Annexin V profile of DMSCs

SOL-DMSCs (n = 8) has a statistically significant higher percentage of necrotic cells (Q1) compared with NIL-DMSCs (n = 7, $p < 0.05$, **Figure 4C**). SOL-DMSCs (n = 8) also had a higher percentage of apoptotic cells i.e., early (Q4), late (Q2), and total apoptotic cells (Q2 + Q4) and a lower percentage of viable/healthy cells (Q3) compared with NIL-DMSCs (n = 7). However, the differences were not statistically significant ($p > 0.05$, **Figure 4C**).

Oxidative stress profile

Aldefluor assay

DMSCs were assessed for their ability to resist oxidative stress based on the percentage of ALDHbr cells in the population using the Aldefluor assay. SOL-DMSCs (n = 8) had a lower percentage of ALDHbr cells compared with NIL-DMSCs (n = 7). However, the differences were not statistically significant ($p > 0.05$, **Figure 5C**).

TBARS (TCA method) lipid peroxidation assay

Oxidative stress, particularly the potential for lipid peroxidation, was assessed by measuring MDA production using TBARS reaction. SOL-DMSCs (n = 8) produced significantly more MDA than NIL-DMSCs (n = 7, $p < 0.05$, **Figure 5D**), thereby suggesting higher potential of lipid peroxidation in SOL-DMSCs.

3.3.5. Pro-inflammatory cytokine (IL-6 and IL-8) secretion

The production of pro-inflammatory cytokines i.e., IL-6 and IL-8 was measured using ELISA. Prior to the assay, DMSCs were serum-starved to synchronize their cell cycle phase, thereby normalising the starting point of the cytokine production. Total removal of FCS from the growth medium (α -MEM complete) was not feasible since DMSCs detached when being cultured in this condition, as shown through the decrease of cell indices in xCELLigence real-time proliferation assay (**Supplementary figure 7**). DMSCs cultured in growth medium supplemented with 1% FCS maintained the viability and the proliferation trend comparable to those cultured in the standard growth medium, which was supplemented with 10% FCS (**Supplementary figure 7**). Therefore, 1% FCS-supplemented medium was used as the serum-starved medium for DMSCs in this study. The ELISAs revealed a statistically significant increase in IL-6 production in SOL-DMSCs ($p < 0.05$, **Figure 6A**). Increased IL-8 production was observed in NIL-DMSCs (n = 7), compared with SOL-DMSCs, but the differences were not statistically significant ($p > 0.05$, **Figure 6B**).

3.4. Lipid analyses

3.4.1. Lipidome identification

Using LipidSearch, 570 unique lipid species was identified based on the MS/MS fragmentation patterns. These lipid species represented five lipid categories i.e., phospholipids (74%), sphingolipids (17%), neutral lipids (9%), fatty acyls (0.2%), and other lipids (0.2%), consisting of 17 lipid classes (**Table 1**). The highest number of lipid species identified was in class phosphatidylethanolamine (PE, 24%), followed by phosphatidylcholine (PC, 23%) and sphingomyelin (SM, 10%). The remaining classes contained $< 10\%$ of the total identified lipid species. The percentages were calculated per total count of lipid species identified. All lipid species were identified in both SOL- and NIL-DMSCs (n = 7 per group).

3.4.2. Lipidome quantification

SOL- and NIL-DMSCs showed significant differences in lipid quantities of various specific lipid species ($p < 0.05$), specifically in the category of: (1) phospholipids i.e., class PE (13%, **Figure 7**), phosphatidylglycerol (PG, 18%, **Figure 8**), phosphatidylinositol (PI, 22%, **Figure 9**), and phosphatidylserine (PS, 33%, **Figure 10**), (2) sphingolipids i.e., class ceramide (Cer, 12%, **Figure 11**) and SM (4%, **Figure 12**), (3) neutral lipids, i.e., class triglyceride (TG, 6%, **Figure 13**), and (4) fatty acyls, i.e., class acyl carnitine (AcCa, **Figure 14**). The percentages were calculated per total count of lipid species identified in particular lipid class. In contrast, all lipid species identified in the following lipid classes: cardiolipin (CL), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylinositol (LPI) from phospholipid category, hexosyl ceramide (Hex1Cer), dihexosyl ceramide (Hex2Cer), trihexosyl ceramide (Hex3Cer) from sphingolipid category, and coenzyme Q (Co) did not show any significant differences in lipid quantities ($p > 0.05$).

DISCUSSION

Proliferation capacity of SOL-DMSCs

Stem cell proliferation is essential to maintain the stem cell pool. In this study, SOL-DMSCs had a significantly lower level of proliferation compared with NIL-DMSCs ($p < 0.05$, **Figure 1A & C**). No significant differences were observed in the attachment level between SOL- and NIL-DMSCs ($p > 0.05$, **Figure 1B**). Numerous studies have reported the association between reduced proliferation levels with MSC ageing (Choudhery et al., 2014, Ksiazek, 2009, Mendes

et al., 2002, Stenderup et al., 2003, Zhou et al., 2008). Nevertheless, to the best of our knowledge, no studies have associated this phenomenon with natural parturition. Studies of the functional decline of DMSCs in pregnancy pathology; preeclampsia are the only evidence currently available that associate stem cell exhaustion (ageing) with parturition. These studies reported DMSC proliferation reduced in preeclampsia samples and this was attributed to the overexpression of miR-16 in preeclampsia-DMSCs (Wang et al., 2012). Accelerated placental ageing was also detected on women with preeclampsia (Cox and Redman, 2017, Manna et al., 2019, Mayne et al., 2017), thereby suggested that functional changes in preeclampsia-DMSCs were strongly associated with ageing. Since the same proliferation trend was observed in SOL-DMSCs, it is highly possible that this particular functional change of SOL-DMSCs also resulted from ageing process of the placenta to facilitate labour at term.

Migration capacity and pro-inflammatory secretion of SOL-DMSCs

In this study, SOL-DMSCs showed a significant increase in migration compared with NIL-DMSCs ($p < 0.05$; **Figure 2**). Increased migration of SOL-DMSCs may be associated with the immune regulatory roles of MSCs during labour. Labour features many inflammatory aspects, particularly in the decidua, as the resident and infiltrating immune cells in this gestational tissue actively secrete a variety of pro-inflammatory cytokines at term (Gomez-Lopez et al., 2014, Norwitz et al., 2015). On the other hand, MSCs display pro-inflammatory phenotypes in highly inflammatory conditions [as reviewed in (Le Blanc and Davies, 2015)]. In responses to inflammatory stimuli, MSCs migrate to the site of infection or injury and stimulate immune induction (Waterman et al., 2010). Through the secretion of various pro-inflammatory cytokines, MSCs then increase the recruitment and functions (i.e., pro-inflammatory state activation and migration) of various immune cells, such as neutrophils and macrophages (Ulivi et al., 2014). In the case of tissue injury, this response is required to augment the initial inflammatory responses, which is the key element in activating wound healing responses (Ulivi et al., 2014). With regard to parturition, increased migration of leukocytes e.g., neutrophils and macrophages, to the labouring decidua was observed at term and this was suggested to contribute to the activation of decidua during the initiation of labour (Hamilton et al., 2012). Based on these data, it is possible that during labour, DMSCs also actively migrate to the inflammatory site in decidua to stimulate immune induction by secreting higher level of pro-inflammatory cytokines. These cytokines then recruited more immune cells, e.g., neutrophils and macrophages, to amplify the initial inflammatory responses required to initiate labour at term. This hypothesis is supported by the finding that SOL-DMSCs secreted a significantly higher level of IL-6 compared with NIL-DMSCs ($p < 0.05$; **Figure 6A**). IL-6, together with IL-8, are principal pro-inflammatory cytokines reported in decidua during labour (Osman et al., 2003). Nevertheless, IL-8 concentration was not significantly changed in SOL-DMSCs ($p > 0.05$; **Figure 6B**), which suggests that DMSCs may not be the major sources of decidual IL-8 during labour at term. Since increased secretion of pro-inflammatory cytokines e.g., IL-6 and IL-8, was also observed in MSCs from aged donors accompanied with the presence of MSC senescence (Gnani et al., 2019), it is possible that increased secretion of pro-inflammatory cytokines in SOL-DMSCs at term also resulted from ageing mechanisms, e.g., cellular senescence and SASPs.

Cell death (necrosis and apoptosis) profile of SOL-DMSCs

The mitochondrial apoptosis assay showed that SOL-DMSCs had a significantly higher percentage of the mitochondrial apoptotic cells ($p < 0.05$, **Figure 3C**). The FITC Annexin V assay also showed that SOL-DMSCs had a higher percentage of apoptotic cells (early, late, and total). However, the differences were not statistically significant ($p > 0.05$, **Figure 4C**). SOL-DMSCs, instead, had a significantly higher percentage of necrotic cells compared with NIL-DMSCs ($p < 0.05$, **Figure 4C**). Mitochondrial apoptosis assay was based on the loss of mitochondrial membrane potential, which was a phenomenon that can occur in both apoptosis and necrosis (Kroemer et al., 1998). Therefore, mitochondrial apoptosis may be indistinguishable with necrosis. In addition, SOL-DMSCs also had a lower percentage of healthy/viable cells compared with NIL-DMSCs, although the differences were not

statistically significant ($p > 0.05$, **Figure 4C**). The data of the lower percentages of viable/healthy SOL-DMSCs are consistent with the findings of reduced SOL-DMSC viability during survival phase (after 250 hours incubation), detected using the Trypan-Blue exclusion method (**Figure 1D**).

The overall decline of SOL-DMSC viability may result from the accumulation of cellular damages resulting from higher exposure of SOL-DMSCs to stress stimuli (e.g., oxidative stress, inflammation) compared with NIL-DMSCs. Since these phenomena are commonly observed in aged organisms (Tower, 2015) and some types of aged stem cells (Koyuncu et al., 2015, Sethe et al., 2006), it can be inferred that these particular changes in SOL-DMSC viability may result from the ageing process during labour at term. Furthermore, based on the findings in this study, it is likely that necrosis was the predominant type of cell death in SOL-DMSCs. This postulate is supported by a study on labouring fetal membranes [as reviewed in (Menon et al., 2017)]. As parturition approaches, fetal membranes undergo senescence and secrete SASPs, which propagate the inflammatory signals for parturition by triggering the release of damage-associated molecular pattern molecules (DAMPs), e.g., high mobility group box (HMGB)-1, heat shock protein (Hsp)-70, uric acid, and fragments of cell-free DNA. The adjacent cells, e.g. decidual cells, recognise these DAMPs through pattern recognition receptors located in their plasma membranes or endosomes, which leads to higher inflammatory responses, the activation of complement system, as well as cell necrosis [as reviewed in (Menon et al., 2017)]. In addition, the inflammatory responses only increase during necrosis, not apoptosis (Wyllie et al., 1980). The necrosis of SOL-DMSCs, thereby, may contribute to the increase of pro-inflammatory responses during labour, as shown through a significant increase of IL-6 level in SOL-DMSCs (Figure 6A). Moreover, a study on the age-dependent cell death of fibroblast cells demonstrated a reduced capacity of fibroblasts to induce apoptosis and an increasing shift from apoptosis to necrosis with age, together with increased level of necrotic inflammation in older fibroblast cells (Miyoshi et al., 2006). Nevertheless, in general, these findings suggest a possible association between ageing, parturition, and cell death, of DMSCs at term.

Oxidative stress profile of SOL-DMSCs

Ageing and parturition are both associated with increased levels of oxidative stress. Gestational tissues including the decidua, are constantly exposed with high levels of oxidative stress, a consequence of elevated pro-inflammatory responses during labour. Decidua were also exposed to physical changes during delivery e.g., sudden exposure to the O₂-rich extra-uterine environment, suppressed utero-placental blood circulation, and higher consumption of O₂. These all contribute to higher production of ROS at term (Diaz-Castro et al., 2015, Poletini et al., 2015). Here, SOL-DMSCs were proposed to have higher levels of oxidative damage, and reduced antioxidant responses, compared with NIL-DMSCs.

With regard to the oxidative damage, SOL-DMSCs had a significantly higher level of lipid peroxidation compared with NIL-DMSCs ($p < 0.05$), as reflected through higher levels of the end product of lipid peroxidation; MDA (**Figure 5D**). Indirect support for this finding was a study of superoxide dismutase (SOD), lipid peroxide (LPO), and prostaglandin F_{2α} (PG-F_{2α}) levels in decidual tissue from patients undergoing spontaneous abortion compared with those at the early stage of normal pregnancies. Levels of SODs were reduced, whereas the level of LPO and PG-F_{2α} increased in decidual tissue from patients undergoing spontaneous abortion (Sugino et al., 2000). It was suggested that parturition was accelerated due to early induction of PG-F_{2α} synthesis as the response to high accumulation of ROS and their end-products, i.e. LPO in the decidua (Hemler and Lands, 1980). This mechanism may also occur in DMSCs during normal term parturition, but at a rate and level that is sufficient to initiate parturition without evoking any pathological effects.

With regard to the antioxidant system, this study assessed and compared the percentage of DMSCs expressing high levels of ALDH (so called ALDH^{br} DMSCs) between SOL- and NIL-DMSCs. The ALDH family of enzymes detoxifies various aldehyde substrates by oxidizing

them into their carboxylic acid forms (Vasiliou and Nebert, 2005). Previous research from our facility reported a reduced ALDH expression in preeclampsia-DMSCs. Similar to SOL-DMSCs, preeclampsia-DMSCs were also shown to exhibit higher level of lipid peroxidation (Kusuma et al., 2017). Nevertheless, in this study, SOL-DMSCs did not show any significant differences in the percentage of ALDH^{br} cells compared with NIL-DMSCs ($p>0.05$; **Figure 5C**). These findings suggest that ALDH may not be the major antioxidant system used by DMSCs during labour at term. Other antioxidant enzymes e.g., SOD, catalase, and peroxidase, which work by different mechanisms and target different types of ROS, may contribute more to the labour process in DMSCs. Nonetheless, further studies are required to fully understand the oxidative stress response of SOL-DMSCs.

Mass-spectrometry based lipid analyses

In this study, SOL- and NIL-DMSCs expressed the same lipid species (a total of 570 lipid species). Most lipid species identified were phospholipids (74%) and sphingolipids (17%). The highest numbers of lipid species identified were in class PE (24%), followed by class PC (23%) and SM (10%). The percentages were calculated per total count of lipid species identified. This was expected since phospholipids and sphingolipids are the major lipid components of plasma membrane. PC, PE, PS, and SM are phospholipids and sphingolipids predominantly found in plasma membrane, contributing to more than half the mass of lipid in most plasma membrane (Alberts et al., 2002).

Generally, the plasma membrane serves as the protective layer for the cells. The plasma membrane also plays an essential role in mediating various biological signals via several signalling pathways e.g., G-protein activators, second messengers, and nuclear receptor activators (Heldin et al., 2016). Given that lipid are the major constituent of most animal cell membranes (~50% of the total mass), changes in lipid profile (i.e., quantities) may greatly affect numerous cellular signalling pathways. In this study, SOL- and NIL-DMSCs significantly differed in the lipid quantities of various specific lipid species, specifically in the category of phospholipids [class PE (13%), PG (18%), PI (22%), and PS (33%)], sphingolipids [class Cer (12%) and SM (4%)], neutral lipids [class TG (6%)], and fatty acyls (class AcCa). The percentages were calculated per total count of lipid species identified in particular lipid class. Compared with NIL-DMSCs, SOL-DMSCs had lower lipid quantities in almost all identified lipid species, except for PE(32:1_20:4) and PE(32:1_22:5), which showed the opposite trend. Based on these findings, it can, therefore, be inferred that these changes in lipid quantities of SOL-DMSCs may be associated with various signalling pathway changes in the decidual tissue in preparation for labour at term.

Major changes in the lipid profile (i.e., increased or decreased quantities of various lipid species) are reported in various parturition-related studies (Birchenall et al., 2019, Herrera and Ortega-Senovilla, 2010, Jo et al., 2017, Moayeri et al., 2017). Specifically, a pilot study by Birchenall et al. (2019) evaluated and compared the metabolite changes in the maternal and fetal plasma following SOL + vaginal delivery at term in humans (with NIL + elective C-section as control group). There were significant changes in the metabolite quantities between SOL and NIL samples that were found mostly in the lipid categories, particularly in class Cer and AcCa (Birchenall et al., 2019). Birchenall et al. (2019) also discussed the high similarity in metabolite changes between labour and strenuous exercise and suggested that the metabolism process of various macromolecules such as, lipids, may change in order to provide the energy required for these high energy consumption activities (i.e., labour and exercise) (Birchenall et al., 2019). Birchenall et al. (2019) also suggested that the energy required for labour was very likely to originate from the maternal tissue (e.g., maternal skeletal muscle, which is derived from osteogenic differentiation of MSCs) since the fetus/neonate is not thought to use its skeletal muscle during labour and delivery (Birchenall et al., 2019). Based on these observations, it is possible that the lipid components of SOL-DMSCs may be catabolised through higher lipolytic activities during labour to provide at least in part, the energy source that fuels the labour process. Interestingly, similar to parturition, major changes

in lipid quantities are reported in several studies of stem cell ageing (Ito and Suda, 2014) and MSC ageing (Kilpinen et al., 2013) with particular changes in the glycerophospholipids (PC, PS, PE, and PI) profile. These studies support the notion that changes in lipid quantities of SOL-DMSCs may also result from the ageing process during term labour.

Changes in DMSC function and lipid profile: a cause or consequence of labour?

In this study, significant ageing-related differences in various important functions (i.e., migration, IL-6 production, necrosis, and lipid peroxidation) and lipid profiles were detected between SOL- and NIL-DMSCs, which suggest that active, spontaneous labour is associated with changes in the properties and behaviours of stem cells at full-term parturition. Some of these molecular and functional changes may be the consequence of labour itself. However, it is also possible that these changes trigger molecular pathways to cause SOL at term. The study design of this project was not sufficient to discriminate between cause or consequence with regard to the relationship between labour and the functional and molecular changes in SOL-DMSCs.

Additional group of samples could provide data to distinguish between cause or consequence. For examples, further studies could include a group of “emergency C-section shortly after SOL” patients as controls. In general, “emergency C-section shortly after SOL” patients are patients who have briefly experienced the labour process before undergoing C-section surgery due to various medical complications. In terms of the length of labour, the sequence of patient groups, from the shortest to the longest, should be patients undergoing (1) NIL + elective C-section, (2) emergency C-section shortly after SOL, and (3) SOL + vaginal delivery. If the functional and molecular profile of DMSCs from placentae of patients undergoing “emergency C-section shortly after SOL” (ECS-DMSCs) resemble those of NIL-DMSCs, it is likely that the changes detected in SOL-DMSCs were the consequence of labour. In other words, if the molecular and functional changes that are detected in SOL-DMSCs, and not in NIL-DMSCs, are not detected in ECS-DMSCs where the donors have undergone the initial phase of labour only, then it is likely that the changes in SOL-DMSCs are a consequence of the normal labour process. However, if the functional and molecular profile of ECS-DMSCs is similar to those in SOL-DMSCs, then it is likely that these changes are the cause of labour since the changes are already occurring in the initial phase of the labour process (**Figure 15**).

Nevertheless, it is worth noting that the collection process of emergency C-section placentae for these analyses may be logistically challenging because of low numbers of samples with suitable medical conditions and the unpredictability of sample availability in normal working hours. Most emergency C-section deliveries are performed due to the fetal and/or maternal medical problem, therefore, the possibility of an underlying pathological condition must be taken into consideration when using these samples. To avoid confounding pathological factors, the ideal patient is one who plans to have an elective C-section, but in whom labour contractions start earlier than expected resulting in the “emergency C-section shortly after SOL” and an uncomplicated outcome for the mother or fetus.

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AUTHORS' ROLES

All authors contributed to the conception and design of the study. JCW and RK performed the experiments for data acquisition. JCW wrote the manuscript and prepared the figures, while

RK, MK, HMG, SPB, and BK critically revised the manuscript. JCW, BK, and HMG analysed and interpreted the data. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The author(s) declared that there is no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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TABLES AND FIGURES

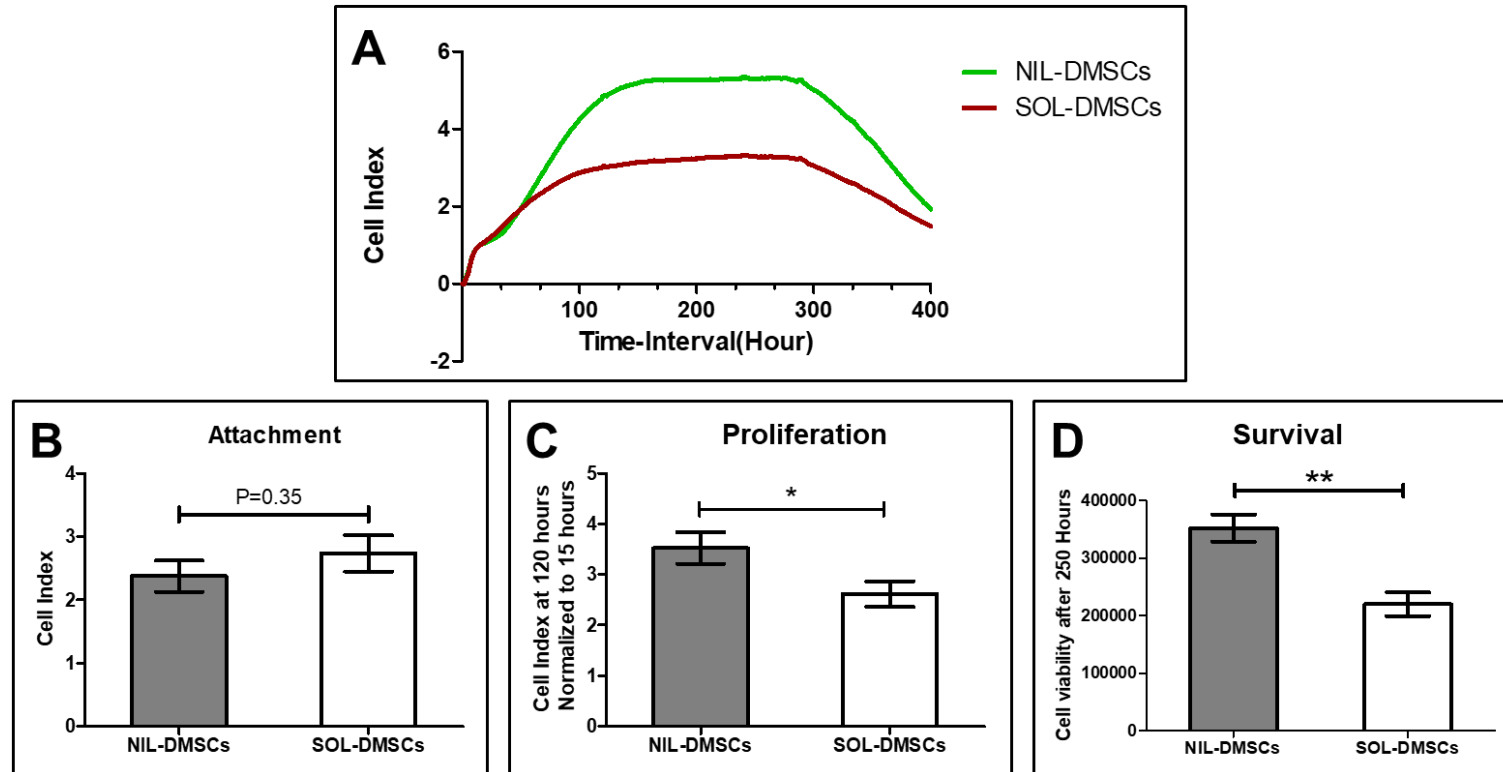


Figure 1. xCELLigence real-time proliferation assay of DMSCs

(A) The lines represent the averaged cell indices of NIL and SOL-DMSCs ($n = 10$ per group). The attachment phase was designated as 0-15 hours, the proliferation phase was 15-120 hours, the survival phase was from 120-300 hours, and the detachment/death phase was 300 hours to the completion of the experiment at 400 hours. **(B)** There were no significant differences in the attachment of SOL- and NIL-DMSCs ($p > 0.05$). SOL-DMSCs showed a statistically significant reduction of **(C)** proliferation and **(D)** cell survivability compared with NIL-DMSCs. Data were presented as mean $\pm$ SEM. P-values deduced from unpaired student t-test. * $p < 0.05$; ** $p < 0.005$.

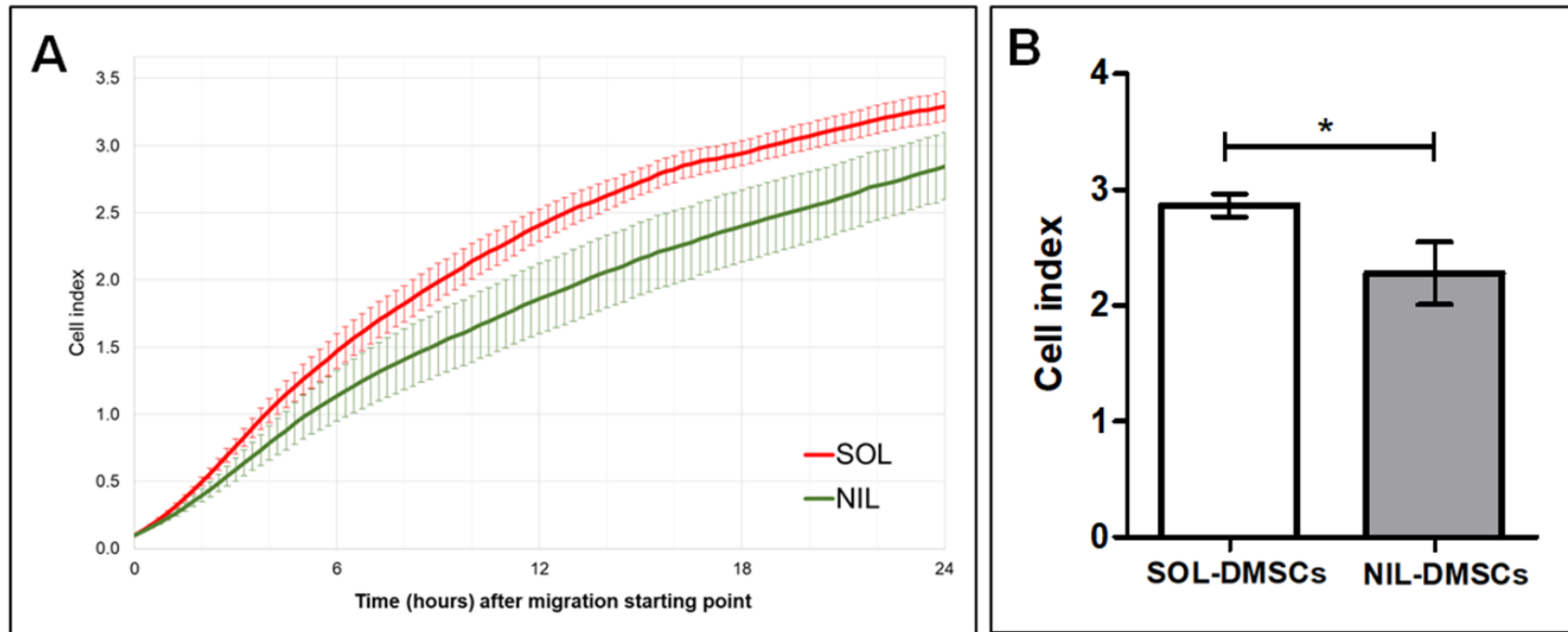


Figure 2. Migration of DMSCs

(A) X-Y plot of xCELLigence real-time migration over 24 hours. The lines represent the averaged cell indices of NIL-DMSCs (n = 7) and SOL-DMSCs (n = 8). **(B)** SOL-DMSCs showed a statistically significant higher level of migration compared with NIL-DMSCs ($p < 0.05$) at 16 hours. Data were presented as mean $\pm$ SEM. P-values deduced from Mann Whitney U-test. * $p < 0.05$.

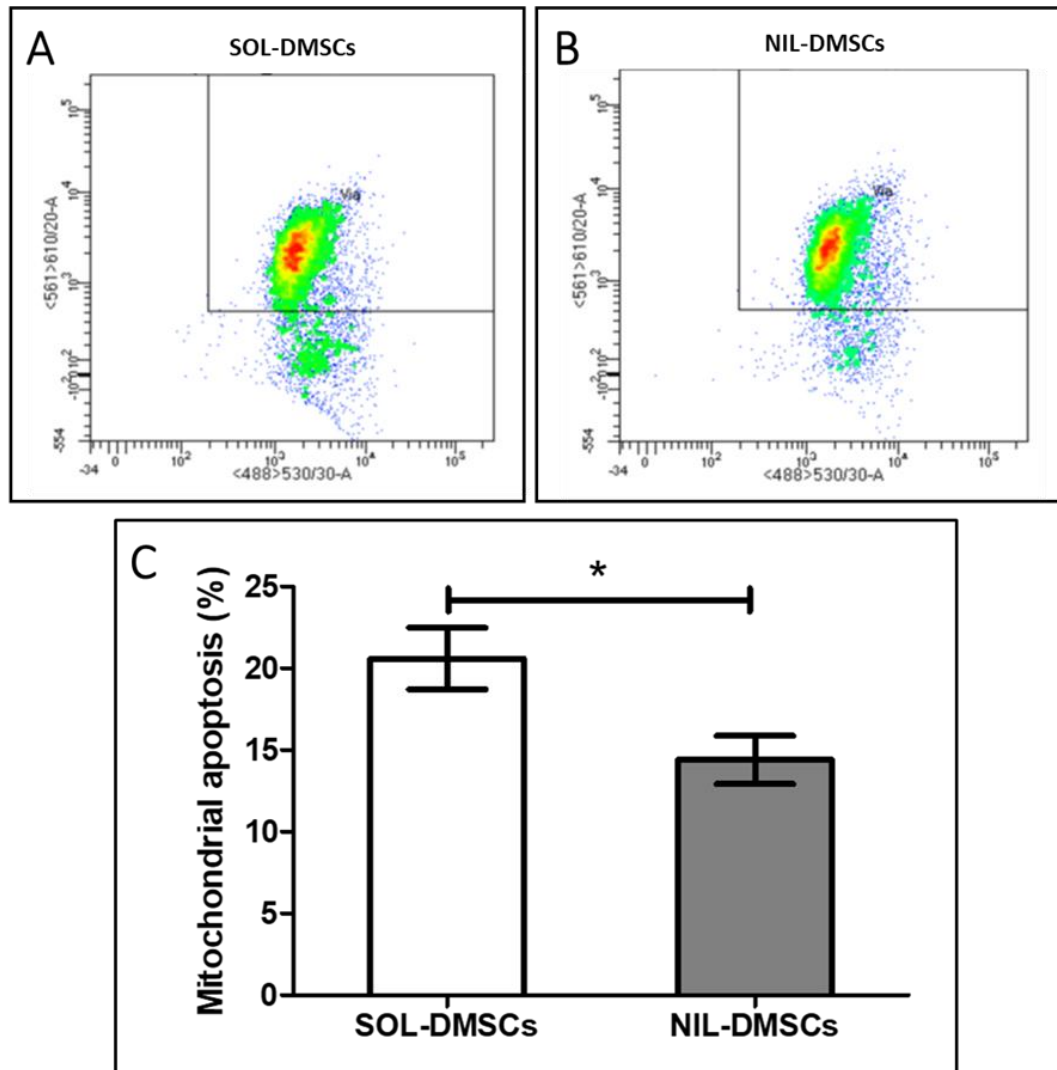


Figure 3. Mitochondrial apoptosis assay of DMSCs

Representative gating adjustment for **(A)** SOL-DMSCs and **(B)** NIL-DMSCs (n = 10 per group). Healthy cells retained JC-1 dye in cytosol and mitochondrial matrix, emitting the green (JC-1 monomeric) and red fluorescence (JC-1 aggregate). Apoptotic cells retained JC-1 dye in cytosol, emitting green fluorescence only (JC-1 monomeric). **(C)** SOL-DMSCs showed a statistically significant higher percentage of mitochondrial apoptotic cells compared with NIL-DMSCs ($p < 0.05$). Data were presented as mean $\pm$ SEM. P-values deduced from unpaired student t-test. * $p < 0.05$.

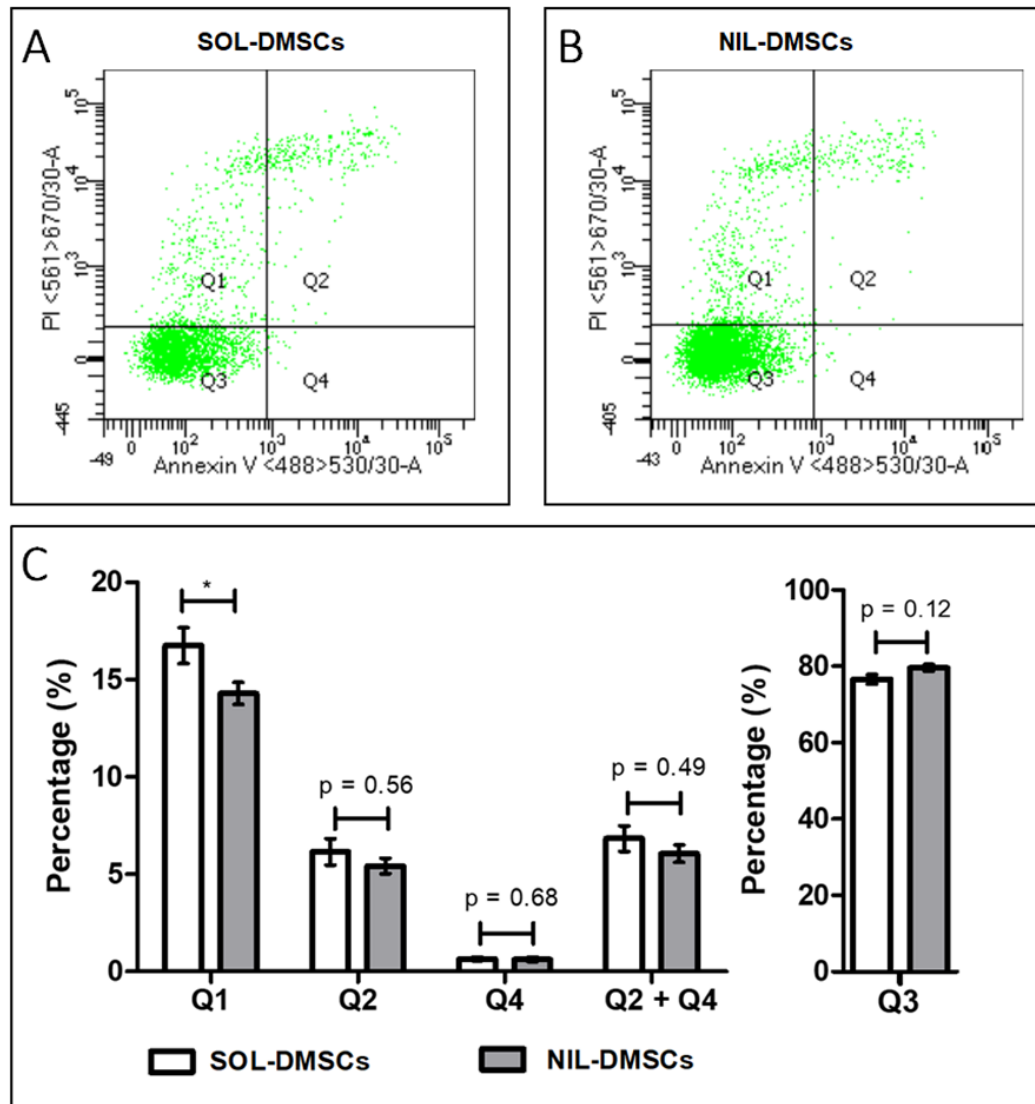


Figure 4. Annexin V profile of DMSCs

Representative gating adjustment of **(A)** SOL-DMSCs (n = 8) and **(B)** NIL-DMSCs (n = 7). Q1: necrosis (Annexin V⁻/Propidium Iodide⁺), Q2: late apoptosis (Annexin V⁺/ Propidium Iodide⁺), Q3: viable/healthy (Annexin V⁻/Propidium Iodide⁻), or Q4: early apoptosis (Annexin V⁺/ Propidium Iodide⁻). X-axis: the fluorescence intensity for Annexin V expression. Y-axis: the fluorescence intensity for propidium iodide expression. **(C)** SOL-DMSCs had a significantly higher percentage of necrotic cells compared with NIL-DMSCs (p < 0.05). An increase in the percentage of apoptotic cells (Q2, Q4, Q2+ Q4) and a decrease in the percentage of healthy/ viable cells (Q3) were detected in SOL-DMSCs compared with NIL-DMSCs. However, the differences were not statistically significant (p > 0.05). Data were presented as mean ± SEM. P-values deduced from Mann Whitney U-test. *p < 0.05.

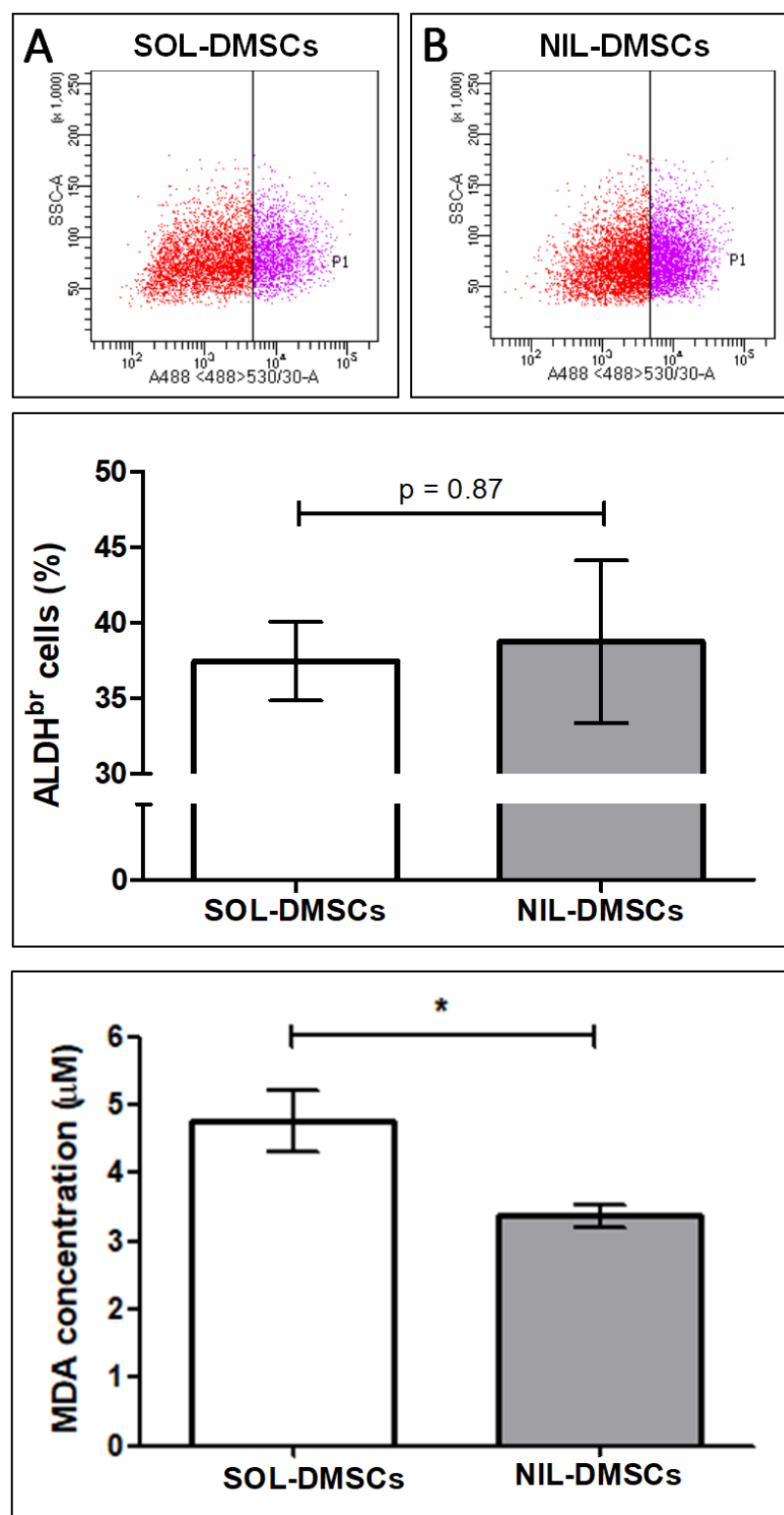


Figure 5. Oxidative stress profile of DMSCs

Representative gating adjustment of **(A)** SOL-DMSCs (n = 8) and **(B)** NIL-DMSCs (n = 7). X-axis: the side scatter profile. Y-axis: the fluorescence intensity of ALDH expression. **(C)** SOL-DMSCs showed a lower percentage of ALDH^{br} cells compared with NIL-DMSCs. However, the differences were not statistically significant (p > 0.05). **(D)** SOL-DMSCs showed a statistically significant higher production of MDA, which is a measure of potential for lipid peroxidation, compared with NIL-DMSCs (p < 0.05). Data were presented as mean ± SEM. P-values deduced from Mann Whitney U-test. *p < 0.05.

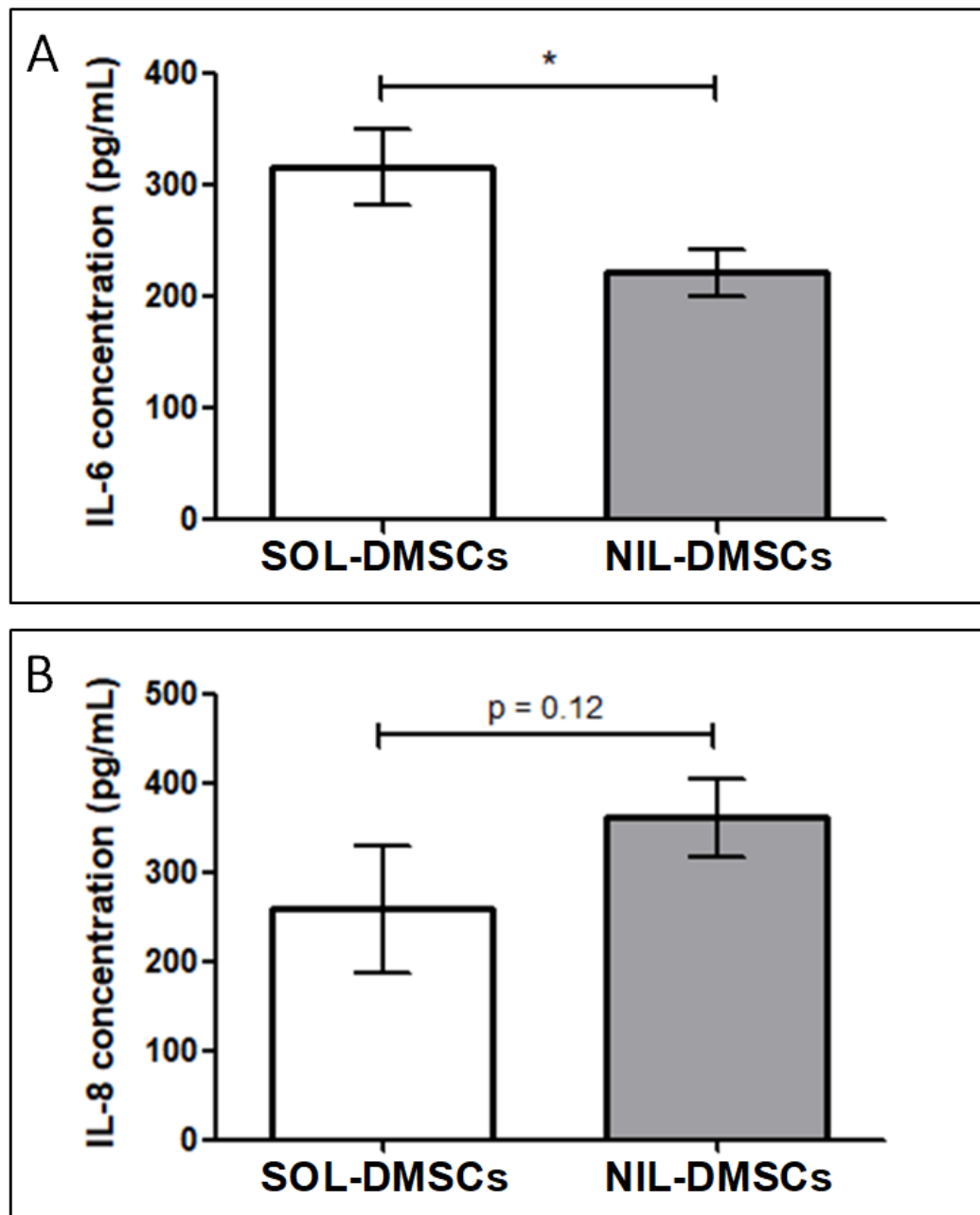


Figure 6. Pro-inflammatory secretion profile of DMSCs

(A) SOL-DMSCs ($n = 8$) produced significantly higher amount of IL-6 compared with NIL-DMSCs ($n = 7$). **(B)** SOL-DMSCs showed a decrease in IL-8 production compared with NIL-DMSCs. However, the differences were not statistically significant. IL-6 and IL-8 concentrations were normalised per 1000 $\mu\text{g/mL}$ cell lysate protein. Data were presented as mean $\pm$ SEM. P-values deduced from Mann Whitney U-test. * $p < 0.05$.

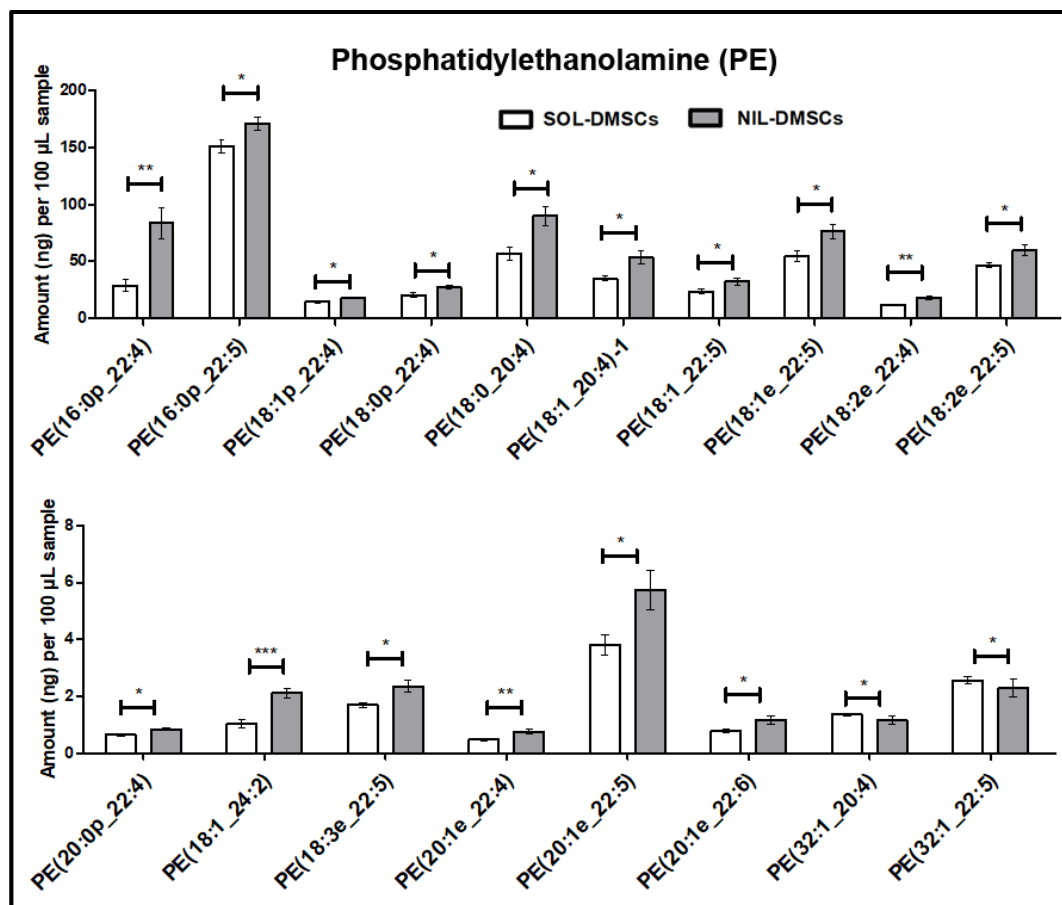


Figure 7. Absolute quantification of Class PE

Only lipid species with p-value < 0.05 between SOL-DMSCs and NIL-DMSCs (n = 7 per group) are shown. Data were presented as mean ± SEM. P-value deduced from Mann-Whitney U-test. *p < 0.05, **p < 0.01, ***p < 0.001.

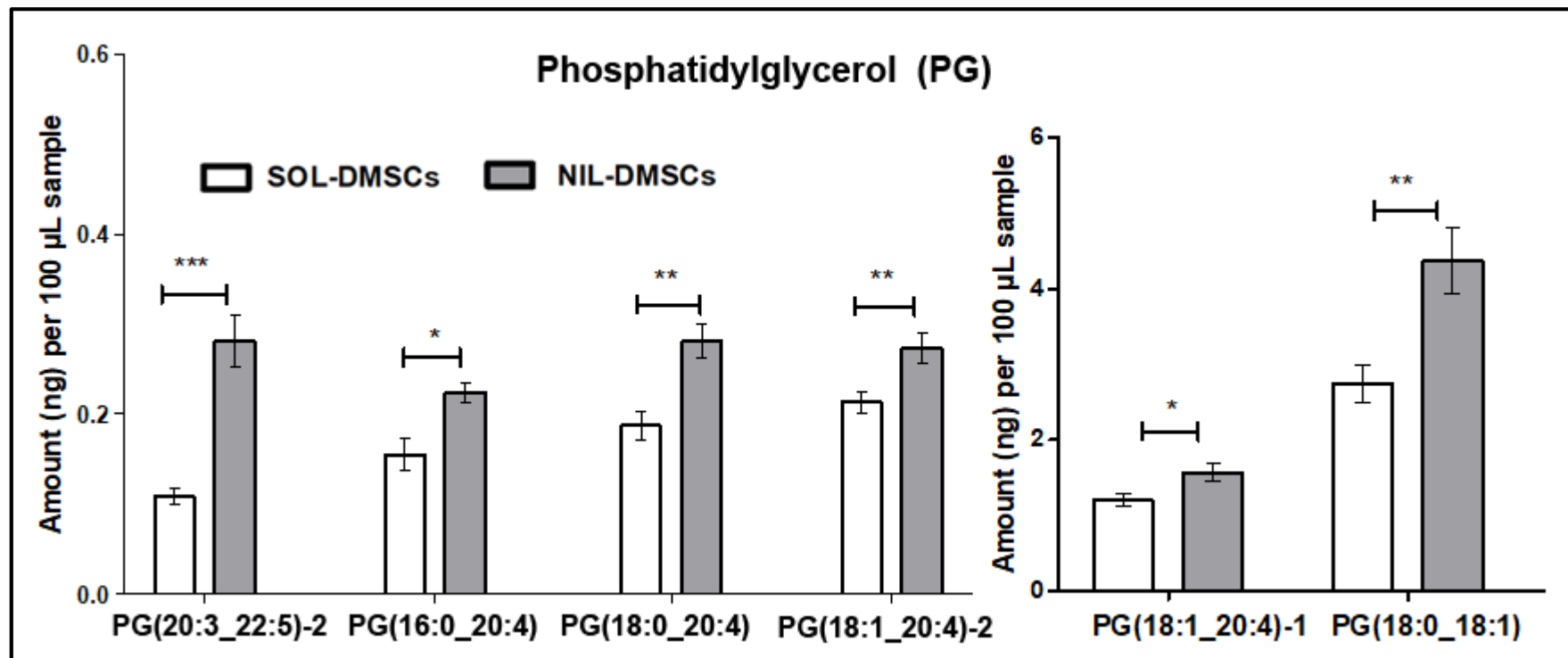


Figure 8. Absolute quantification of Class PG

Only lipid species with p-value < 0.05 between SOL-DMSCs and NIL-DMSCs (n = 7 per group) are shown. Data were presented as mean $\pm$ SEM. P-value deduced from Mann-Whitney U-test. *p < 0.05, **p < 0.01.

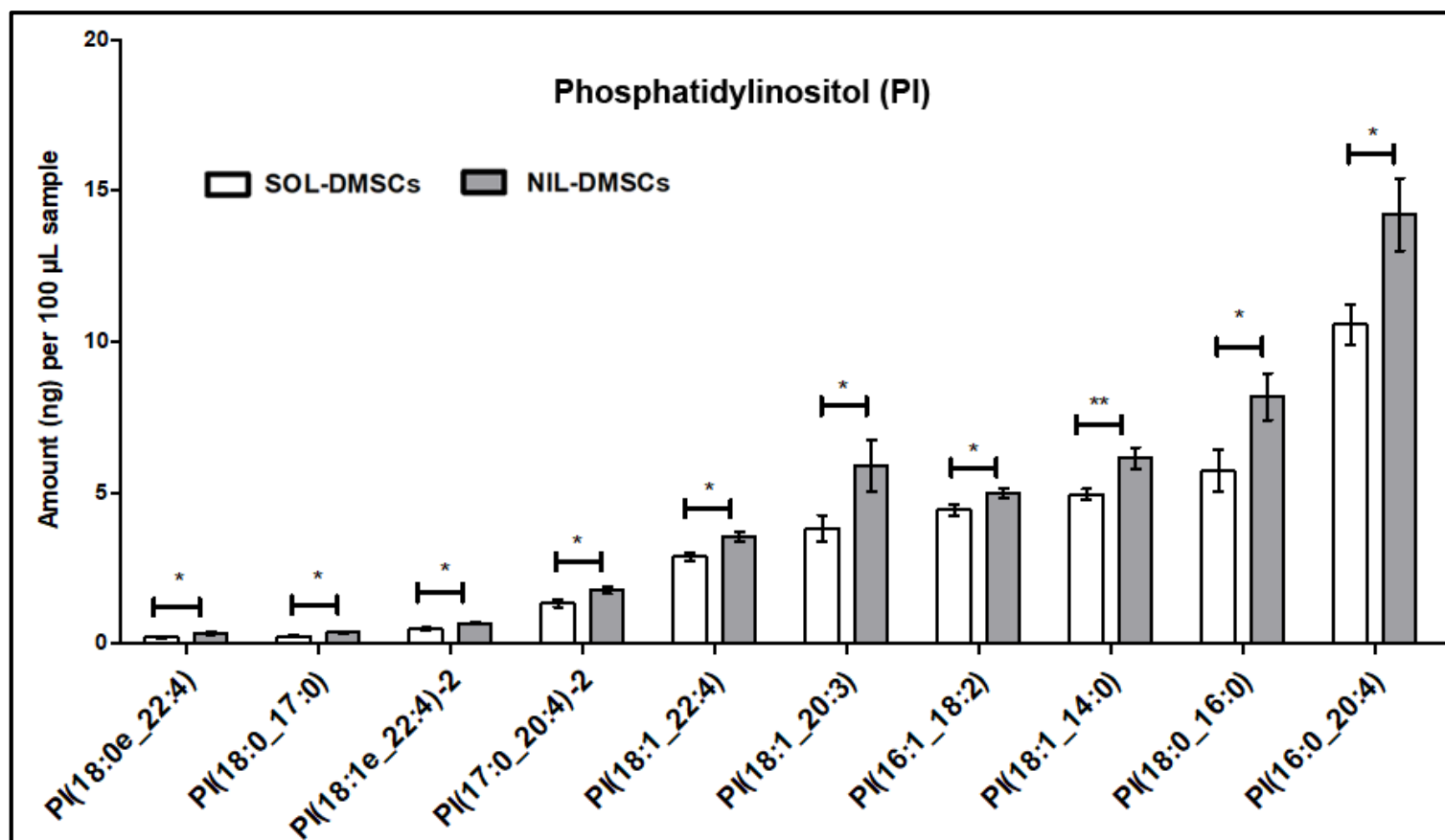


Figure 9. Absolute quantification of Class PI

Only lipid species with p-value < 0.05 between SOL-DMSCs and NIL-DMSCs (n = 7 per group) are shown. Data were presented as mean ± SEM. P-value deduced from Mann-Whitney U-test. *p < 0.05, **p < 0.01.

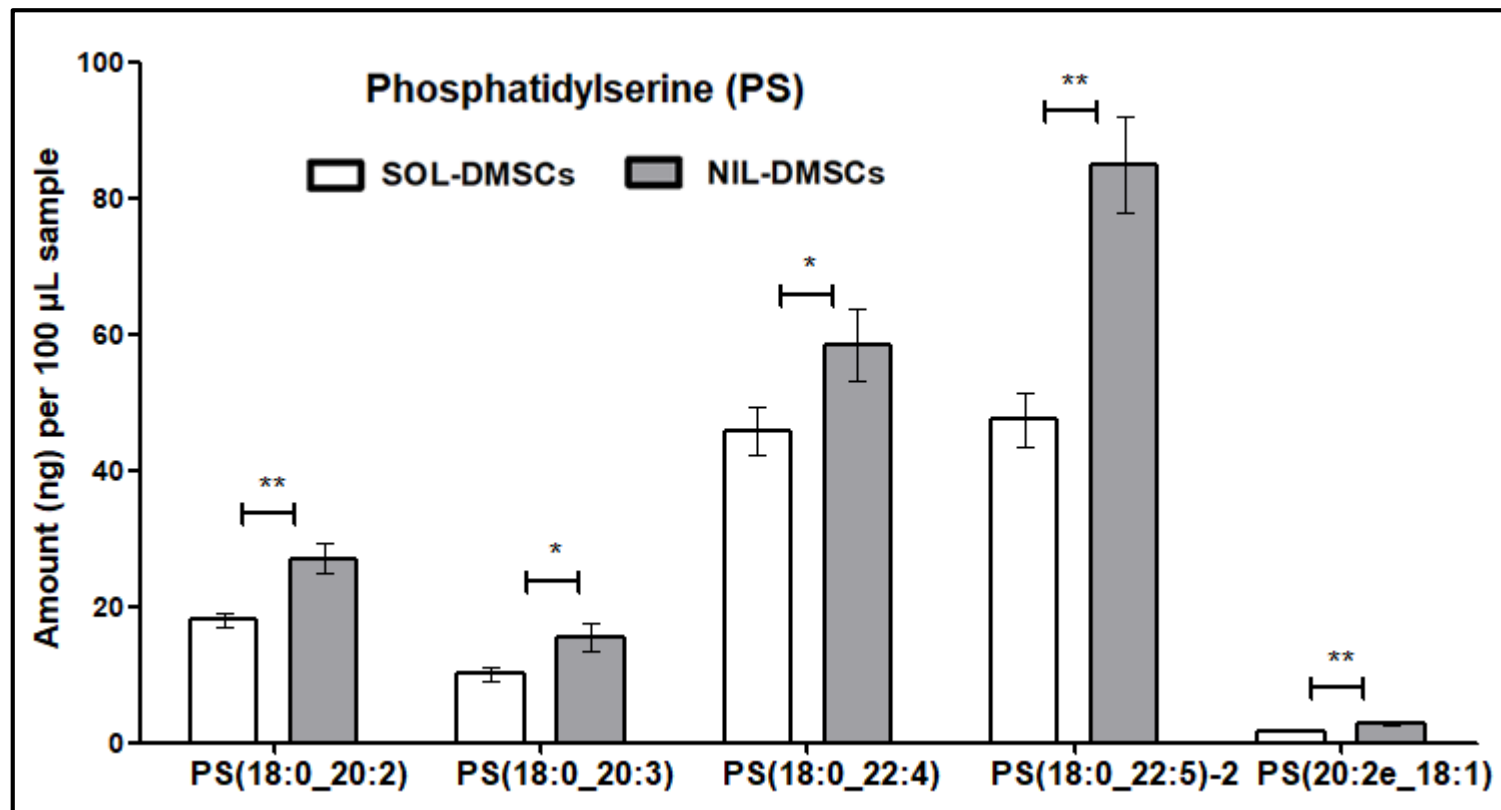


Figure 10. Absolute quantification of Class PS

Only lipid species with p-value < 0.05 between SOL-DMSCs and NIL-DMSCs (n = 7 per group) are shown. Data were presented as mean $\pm$ SEM. P-value deduced from Mann-Whitney U-test. *p < 0.05, **p < 0.01.

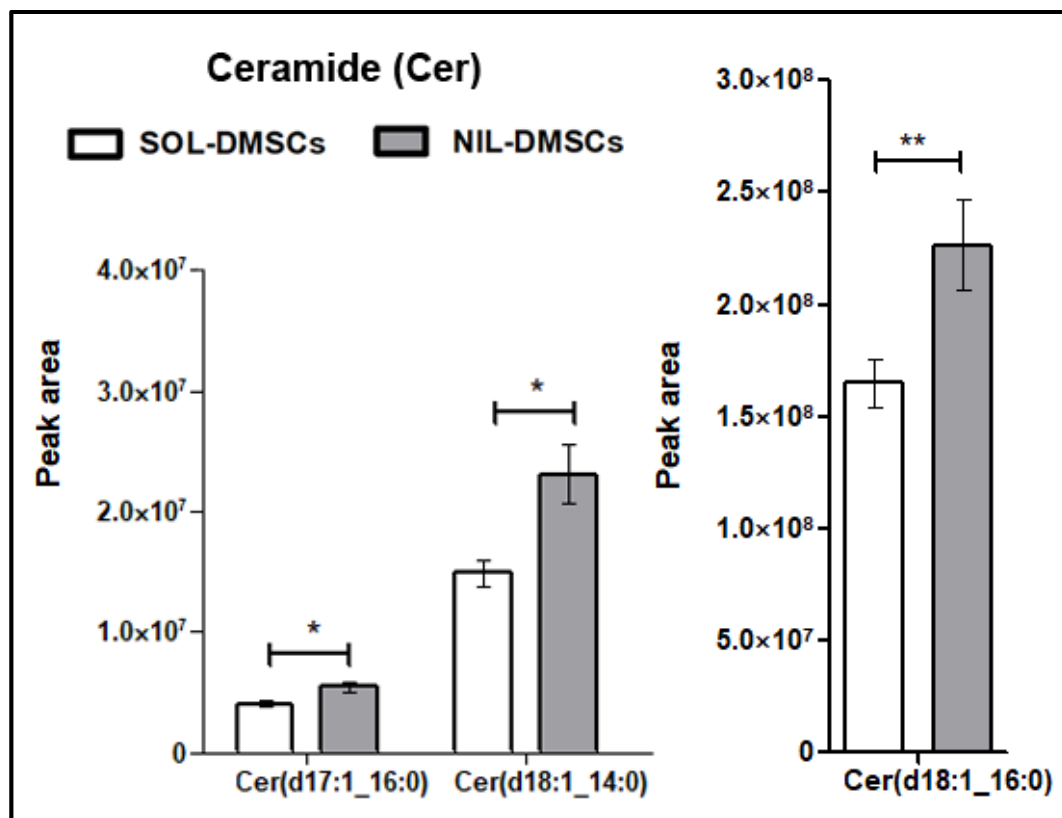


Figure 11. Relative quantification of Class Cer

Only lipid species with p-value < 0.05 between SOL-DMSCs and NIL-DMSCs (n = 7 per group) are shown. The peak area was per 100 μ L sample. Data were presented as mean $\pm$ SEM. P-value deduced from Mann-Whitney U-test. *p < 0.05, **p < 0.01.

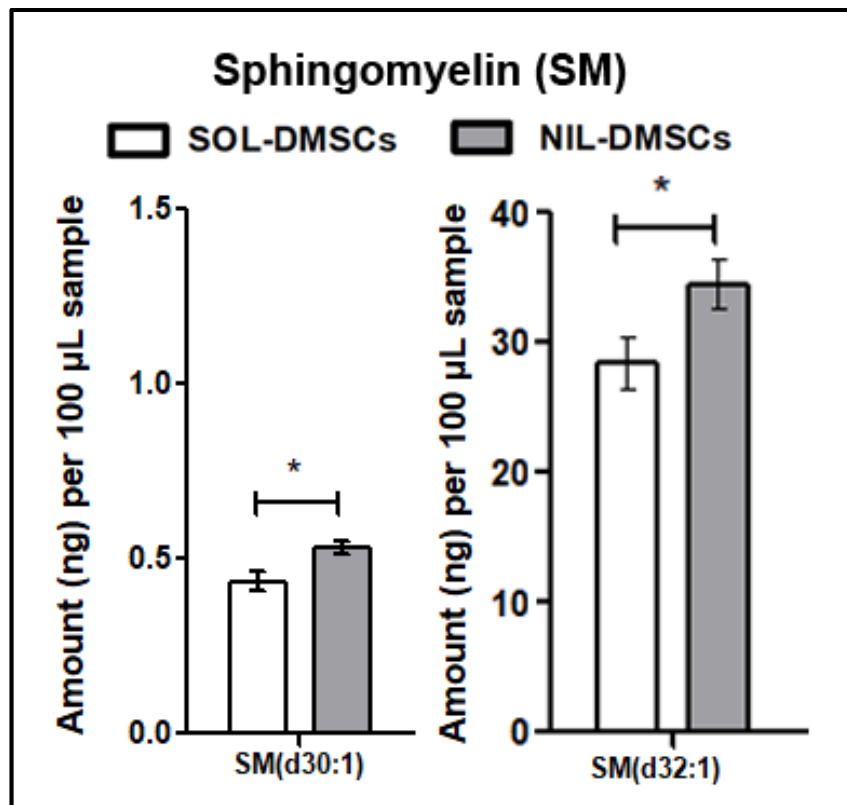


Figure 12. Absolute quantification of Class SM

Only lipid species with p-value < 0.05 between SOL-DMSCs and NIL-DMSCs (n = 7 per group) are shown. Data were presented as mean ± SEM. P-value deduced from Mann-Whitney U-test. *p < 0.05.

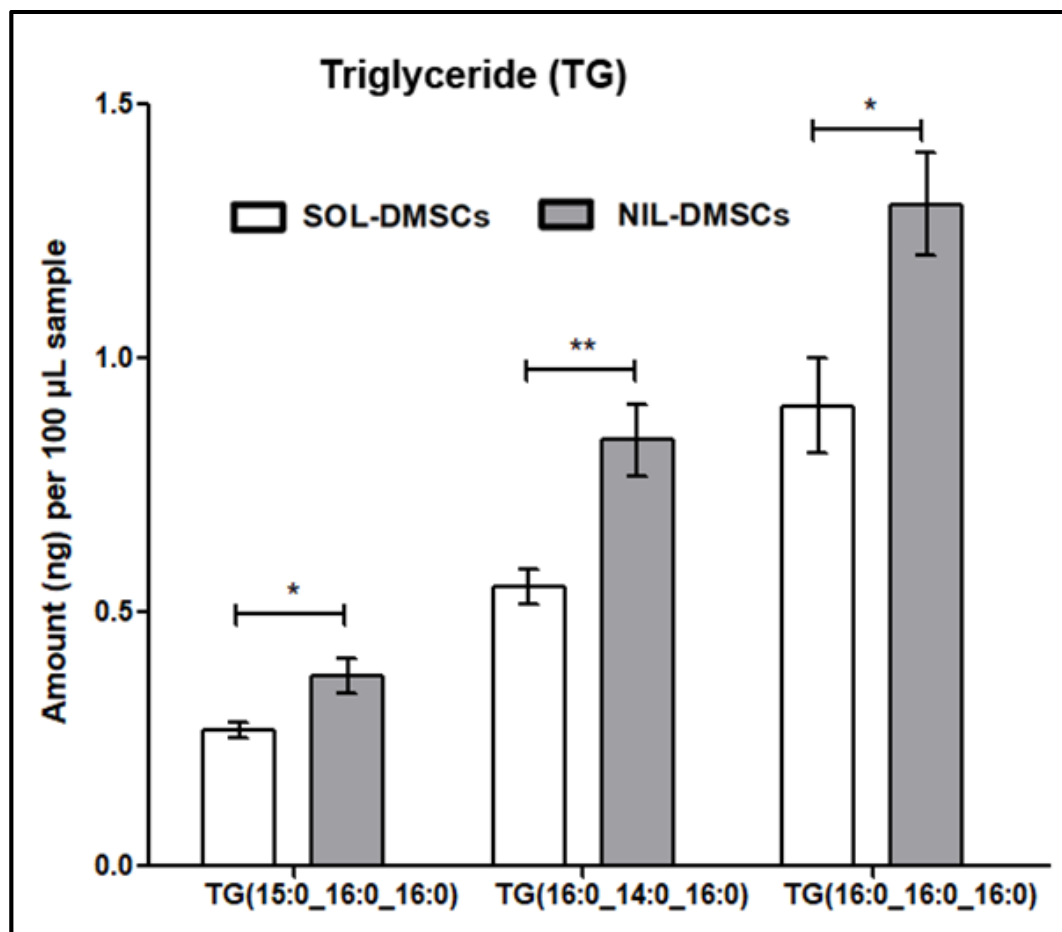


Figure 13. Absolute quantification of Class TG

Only lipid species with p-value < 0.05 between SOL-DMSCs and NIL-DMSCs (n = 7 per group) are shown. Data were presented as mean $\pm$ SEM. P-value deduced from Mann-Whitney U-test. *p < 0.05, **p < 0.01.

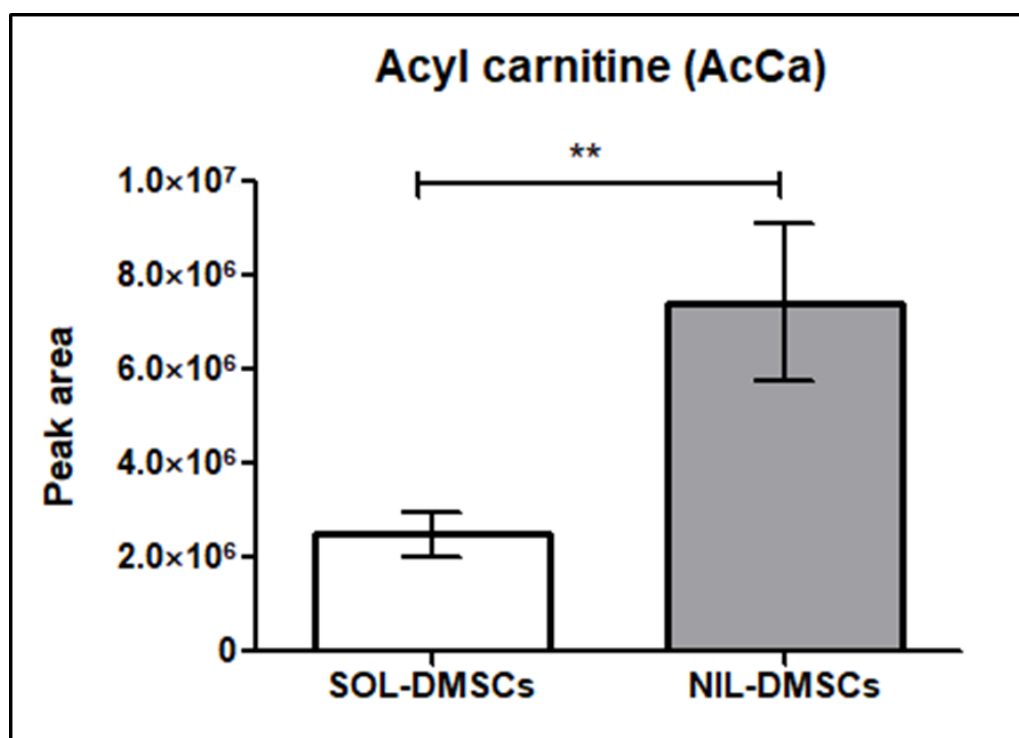


Figure 14. Relative quantification of Class AcCa

Only 1 lipid species was identified in Class AcCa, i.e., AcCa(16:0). The peak area was per 100 μ L sample ($n = 7$ for SOL-DMSCs and NIL-DMSCs each). Data were presented as mean $\pm$ SEM. P-value deduced from Mann-Whitney U-test. ** $p < 0.01$.

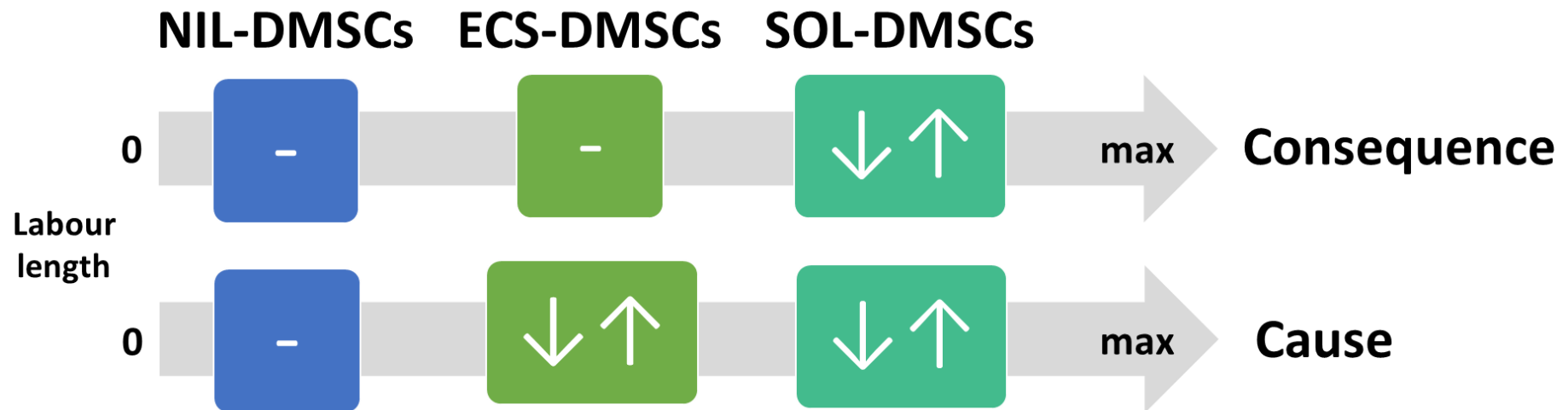


Figure 15. The rationale of the cause-consequence analyses

NIL-DMSCs: DMSCs from placentae of not-in-labour patients undergoing elective C-section delivery. ECS-DMSCs: DMSCs from placentae of patients who undergo a brief labour process before emergency C-section delivery is performed. SOL-DMSCs: DMSCs from placentae of patients undergoing spontaneous onset of labour + vaginal delivery. “-” indicates a control level of DMSC function and lipid profile, while “↓↑” indicates altered levels of DMSC function and lipid profile.

Table 1. Summary of the number of identified lipid in SOL- and NIL-DMSCs

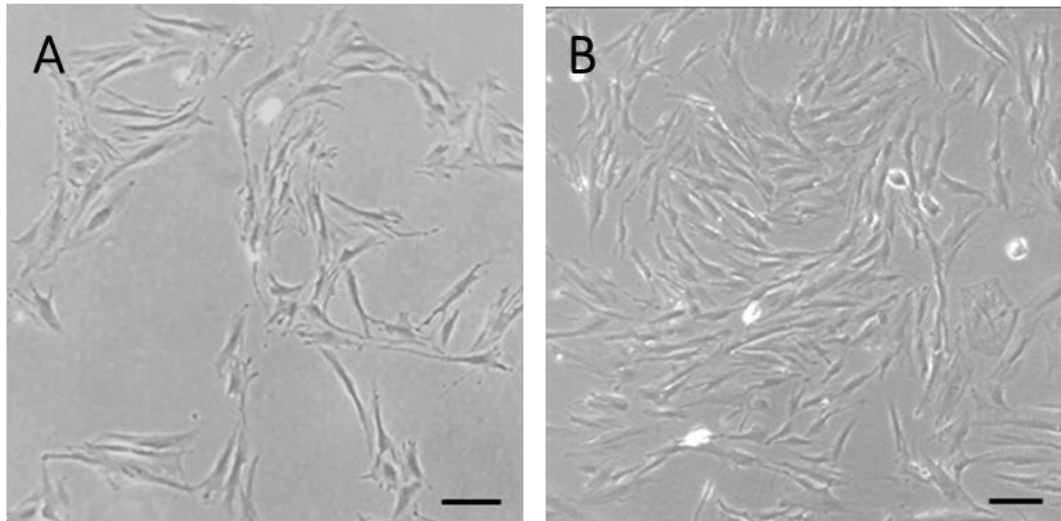
Lipid category/class		Count
Phospholipid (n = 422)		
CL	Cardiolipin	31
LPC	Lysophosphatidylcholine	20
LPE	Lysophosphatidylethanolamine	9
LPI	Lysophosphatidylinositol	1
PC	Phosphatidylcholine	129
PE	Phosphatidylethanolamine	137
PG	Phosphatidylglycerol	34
PI	Phosphatidylinositol	46
PS	Phosphatidylserine	15
Sphingolipids (n = 94)		
Cer	Ceramide	25
Hex1Cer	Hexosyl ceramide	6
Hex2Cer	Dihexosyl ceramide	2
Hex3Cer	Trihexosyl ceramide	4
SM	Sphingomyelin	57
Neutral lipids (n = 52)		
TG	Triglyceride	52
Fatty acyl and other lipids (n = 2)		
AcCA	Acyl Carnitine	1
Co	Coenzyme Q	1
Total		570

SUPPLEMENTARY DATA

Supplementary table 1. Summary of patient characteristics

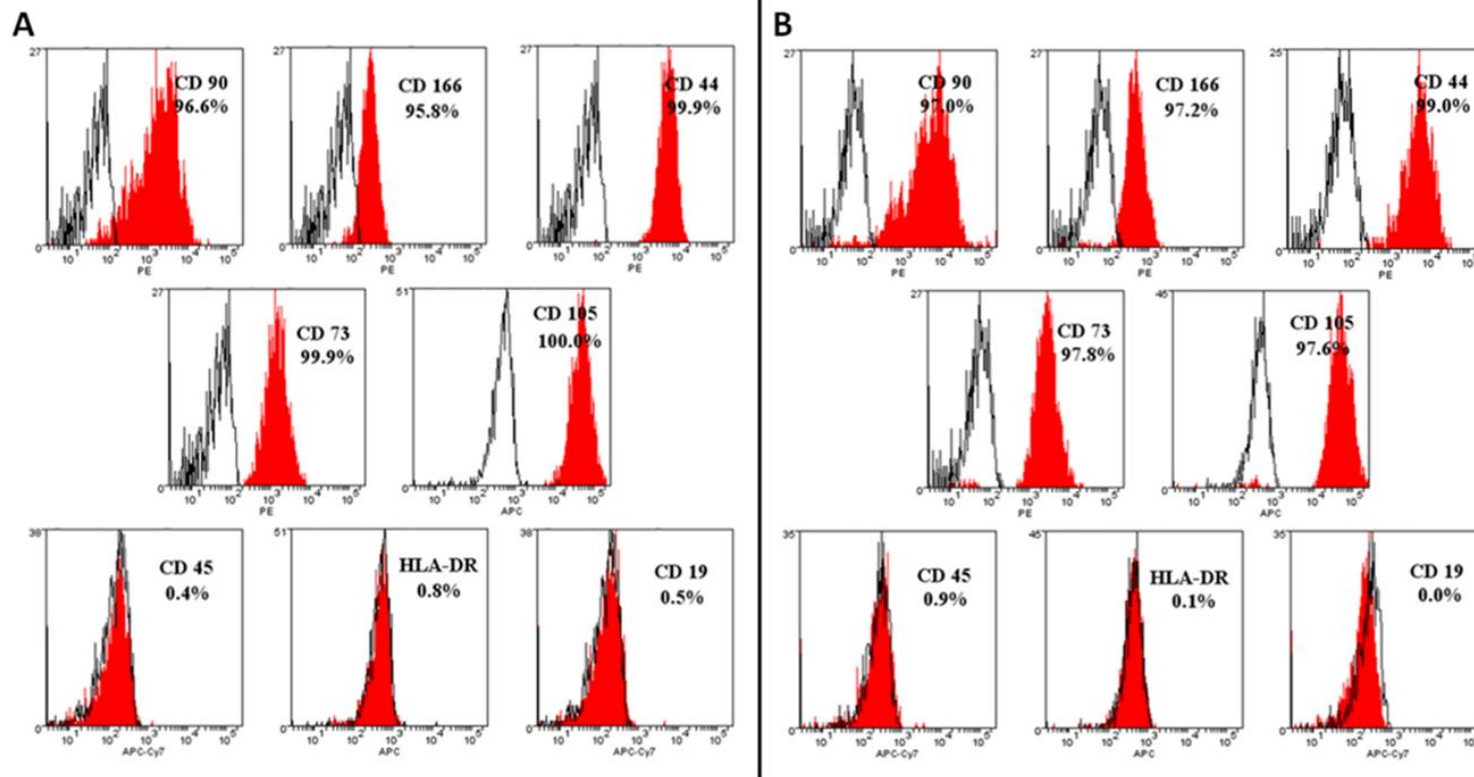
Patient characteristics	Labour status		p-value
	SOL (n = 19)	NIL (n = 17)	
Maternal			
Age (years)	30.9 ± 0.9	30.5 ± 1.1	0.70 ^a
BMI (kg/m ²)	21.7 ± 0.6 [*]	23.5 ± 1.2	0.32 ^a
Labour			
Total length of labour (h)	5.79 ± 0.83	N/A	N/A
Birth and Neonate			
Gestational age (weeks)	38.83 ± 0.29	38.69 ± 0.22	0.80 ^a
Placental weight (g)	616 ± 31 ^{**}	678 ± 38	0.40 ^a
Infant birth weight (g)	3398 ± 95	3604 ± 94	0.14 ^a
Infant gender	4 Male and 15 Female	7 Male and 10 Female	0.28 ^b

Numerical data were presented as mean ± SEM. P-values deduced from (a) Mann Whitney U test and (b) Fisher's exact test. The differences between groups are considered statistically significant when p-value < 0.05. ARM: artificial rupture of membrane. SROM: spontaneous rupture of membrane. *BMI of one patient was not measured (n = 18). **Placental weights of four patients were not measured (n = 15).



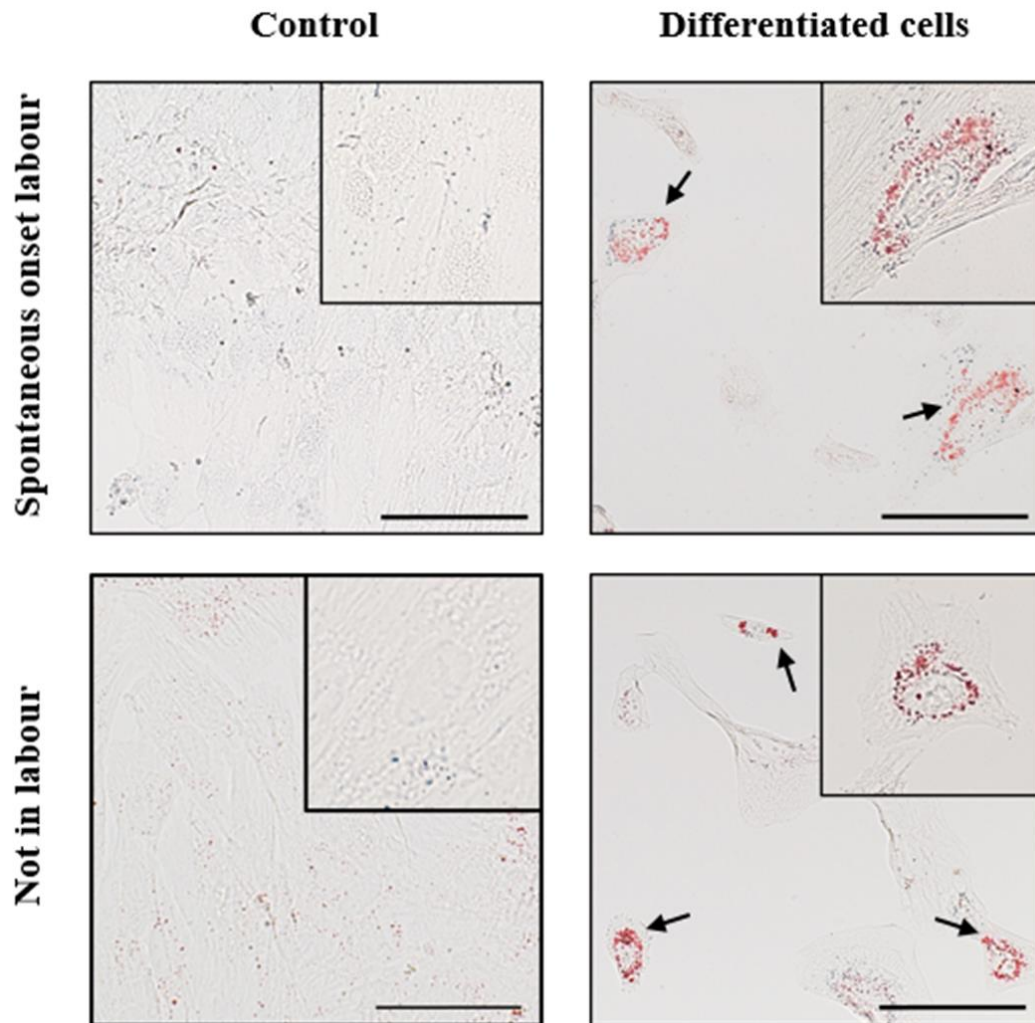
Supplementary figure 1. *In vitro* morphology of the representative primary cells

Primary cells were isolated from term placentae of patients undergoing **(A)** SOL and **(B)** NIL. Cells from both patients demonstrated typical fibroblastic morphology of MSCs. Morphology was observed at passage 2, using a bright field inverted microscope (Leica DM E) at a magnification of 100X. Scale bar = 50 μ m.



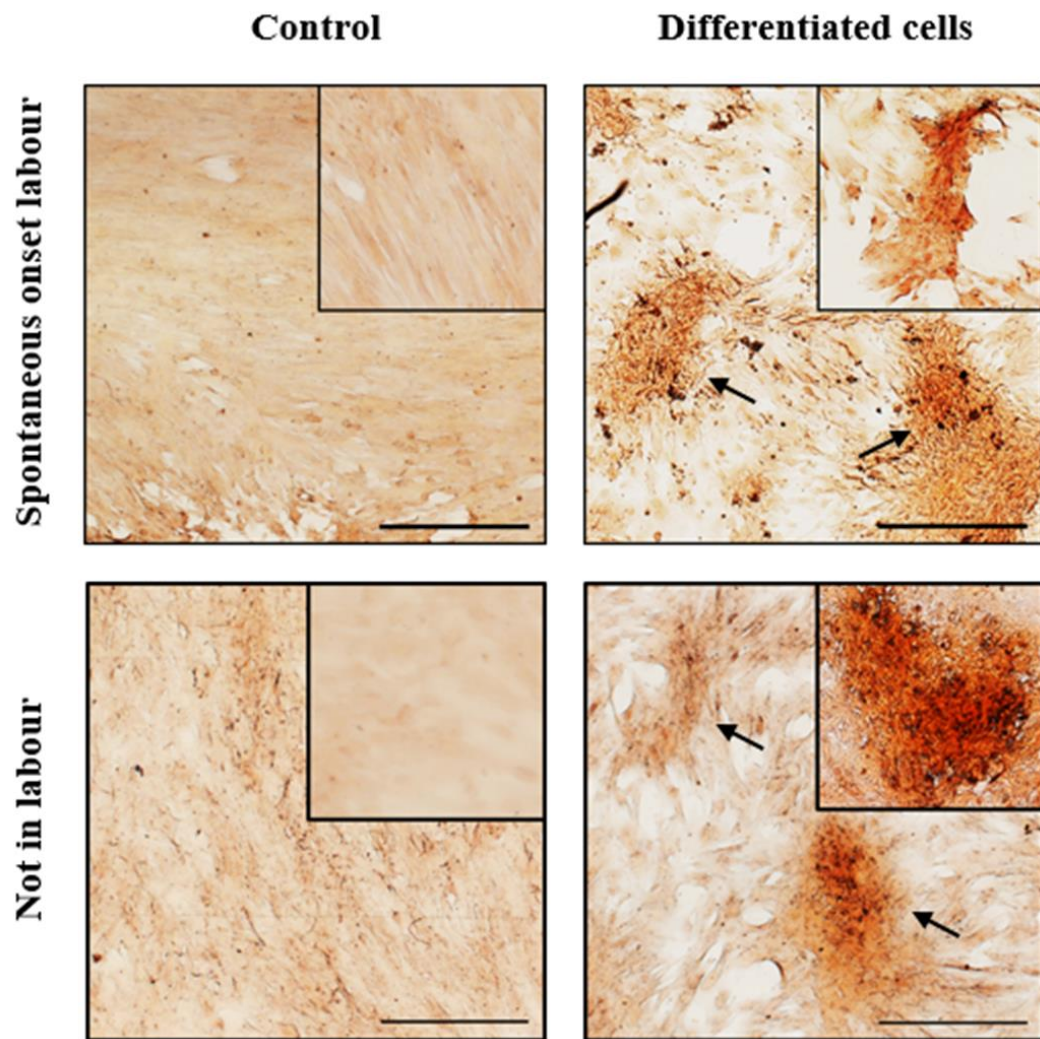
Supplementary figure 2. Flow cytometry analyses of the representative primary cell surface marker expression

Primary cells isolated from both **(A)** SOL and **(B)** NIL patients satisfied MSC criteria by positively expressed CD90, CD166, CD44, CD73, and CD105 ($\geq 95\%$ positive) and negatively expressed the hematopoietic markers CD45, HLA-DR, and CD19 ($\leq 5\%$ positive). Red and white histograms represent the expression of MSC-specific surface markers and their associated isotype respectively. X-axis: fluorescence intensity. Y-axis: number of recorded events, set to 10^4 cells per run. The percentages of cells positively expressed the surface markers are shown. PE: Phycoerythrin; APC: Allophycocyanin; Cy7: tandem conjugate.



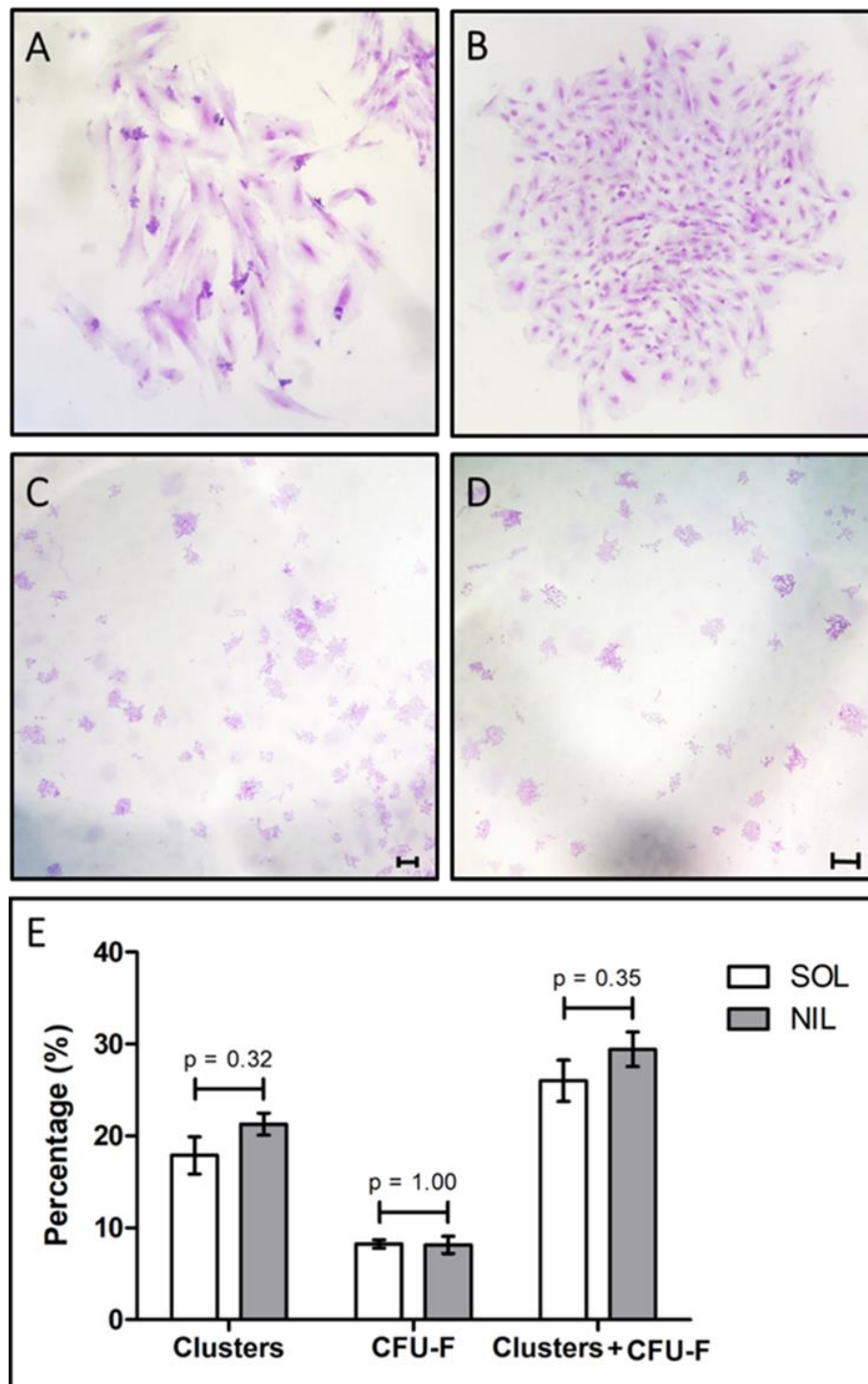
Supplementary figure 3. Adipogenic potential of the representative primary cells

Primary cells from both SOL and NIL patients showed potential to differentiate into the adipogenic lineage, as shown through the formation of lipid droplets (indicated by arrows) after 14 days incubation with adipogenic induction media. Cells were stained with Oil Red O prior to the microscopic observation at 200X magnification (Axioskop 2 microscope equipped with Nikon DXM 1200C camera). Scale bar = 50 μ m. Insets show the differentiated cells at 400X magnification.



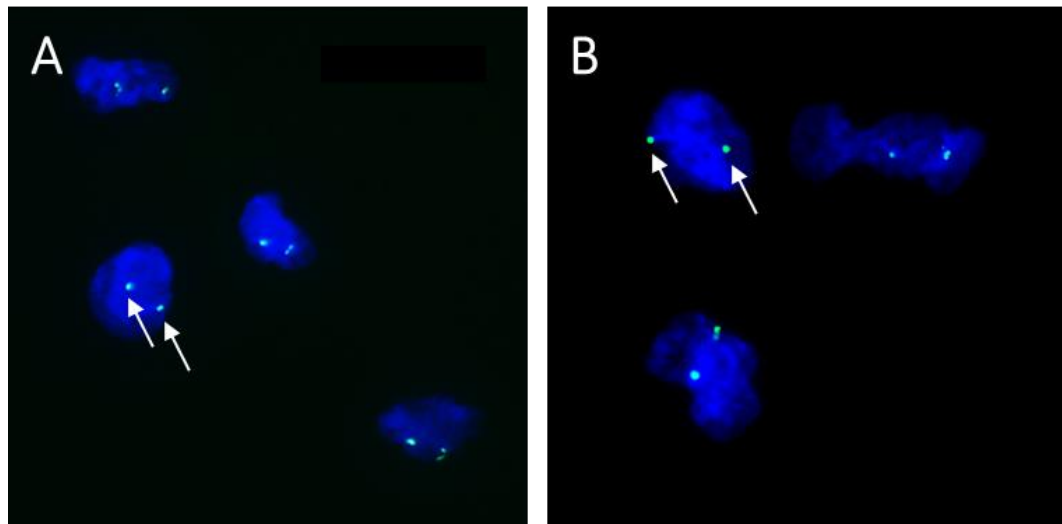
Supplementary figure 4. Osteogenic potential of the representative primary cells

Primary cells from both SOL and NIL patients showed potential to differentiate into the osteogenic lineage, as shown through the formation of calcium deposits (indicated by arrows) after 21 days incubation with osteogenic induction media. Cells were stained with Alizarin Red prior to the microscopic observation at 40X magnification (Axioskop 2 microscope equipped with Nikon DXM 1200C camera). Scale bar = 100 μ m. Insets show the differentiated cells at 100X magnification.



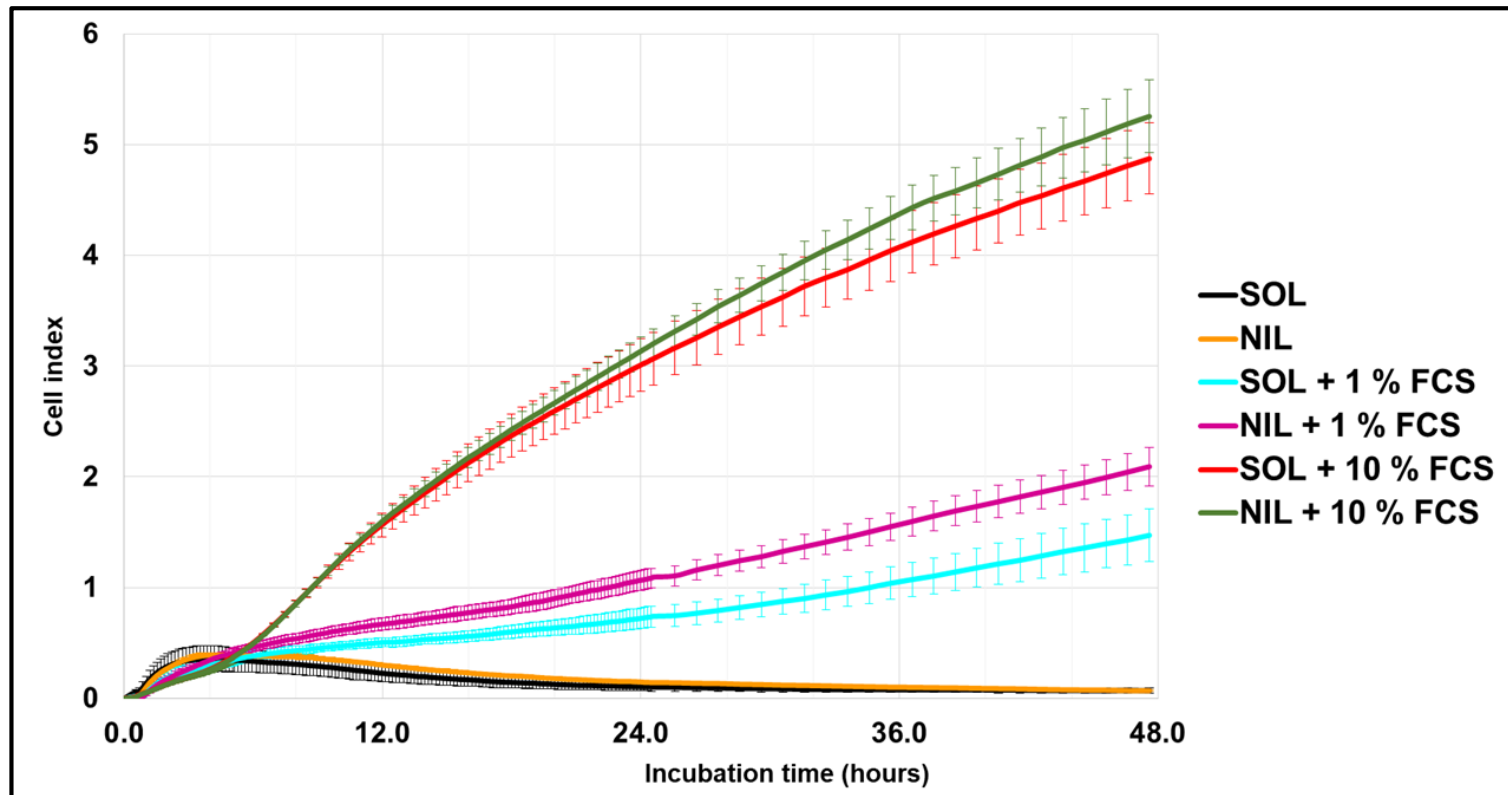
Supplementary figure 5. Clonogenicity of the isolated primary cells

Morphology of a representative **(A)** cell cluster containing fewer than 50 cells and **(B)** CFU-F colony of greater than 50 cells at 400X magnification. Clonogenicity of DMSCs from patients undergoing **(C)** SOL and **(D)** NIL observed at 64 X magnification. Cells were observed with a stereo microscope (Leica/Wild M3C). Scale bar: 1 mm. **(E)** Clonogenic efficiency of SOL- and NIL-DMSCs were not significantly different ($p > 0.05$). Data were presented as mean $\pm$ SEM. P-values deduced from Mann-Whitney U test. Samples were tested in triplicate.



Supplementary figure 6. FISH analyses of the representative primary cells

FISH analyses were performed on a minimum of 200 cell nuclei per sample of primary cells from placentae of male babies. Primary cells from **(A)** SOL and **(B)** NIL patients were confirmed as maternal in origin by the presence of two Spectrum Green X chromosome signals (arrows) and the absence of Spectrum Orange Y chromosome signals. Cell nuclei were counterstained with DAPI. Nuclei were observed with a fluorescence microscope (Olympus BX51 microscope equipped with JAI M300 CCD camera) at 600X magnification. Interphase FISH images were processed using Metasystems ISIS image analysis software v5.3.16 (MetaSystems).



Supplementary figure 7. Optimisation of serum concentration of the serum starvation medium

Real-time xCELLigence analysis of cell proliferation. The lines represent the cell indices of SOL- and NIL-DMSCs resuspended in growth medium supplemented with 0 (n = 3 per group), 1 (n = 3 per group), and 10% FCS (n = 8 for SOL-DMSCs and n = 7 for NIL-DMSCs). Data were presented as mean $\pm$ SEM. Cell indices were measured for 48 hours. Cells seeded in serum-starvation medium (1% FCS) showed a similar proliferation trend to those seeded in α -MEM complete medium (10% FCS), but at a reduced level of cell indices.