

# **Isolation and characterization of mesenchymal stem cells from human tissues**

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## Summary

Mesenchymal stem cells (MSCs) derived from human adipose tissue and umbilical cord (Wharton's jelly, UCB) represent a useful source of adult stem cells for cellular therapy and tissue engineering. The biggest concern with the use of MSCs therapeutically relates to their isolation and growth/manipulation *ex vivo*. This study aimed to establish methods for the routine isolation and characterization of MSCs from human tissues. The objectives were (1) to show that MSCs could be isolated from different human tissues, namely adipose tissue, Wharton's jelly, and UCB; (2) to confirm the MSC phenotypic profile over at least 10 passages; and (3) to show the multilineage differentiation capacity of the isolated cells. The minimal criteria as defined by the International Society for Cellular Therapy (ISCT) were used to determine whether MSCs were successfully isolated from various human tissues. Two different techniques involving enzymatic digestion or explant cultures were utilized, and compared for isolating MSCs from Wharton's jelly. Umbilical cord blood has been suggested as another source of MSCs. However, we were unable to grow MSCs from UCB. Proliferation kinetics of isolated MSCs revealed that cords, either from digested cords or cord pieces had a mean PDT from passage 1 to 4 that was approximately 3 fold lower than for the ASCs. Mesenchymal stem cells from adipose tissue and Wharton's jelly expressed the classical MSC phenotype (CD73+, CD90+, CD105+, CD34-, and CD45-). The cells from Wharton's jelly showed a more uniform MSC profile over passages, with higher levels of marker expression when compared to ASCs. Variability in phenotype was observed in early ASC passages, whereas WJ-MSCs seemed to attain the MSC phenotype as early as passage 0 for both isolation techniques. Low levels of CD34 positive cells remained in the ASCs. Oil red O staining was used for identifying the lipid droplets in adipogenic differentiation cultures. A colorimetric assay as well as image analysis was used to quantify the differentiation. For the cord samples, both assays produced positive results. Histological examination, however, revealed that the cords did not form lipid droplets. The ASCs showed a statistically significantly greater differentiation capacity into adipocytes compared with the cords (pooled digested and pieces data). Alizarin red S staining was used for identifying calcium deposition during matrix mineralization in osteogenic differentiation cultures. No significant differences in osteogenic differentiation were observed between ASCs and WJ-MSCs. Chondrogenic differentiation was observed for both MSC sources by positive staining of glycosaminoglycans using toluidine blue O. The main findings of the study showed that MSCs, according to the ISCT guidelines, were successfully harvested from adipose tissue. However, due to the lack of adipogenic differentiation of WJ-derived cells, they did not meet the ISCT guidelines to be classified as MSCs, and were referred to as MSC-like cells. Regardless of the isolation technique used, Wharton's jelly yielded cells with similar

proliferation capacity, phenotype, and differentiation capacity. This study did, however, reveal that biological differences do exist between stem cells from different sources.

Key words: MSCs, adipose tissue, Wharton's jelly, stem cells, isolation, characterization, differentiation, growth kinetics, flow cytometry, phenotype

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## List of Abbreviations and symbols

%	Percentage
ADAS	Adipose-derived adult stem cell
AdMSC	Adipose mesenchymal stem cell
ADSC	Adipose-derived stromal cell
APC	Allophycocyanin
ARS	Alizarin red S
ASC	Adipose derived-stem cell
BM	Bone marrow
BMMC	Bone marrow mononuclear cells
BMP	Bone morphogenetic protein
Ca <sup>2+</sup>	Calcium <sup>2+</sup>
cAMP	Cyclic adenosine monophosphate
CBE	Cord blood-derived embryonic like stem cells
CD	Cluster of differentiation
CEBP	CCAAT/enhancing binding protein
CFU-F	Colony forming unit fibroblast
CGM	Complete growth medium
CO <sub>2</sub>	Carbon dioxide
CPC	Cetylpyridinium or hexadecylpyridinium chloride
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle's medium

DNA	Deoxyribonucleic acid
EB	Embryoid body
EC	Embryonic carcinoma
ECD	Electron coupled dye
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGSC	Embryonic germ stem cells
ESC	Embryonic stem cell
FAH	Fumarylacetoacetate hydrolase
FBS	Fetal bovine serum
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
G-CSF	Granulocyte colony-stimulating factor
GMP	Good manufacturing practice
GvHD	Graft-versus-host disease
hESC	Human embryonic stem cell
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
hUC	Human umbilical cord
hUCMSC	Human umbilical cord matrix stem cell
ICM	Inner cell mass
IDO	Indoleamine 2,3-dioxygenase

IFN-γ	Interferon gamma
IFN-β	Interferon beta
IL-6	Interleukin-6
iPSC	Induced pluripotent stem cell
ISCT	International Society for Cellular Therapy
ISHAGE	International Society for Hematotherapy and Graft Engineering
IVF	<i>in vitro</i> fertilization
Klf4	Kruppel-like factor 4
Lipo_asp	Lipoaspirate
MCSF	Macrophage colony stimulation factor
Mg <sup>2+</sup>	Magnesium <sup>2+</sup>
MI	Myocardial infarction
MNC	Mononuclear cell
MPC	Multipotent progenitor cell
MSC	Mesenchymal stem cell
Na <sub>2</sub> CO <sub>3</sub>	Sodium carbonate
NHL	Non-Hodgkin's lymphoma
NTBC	2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione
°C	Degrees Celsius
Oct3/4	Octamer-binding transcription factor 3/4
OI	Osteogenesis imperfect
OPG	Osteoprotegerin
ORO	Oil red O

p/s	Penicillin/ streptomycin
PBS	Phosphate buffered saline
PC5	Phycoerythrin-cyanine 5.1
PC7	Phycoerythrin-cyanine 7
PD/T	Population doubling/time
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
pH	Potential of hydrogen
PI	Proliferation index
PLA	Processed lipoaspirate
PPAR-γ	Peroxisome proliferator-activated receptor gamma
RANK	Receptor activator of nuclear factor-κB
RANKL	Receptor activator of nuclear factor-κB ligand
RBC	Red blood cell
RT	Room temperature
RTC	Randomized clinical trial
Runx2	Runt family transcription factor 2
SANBS	South African National Blood Services
SCID	Severe combined immunodeficient
SCNT	Somatic cell nuclear transfer
SD	Standard deviation
SDF-1	Stromal-derived factor-1
SOX	Sex-determining region Y-type high motility group box

SVF	Stromal vascular fraction
TBO	Toluidine blue O
TGF- $\beta$	Transforming growth factor beta
UC	Umbilical cord
UCB	Umbilical cord blood
USSC	Unrestricted somatic stem cell
WJ-MSC	Wharton's jelly mesenchymal stem cells
WJSC	Wharton's jelly stem cells
yr	Year
$\alpha$ - MEM	Modified Eagle's medium- Alpha



## Chapter 1: Introduction

---

Cell based therapy describes the process of introducing cells into tissue for treating diseases or damaged tissues. In particular, the use of autologous cells for the repair of damaged or defective tissue has enormous potential for the treatment of a wide variety of diseases and tissue defects. The cells of interest include, but are not limited to stem cells, especially because of their therapeutic potential as candidates for replacement or reparative therapy of diseases including gene or cell engineering (Nerem, 2007).

Replacement involves growing or producing tissues and organs outside the body followed by implanting the newly engineered tissue or organ. Repair refers to biological repair at a cellular level. Regeneration is the ability to grow new tissues and organs *in vivo* (Nerem, 2007). A better understanding of basic biology will contribute to the success of bringing more biological treatment approaches to patients. Cells and/or stem cells are believed to reside in specific tissue microenvironments known as niches (Ohlstein *et al.*, 2004). Cells are not independent entities, but rather exert functions as directed by the specific microenvironment they reside in. The therapeutic use of cells will rely on initiating signals, the right signal at the right place and at the right time, which will direct the cells' function in a given direction.

When using exogenous cells as a therapy, the source of cells needs to be considered. The choice between using differentiated cells, stem cells, or progenitor cells will depend on the desired function one wishes to achieve. Autologous cell therapy implies that the donor and recipient is the same person. The cells are harvested, cultured *ex vivo* to obtain therapeutic cell numbers, and then re-introduced to repair the damaged tissue without the need for immunosuppressive drugs (Fodor, 2003). Limitations in harvesting autologous cells (such as the amount of tissue that can be harvested, and the need for surgery or a biopsy which could lead to a secondary site of pain) and the need for culture expansion are still problematic. The development of immunosuppressive drugs has facilitated the use of allogeneic cells. Cells are defined as being allogeneic when the donor and recipient

is not the same person. Treatment using allogeneic cells would have broader therapeutic applications as cells could potentially be harvested from a single donor or from a few donors, expanded, and then stored until needed. However, allogeneic treatment requires the cells to be human leukocyte antigen (HLA) matched along with the use of immunosuppressive drugs to prevent immune rejection of donor cells by the host. Bone marrow (BM) transplantation is the most widely utilized and universally accepted form of cell-based therapy often used to treat hematologic disorders and cancers. It is applied in either an autologous or allogeneic manner. Xenotransplantation (cross-species transplantation) is not widely accepted as cross-species pathogen infection may occur (Kaiser, 2004). For cell-based approaches, cells are often used in combination with a scaffold. Biological or synthetic scaffolds provide the appropriate structural support to direct regeneration of tissues and organs.

Problems with cell-based therapeutic strategies in the clinical setting are the variability between individuals, the disease state of the individual, and immunogenicity when not using autologous cells. Cell-based therapies may need to be customized for specific patients, leading to a labour-intensive and individualized form of medical treatment. However, the possibility of producing cells in bulk from a master stock, banking these cells and then delivering the cell treatment from the banked cells is also being considered (Daley and Scadden, 2008). Currently, international registries of BM donors enable the transplantation of allogeneic hematopoietic stem cells (HSCs) for treating blood cancers and genetic diseases.

In South Africa, cell-based therapy using non-hematopoietic adult stem cells is still in a very early phase, despite the significant progress that has been made in many other parts of the world. Over the years the focus of stem cell research has shifted from interest in embryonic stem cells (ESCs) to adult stem cells, with increasing interest arising in the non-hematopoietic adult stem cells known as mesenchymal stem cells (MSCs). Mesenchymal stem cells are being proposed as the most appropriate adult stem cell candidate for therapeutic purposes as they are known to be immune privileged (Bartholomew *et al.*, 2002, Chamberlain *et al.*, 2007). Currently the *in vivo* location, identification, and activities of MSCs are still poorly understood. One of the concerns related to the routine therapeutic use of MSCs is that they

cannot be reproducibly isolated. The identification of MSCs is based on morphologic or phenotypic characteristics along with their differentiation capacity into lineages of mesenchymal origin. However, there is no one specific MSC marker or set of markers that permits the isolation of a pure population of MSCs.

The mechanisms underlying the therapeutic benefits of MSCs are still poorly understood. Mesenchymal stem cell therapy is not yet considered as a routine form of clinical treatment. Proof of concept pre-clinical data and clinical trials addressing the safety and efficacy of MSCs are therefore urgently needed. The challenges preventing clinical use of MSCs are (1) the link between the phenotypic characteristics and biological functions of MSCs; (2) the lack of clinical data to support the safety of MSCs; (3) a lack in understanding of the biology of MSCs following *in vivo* transplantation (*i.e.* mechanisms responsible for survival); (4) a lack in understanding of the mechanisms of MSC homing; and (5) the lack of data showing MSC efficacy following transplantation (Si *et al.*, 2010).

The proposed study aims to establish methods for the routine isolation and *in vitro* characterization of MSCs from three different human tissues. Stem cells should meet the criteria as proposed by Gimble *et al.*, (2007) before they can be considered as a therapy for regenerative applications. Stem cells need to:

- i. be available in abundant quantities
- ii. be harvested by minimally invasive procedures
- iii. have multipotent differentiation capacity
- iv. be safely and effectively transplanted in autologous and/or allogeneic settings
- v. be expandable in culture under Good Manufacturing Practice (GMP) conditions
- vi. lack formation of teratomas or tumours following transplantation

Mesenchymal stem cells conform to the above mentioned criteria. Therefore, investigation into their routine isolation and characterization from human tissues is an essential first step before they can be considered for clinical applications.

The biggest concern with the use of MSCs therapeutically relates to their isolation. Literature shows a lack of standardization when isolating and characterizing these cells. Bone marrow derived MSCs have been the cells of choice in research and for

clinical applications. However, their isolation involves painful invasive harvesting with low cell yield (Fong *et al.*, 2011). Reports on tissues which contain MSCs include adipose tissue, umbilical cord (Wharton's jelly), and umbilical cord blood (UCB).

The biological and clinical interest in MSCs has risen dramatically, which will undoubtedly accelerate scientific discovery and the development of novel cellular therapies. Many ambiguities and inconsistencies have arisen with this increasing interest. To address the problem, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) has proposed a set of standards to define MSCs for both laboratory-based scientific investigations and for pre-clinical studies. They propose three minimum criteria: (1) adherence to plastic under standard culture conditions; (2) positive expression ( $\geq 95\%$ ) for cell surface antigens CD105, CD73, CD90 and negative expression ( $\leq 2\%$ ) for CD45, CD34, CD14, or CD11b, CD79 $\alpha$  or CD19 and HLA-DR; and (3) *in vitro* differentiation into adipocytes, osteoblasts, and chondrocytes (Dominici *et al.*, 2006). Many papers have been published stating the successful isolation of MSCs; however, ambiguities around their characterization still exist.

The present study aimed to establish methods for the routine isolation and characterization of MSCs from human tissues. The study objectives were (1) to establish whether MSCs could be isolated from different human tissues, namely adipose tissue, Wharton's jelly, and UCB; (2) to determine the MSC phenotypic profile over at least 10 passages; and (3) to determine the multilineage differentiation capacity of the isolated cells. The minimal criteria as defined by the ISCT were used in determining whether MSCs were successfully isolated from various human tissues.

The isolation of MSCs from the various tissues will be described in Chapter 3. Isolation methods commonly described in literature were followed and standardized. For the isolation of Wharton's jelly-derived MSCs (WJ-MSCs), two different isolation processes were followed and compared to determine the most appropriate method. The proliferation rates between passages and tissue sources were compared. Following the isolation of the cells, phenotypic characterization was done. Cells were characterized over at least 10 passages for the expression of MSC markers. Chapter

4 describes phenotypic profiles of the three different tissues and between the same tissues obtained from different donors. The multilineage differentiation capacity of the isolated cells will also be described. Chapter 5 reports on experiments which examined the ability of all sources of MSCs to differentiate into adipocytes, osteoblasts, and chondrocytes. Histology, image analysis software, and colorimetric assays were employed to quantitate the differentiation capacity between samples.

The following hypotheses were formulated for the present study:

1. Cells that conform to the minimal characterization criteria to be classified as MSCs can be isolated from adipose tissue, UCB, and Wharton's jelly.
2. MSCs isolated from Wharton's jelly, either by enzymatic digestion or by explant cultures, will show no differences phenotypically or in differentiation capacity.
3. MSCs isolated from adipose tissue and Wharton's jelly will show differences phenotypically and in differentiation capacity.
4. Wharton's jelly is a better source of MSCs than adipose tissue.

This dissertation concludes with a summary of the outcomes of the study and the implications this might have for future clinical applications of MSCs.

## Chapter 2: Literature review

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### 2.1 Overview of Stem Cells

Stem cell therapy has the potential to be applied to patients suffering from degenerative diseases and tissue defects due to trauma and/or disease. This procedure may also offer the only therapeutic option to various diseases that remain incurable at present. Stem cells also show promise for the repair of cellular defects associated with numerous degenerative disorders of genetic origin.

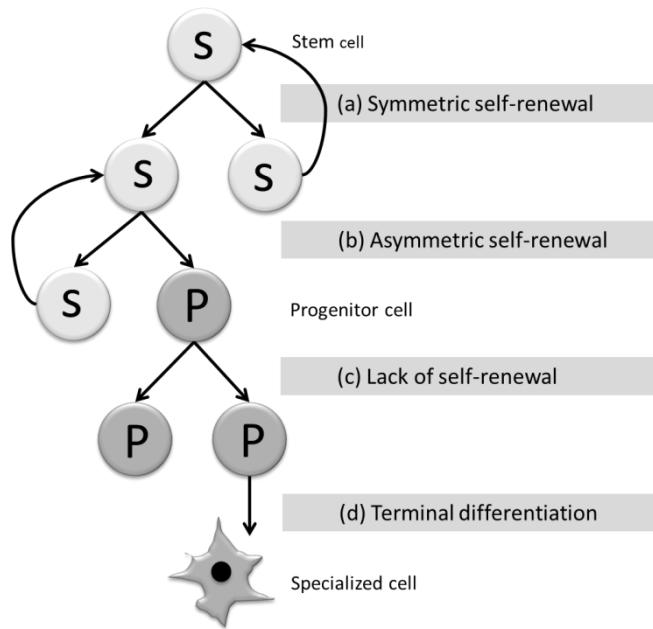
#### 2.1.1 Stem cell definition

The human body is made-up of three categories of cells: somatic cells, germ cells, and stem cells. Somatic cells consist of every cell type forming the human body except the germ cells, gametes, gametocytes, and the undifferentiated stem cells. These cells make up all internal organs, bones, skin, blood, and connective tissue. Germ cells give rise to gametes, the cells that fuse during sexual reproduction, namely the egg and sperm (Bongso and Richards, 2004). Stem cells are primitive unspecialized cells capable of replicating themselves for indefinite periods while still maintaining the potential to generate specialized cells. Progenitor cells are distinguished from stem cells by their lack of self-renewal. Scientists define cells as stem cells if they have the ability to self-renew, show high proliferative potential, and show multi-lineage differentiation into specialized cells (Verfaillie *et al.*, 2002). The process of self-renewal occurs when the stem cell divides to generate one (asymmetric division) or two daughter cells (symmetric cells). The daughter cells retain the developmental potential of the mother stem cell (depicted in Figure 1). Examples of multi-lineage differentiation include HSCs which form blood cells, and MSCs that can differentiate into cells of the mesenchymal lineage, namely adipocytes, osteoblasts, and chondrocytes.

#### 2.1.2 Origin of the term “stem cell”

The term “stem cell” can be dated back to the 19<sup>th</sup> century. The German biologist Ernst Haeckel used the term “stammzelle” to describe the ancestor unicellular organism from which all multicellular organisms were presumed to have evolved. He also used it to describe the fertilized egg that gave rise to all cells of an organism.

Theodor Boveri and Valentin Häcker described cells committed to give rise to the germline as stem cells, while Artur Pappenheim, Alexander Maximow and Ernst Neuman named the proposed progenitor giving rise to the blood system, a stem cell (Reviewed in Ramalho-Santos and Willenbring, 2007).



**Figure 1. Scheme showing the self-renewal ability of stem cells.**

A stem cell has the ability to self-renew by symmetrically dividing into two daughter stem cells, increasing the stem cell pool (a), or asymmetrically dividing to form a stem cell and a restricted progenitor cell, maintaining the stem cell pool as well as generating differentiated progeny (b). The progenitor cell cannot self-renew (c), but can undergo terminal differentiation into a specialized cell (d). Figure prepared by Karlien Kallmeyer. Modified from Figure 1 in Molofsky et al. (2004).

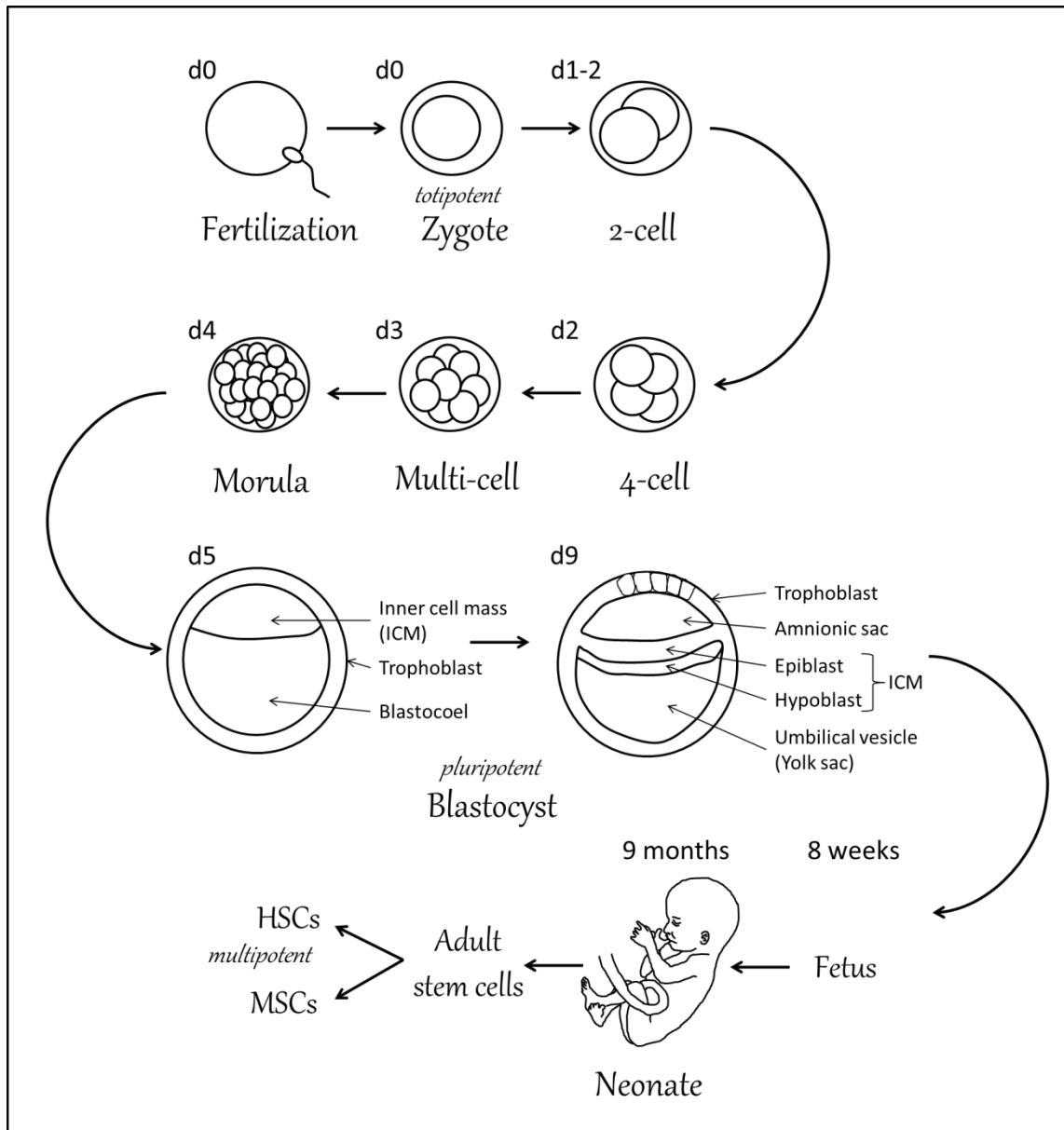
### 2.1.3 Embryonic stem cells

Stem cells are classified according to their origin, either as ESCs, embryonic germ stem cells (EGSCs), or adult stem cells. Stem cell research can be traced back to 1967, when murine embryonic carcinoma (EC) cells were first derived, followed by the establishment of murine ESCs in 1981 (Martin, 1981, Evans and Kaufman, 1981). However, research in this area actually dates back to 1953 when Leroy Stevens, a researcher working on mice at the Jackson Laboratory (in Bar Harbor, Maine), investigated teratomas in mice (Stevens and Little, 1954). He found that a specific mouse strain showed an incidence of testicular teratomas. Teratomas are

benign tumours that show rapid growth *in vivo* and are made up of a mixture of tissues (usually from all three germ layers). This finding led to further teratoma investigations. When a single cell derived from such tumours was injected into adult living tissue it was able to form a benign tumour consisting of all three germ layers (Kleinsmith and Pierce, 1964). This was the first insight into a unique cell type capable of growing indefinitely and of differentiating into multiple adult cell types.

#### 2.1.3.1 Embryonic development

Once fertilization of the oocyte has occurred it is called a zygote. The zygote is totipotent as it has the capacity to produce any cell type in the body. The zygote undergoes a series of cell divisions. By the 16-cell stage, the compacted embryo is termed a morula. The outer cells give rise to the trophectoderm and the inner cells become the inner cell mass (ICM). By day five the embryo develops into a blastocyst consisting of a sphere of flattened trophectoderm cells (which become the trophoblast), small round cells forming the ICM, and the blastocoel. The ICM is pluripotent as it can give rise to all three germ layers but is unable to form extra-embryonic tissue. As development continues, the trophoblast directs implantation into the uterus. Inside the blastocyst, a thin layer of cells, called the hypoblast delaminates from the ICM. These cells migrate and divide, lining the blastocoel. This newly formed cavity becomes the yolk sac. The remaining cells of the ICM become the epiblast. They form a second cavity, opposite to the hypoblast, which is filled with amniotic fluid. The remaining ICM cells positioned between the yolk sac and amniotic cavity will give rise to the embryo. During the process of gastrulation, cells of the embryo establish the three germ layers and differentiate into different cell types that make-up the organism. Following birth, the neonate's stem cells are now classified as adult stem cells. Adult stem cells are multipotent as they give rise to cells from only a single germ layer. This description of embryonic development summarized by Zerucha (2009) is depicted in Figure 2.



**Figure 2. Overview of embryonic development.**

After fertilization, the zygote is referred to as being totipotent. It undergoes a series of mitotic divisions and by day five the compacted embryo, formally referred to as the morula, develops into a blastocyst. The blastocyst consists of a sphere of cells named the trophoblast, an inner cell mass (ICM), and a cavity called the blastocoel. At this stage the blastocyst is pluripotent. As development continues, the trophoblast directs implantation into the uterus. The ICM gives rise to the hypoblast which forms the yolk sac, the epiblast which forms the amniotic fluid, and the embryo. The process of gastrulation further develops the embryo to form the three germ layers, making up the organism. After birth, adult stem cells can be derived from the neonate. These adult stem cells, namely hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) are referred to as being multipotent. Figure prepared by Karlien Kallmeyer.

### 2.1.3.2 Derivation of human ESCs

Embryonic stem cells are harvested from the ICM of 5-day-old blastocysts generated by *in vitro* fertilization (Bongso *et al.*, 1994). When grown in culture, ESCs have the potential to differentiate into cells of all three germ layers. The harvested ICM can be propagated in culture when grown on feeder layers (Richards *et al.*, 2002). A feeder layer provides the appropriate surface for cell attachment and supports ESC growth. *In vitro*, ESCs can undergo spontaneous differentiation forming a wide variety of cell types. Controlled *in vitro* differentiation can be performed using embryoid body (EB) formation. Induction of EBs can be achieved through removal of the feeder layer and disaggregation into single cells in suspension culture.

Embryonic stem cells were initially derived from mice (Evans and Kaufman, 1981, Martin, 1981) and later from primates (Thomson *et al.*, 1995). It was not until 1998 that human ESC lines were derived from human blastocysts (Thomson *et al.*, 1998). These human ESC (hESC) lines fulfilled the criteria of being human blastocyst-derived, showed undifferentiated proliferation, could form all three germ layers (Itskovitz-Eldor *et al.*, 2000), displayed a normal karyotype, and showed high telomerase activity.

### 2.1.3.3 Somatic cell nuclear transfer

Human embryonic stem cells have the potential to be used for degenerative diseases and tissue repair. Somatic cell nuclear transfer (SCNT) has been investigated for creating cells that are genetically identical to the nuclear donor. Thus it involves introducing a nucleus from an adult donor cell into an enucleated oocyte. Cell stimulation gives rise to the fusion of the donor nucleus with the oocyte, which can then be cultured *in vitro* to form a blastocyst. The resulting blastocyst can further be used for therapeutic cloning, the process in which a cloned blastocyst is explanted in culture to give rise to ESCs that are genetically identical to the donor. However, this has also opened up the controversial field of reproductive cloning. In 1996 the first sheep named Dolly was cloned by SCNT (Campbell *et al.*, 1996).

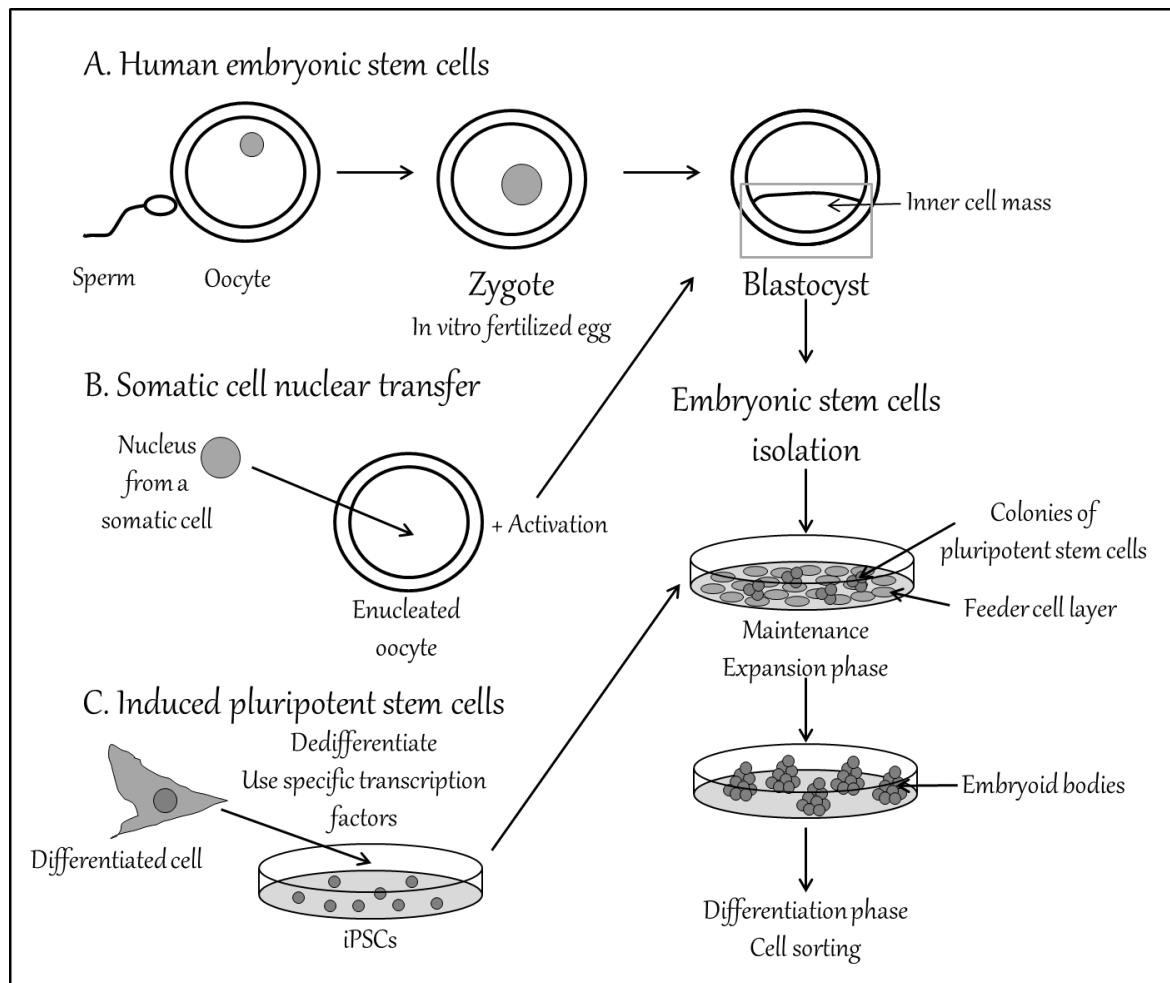
### 2.1.3.4 Induced pluripotent stem cells

Induced pluripotent stem cells (iPSCs) are generated by reprogramming somatic cells to an embryonic-like state, gaining back a state of pluripotency.

Reprogramming can be induced using specific transcription factors. Induction to iPSCs was first reported using mouse fibroblasts (Takahashi and Yamanaka, 2006). They were generated by using retroviral-mediated expression of core transcription factors required for maintaining ESC pluripotency and proliferation. The newly formed iPSCs exhibited morphology and growth properties of ESCs and expressed ESC marker genes. When implanted they showed tumour formation containing tissues from all three germ layers. Following injection of mouse iPSCs into blastocysts, they contributed to mouse embryonic development. Human adult dermal fibroblasts were successfully reprogrammed into iPSCs using the same four factors as for mice: Oct3/4, Sox2, Klf4, and c-Myc (Takahashi *et al.*, 2007). The most common source from which human iPSCs have been derived is skin fibroblasts (Lowry *et al.*, 2008), while peripheral blood has been suggested as an alternative source (Staerk *et al.*, 2010). Figure 3 shows how pluripotent stem cells can be generated.

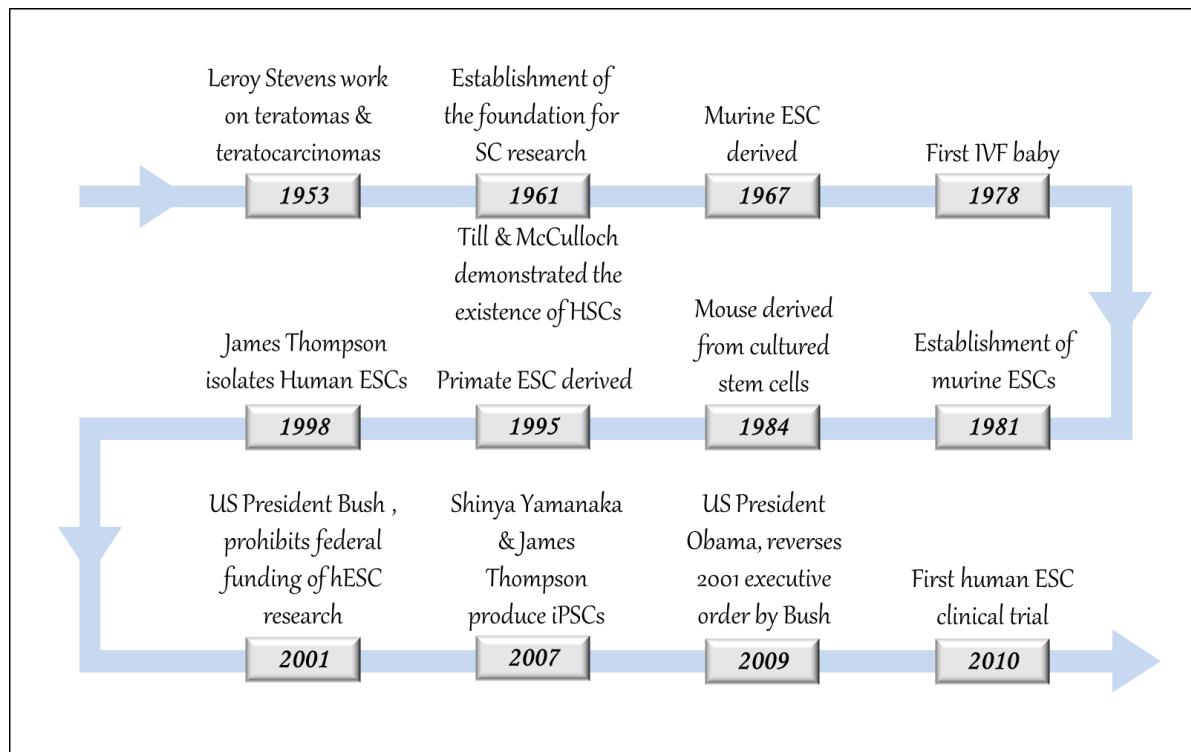
#### 2.1.3.5 The ethical dilemma

The use of ESCs has been central in ethical debates. It poses a moral dilemma which arises from our duty to prevent or alleviate suffering, while still respecting the value of human life (Wert and Mummery, 2003). Embryonic stem cells are highly controversial because they are derived from human pre-implantation embryos. Harvesting these cells result in the destruction of a potential human life. The controversy is based on the definition of when life begins and on the moral status of the embryo. Due to the controversy around ESCs, alternative stem cell sources have been sought. Alternatives include the use of iPSCs and adult derived stem cells. Induced pluripotent stem cells are expected to offer the same therapeutic potential as human ESCs without the ethical concerns. In 2012, Sir John Gurdon and Dr. Shinya Yamanaka won the Nobel Prize for their discovery that adult cells can be reprogrammed to become pluripotent. Adult stem cells are more restricted in their differentiation potential. However, they are not surrounded by ethical issues and have potential for the regeneration of damaged tissues and organs. Figure 4 shows some of the highlights in the history of ESC development.



**Figure 3. Isolation, generation, and culture of pluripotent stem cells.**

(A) Embryonic stem cells (ESCs) are derived from the inner cell mass of the blastocyst which is formed by *in vitro* fertilization. They are grown on feeder layers to maintain their undifferentiated state during expansion. When removed from feeder layers and transferred to suspension culture, multicellular aggregates form termed embryoid bodies. Plated embryoid bodies can spontaneously differentiate into the three germ layers. (B) Somatic cell nuclear transfer involves introducing a nucleus from a somatic cell into an enucleated oocyte, followed by activation stimuli to facilitate fusion. The generated embryo can be used to generate ESC lines. (C) Induced pluripotent stem cells (iPSCs) are generated from differentiated somatic cells that have been reprogrammed to a pluripotent state using specific transcription factors. Figure prepared by Karlien Kallmeyer. Modified from Figure 1 by Brignier and Gewirtz (2010).



**Figure 4. Timeline showing stem cell history highlights.**

The history of embryonic stem cells can be dated back as far as 1953. The field of stem cells is growing, and future focus is likely to be directed more to adult derived stem cells rather than to ESCs. Figure prepared by Karlien Kallmeyer.

#### 2.1.4 Adult stem cells

Adult stem cells are found in adult tissues and organs and play a role in tissue regeneration. They are undifferentiated and unspecialized cells capable of self-renewal and multipotent differentiation. Compared to ESCs they have less self-renewal ability as they lack high levels of telomerase (Verfaillie *et al.*, 2002, Hiyama and Hiyama, 2007). Most normal human cells are unable to divide indefinitely but are programmed for a given number of cell divisions (Hayflick and Moorhead, 1961).

##### 2.1.4.1 Adult stem cell plasticity

Stem cell plasticity refers to the ability of tissue specific stem cells to acquire the fate of cell types different from the tissue of origin. Under specific conditions adult stem cells may “transdifferentiate” to contribute differentiated progeny from a different tissue source. Some criticism regarding stem cell plasticity exists due to the low frequency of transdifferentiation, lack of proof that plasticity results from a single

stem cell, and that this lineage-switch goes against established developmental biology (Verfaillie *et al.*, 2002).

Stem cell plasticity studies have mostly been performed using adult BM- or peripheral blood-derived HSCs. Studies defining HSC plasticity were based on their differentiation into cells/tissues showing non-hematopoietic morphology and phenotype (Verfaillie *et al.*, 2002). In order to prove that adult stem cell plasticity exists, it is necessary to show that the cell was donor-derived, has a phenotypic profile identical to the resident cells into which it has been differentiated, and is functional within the tissue (Krause, 2002). An example is provided by a study conducted by Lagasse *et al.* (2000) which showed that intravenous administration of wild-type  $\beta$ -galactosidase expressing marrow cells enriched for HSCs in the FAH-/- mouse rescued the mice and restored liver function. An animal model for tyrosinemia type I was used, where the gene encoding fumarylacetoacetate hydrolase (FAH) was deleted (Nakamura *et al.*, 2007). This mutation is lethal and animals can be kept alive by administration of 2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC). The transplanted mice were weaned off NTBC, while the functional donor cells, identified histochemically by staining positive for  $\beta$ -galactosidase, differentiated into hepatocytes and rescued the otherwise lethal phenotype. As a final approach, the expression of hepatocyte and hematopoietic markers was determined in the regenerated liver nodules that originated from the transplantation, confirming that most donor cells were mature hepatocytes.

#### 2.1.4.2 Tissue specific stem cells

Bone marrow transplantation is a well-established form of treatment for hematologic malignancies. It involves isolating HSCs from mononuclear cells found within the BM. Hematopoietic stem cells have the ability to completely and permanently regenerate a lymphohematopoietic system after myeloablation treatment. Transplantation using HSCs is the gold standard for restoring tissue function by stem cell engraftment (Lagasse *et al.*, 2001). Harvesting of peripheral blood is a less invasive source of HSCs. In 1986 peripheral blood started to replace BM as a stem cell source (Körbling and Freireich, 2011). It has become the preferred source of HSCs, especially for allogeneic transplants, and involves the mobilization of HSCs from the BM following administration of granulocyte colony-stimulating factor (G-

CSF) to the donor. International BM donor registries provide a source of HSCs for allogeneic transplantation for the treatment of blood cancers and genetic diseases.

The blood remaining in the umbilical cord and placenta after birth also harbours HSCs. This routinely discarded medical waste has been accepted as a rich alternative source of HSCs (Rubinstein *et al.*, 1993). The first UCB transplantation was performed in 1988 from an HLA matched sibling donor to treat a child with Fanconi anaemia (Gluckman and Rocha, 2005). Umbilical cord blood also contains other stem cells such as MSCs (Lee *et al.*, 2004a), unrestricted somatic stem cells (USSCs) (Kögler *et al.*, 2004), cord blood-derived embryonic like stem cells (CBEs) (Forraz *et al.*, 2004, McGuckin *et al.*, 2004), and multipotent progenitor cells (MPCs) (Lee *et al.*, 2007). Whether these cells are truly different cell types, or merely at different stages of differentiation still needs further investigation. The interest of the current study lies in UCB as a source of non-hematopoietic stem cells, namely MSCs.

The adult stem cell exists in various specialized and differentiated tissues and organs (Table 1). These tissue-specific stem cells self-renew and differentiate into the specialized cell types that constitute the tissue in which they reside.

**Table 1. Stem cells in adult organs and tissues**

Body Part	Stem cells	References
Skin	Contains stem cells related to epidermis, epithelium, and hair follicles and is associated with the repair and replacement of these tissues.	Alonso and Fuchs (2003), Blanpain and Fuchs (2006)
Muscle	Satellite stem cells reside beneath the basal lamina of mature muscle fibers. They are responsible for postnatal skeletal muscle growth, hypertrophy, and for myofiber repair and regeneration.	Brack and Rando (2012), Mauro (1961), Zammit <i>et al.</i> (2006)
Digestive system	Intestinal stem cells reside near the base of intestinal crypts. They give rise to progenitor cells capable of differentiating into mature cell types required for normal gut function.	Leedham <i>et al.</i> (2005), Umar (2010)
Brain	Neural stem cells from the nervous system are able to self-renew and generate both neurons and glia.	Butcher (2004), Stemple and Anderson (1992)
Teeth	Dental pulp stem cells regenerate dental pulp tissue. They have also been suggested to show neuronal and muscular differentiation properties.	Kawashima (2012), Stanley (1962)
Liver	Hepatic stem cells restore liver mass in response to parenchymal cell loss. Hepatic stem cells are obtainable from hepatocytes or cholangiocytes in the liver, or from HSCs in BM.	Alison (1998), Forbes <i>et al.</i> (2002)
Pancreas	Clearly identifiable pancreatic stem cells have not yet been found. Pancreatic stem cell origin/existence has been debated since the demonstration that functional beta-cells can self-duplicate themselves.	Bonner-Weir and Sharma (2002), Dor <i>et al.</i> (2004), Jiang and Morahan (2012)

### 2.1.5 International Society for Cellular Therapy

The International Society for Cellular Therapy is a global society. Their mission statement is “driving the translation of scientific research to deliver innovative cellular

therapies to patients" (Maziarz *et al.*, 2011). Members and leaders of the society include world-class scientists, clinicians, technologists, and regulatory professionals from 40 countries involved in developing cell therapies from the preclinical to the clinical setting. The society was founded in 1992 as the International Society for Hematotherapy and Graft Engineering (ISHAGE), and was later renamed to the ISCT to allow the focus to be broadened to include non-hematopoietic stem cells, specifically mesenchymal stem/stromal cells (MSCs). The growing field of MSCs in cellular therapy led to confusion regarding the definition of MSCs. In response, the ISCT generated two international position statements regarding the nomenclature and criteria for defining mesenchymal stromal cells (Dominici *et al.*, 2006, Horwitz *et al.*, 2005) which are still applied today.

### **2.1.6 Confusion around the term “Mesenchymal stem cell”**

The term “mesenchymal stem cell” is used to describe the plastic-adherent cells commonly isolated from BM and adipose tissue, with *in vitro* multipotent differentiation capacity. However, these cell populations are heterogeneous and are believed to be too crude to be termed MSCs. The early work of Alexander Friedenstein proposed the term “marrow stromal cells or stem cells” to describe the fibroblastic colonies from BM cells cultured *in vitro* (Friedenstein *et al.*, 1968, Friedenstein *et al.*, 1966, reviewed in Wagner and Ho, 2007). He also started using the term “osteogenic stem cells” to describe the clonal osteogenic progenitors identified in BM stoma (Owen and Friedenstein, 1988). The term “mesenchymal stem cells” was coined by Caplan (1991) and popularized by Pittenger (1999). To clarify the terminology, the ISCT proposed that the fibroblast-like plastic adherent cells, regardless of tissue origin, be named multipotent mesenchymal stromal cells, while reserving the term mesenchymal stem cell for a subset of these cells that clearly demonstrate stem cell activity (Horwitz and Keating, 2000, Horwitz *et al.*, 2005). The acronym MSC is currently used for both mesenchymal stromal- and stem cells and it is left up to the scientist to clearly state which nomenclature is being referred to in order to avoid any confusion. This study refers to mesenchymal stem cells as MSCs.

## 2.2 Mesenchymal stem cells

Mesenchymal stem cells are a heterogeneous population of multipotent adult stem cells. They were first isolated from BM more than 40 years ago (Friedenstein *et al.*, 1968). Although originally found in the BM, MSCs have been shown to reside in virtually all post-natal human tissues and organs, including adipose tissue, peripheral blood, cord blood, Wharton's jelly, liver, and fetal tissue, to name just a few (da Silva Meirelles *et al.*, 2006). They have the ability to self-renew and are capable of differentiation into mesenchymal tissues such as adipose, bone, cartilage and muscle (Pittenger *et al.*, 1999, Zuk *et al.*, 2002). They have also been shown to exhibit non-HLA restricted immunosuppressive properties, supporting the view that differentiated and undifferentiated MSCs can be transplanted between HLA-incompatible persons (Le Blanc *et al.*, 2003).

### 2.2.1. Differences between MSCs from different sources

Mesenchymal stem cell populations can be isolated from various tissues. These tissues may differ developmentally (fetal or adult tissue), as well as anatomically (bone marrow or fat) (Christodoulou *et al.*, 2013). Although BM-MSCs and adipose-derived stem cells (ASCs) share biological characteristics, differences in their phenotype, in their differentiation potential, in their gene expression profile and in their immunomodulatory activity have been noted (Kern *et al.*, 2006, Strioga *et al.*, 2012, Wagner *et al.*, 2005). These differences may be due to tissue specific features, the heterogeneity of the cell population, or could be related to isolation and culturing techniques. Generally, ASCs are more easily isolated, resulting in higher yields of stem cells compared to BM (Fraser *et al.*, 2006, Hass *et al.*, 2011). Collection of BM aspirates is a highly invasive procedure. Also, BM-MSCs show decreased cell number and differentiation capacity with age (Kern *et al.*, 2006, Stolzing *et al.*, 2008). Fetal tissues, such as UCB (Bieback *et al.*, 2004, Lee *et al.*, 2004a) and Wharton's jelly (De Bruyn *et al.*, 2011, Pereira *et al.*, 2008) of human umbilical cord have been proposed as additional sources of MSCs.

#### 2.2.1.1 Bone marrow

Bone-marrow derived stromal cells retain a subpopulation of cells capable of differentiation into mesenchymal tissues. These cells are referred to as marrow stromal cells or MSCs. Bone-marrow derived MSCs were first isolated as cultures of

plastic-adherent cells displaying a fibroblastic morphology from BM more than 40 years ago (Friedenstein *et al.*, 1968, Friedenstein *et al.*, 1976). They were also referred to as colony forming unit-fibroblasts (CFU-Fs). The idea of resident non-hematopoietic adult stem cells in the BM was later proposed by Owen and Caplan (Caplan, 1991, Owen, 1988). In 1999, the first definitive markers for isolating human MSCs were proposed, along with the development of *in vitro* differentiation assays (Pittenger *et al.*, 1999).

#### 2.2.1.2 Adipose tissue

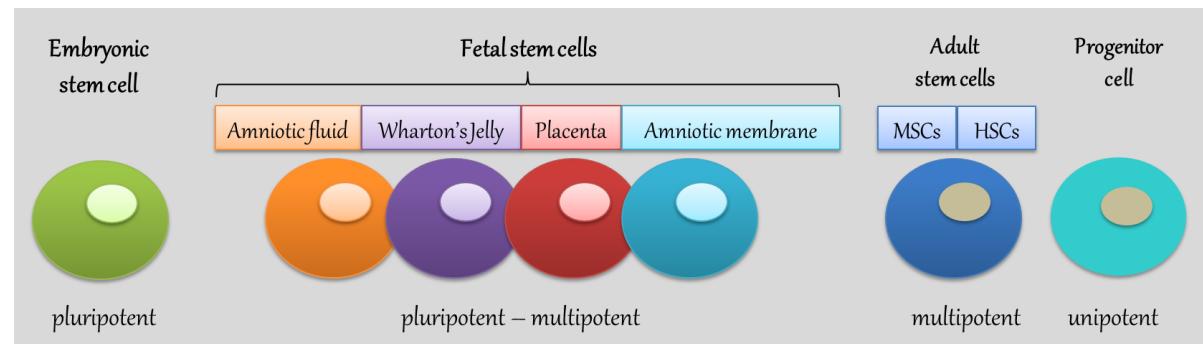
In 1964, Rodbell pioneered the initial methods for isolating cells from rat adipose tissue (Rodbell, 1964). This technique was later successfully applied to human tissue (Deslex *et al.*, 1987). Isolation of adipose cells from adipose tissue involves enzymatic digestion with collagenase to liberate the fat cells, followed by density centrifugation to further separate the fat cells from the denser stromal vascular fraction (SVF) (Zuk *et al.*, 2002, Zuk *et al.*, 2001). Cells within the SVF readily adhere to plastic tissue culture flasks and consist of a heterogeneous mixture of endothelial cells, smooth muscle cells, pericytes, fibroblasts, mast cells, and preadipocytes, as well as a population of multipotent adipose tissue-derived MSCs/ASCs (Zuk *et al.*, 2001). To obtain a homogeneous cell population of ASCs, the SVF can be expanded in culture for extended periods. Liposuction is a popular surgical procedure in which large volumes of adipose tissue are routinely discarded as waste. Utilizing this waste product as a potential source of stem cells could yield large numbers of stem cells with therapeutic potential. In 2001, ASCs were identified and characterized in lipoaspirate, leading to the recognition that adipose tissue may provide an alternative source of MSCs to BM (Zuk *et al.*, 2001).

Confusion around the nomenclature used to describe the plastic adherent cell population isolated from adipose tissue exists. Various terms such as adipose-derived stem/stromal cells (ASCs), adipose-derived adult stem (ADAS) cells, adipose-derived adult stromal cells, adipose-derived stromal cells (ADSCs), adipose stromal cells (ASCs), adipose mesenchymal stem cells (AdMSCs), lipoblast, pericyte, preadipocyte, and processed lipoaspirate (PLA) cells have been used interchangeably to describe these cells (Gimble *et al.*, 2007). To simplify the current nomenclature, the International Fat Applied Technology Society has proposed the

term “adipose-derived stem cells” (ASCs) to identify the isolated, plastic-adherent, multipotent cell population (reviewed in Gimble *et al.*, 2007).

### 2.2.1.3 Fetal stem cells

Fetal stem cells can be isolated from aborted fetuses or from supportive extra-embryonic structures. Multiple extra-embryonic tissues, such as UCB, amniotic fluid, Wharton's jelly, amniotic membrane, and placenta develop during gestation. These tissues harbour stem cells that portray characteristics between ESCs and adult stem cells (Figure 5) (reviewed in Pappa and Anagnou, 2009).



**Figure 5. Model depicting stem cell developmental hierarchy.**

Fetal stem cells originate from several fetal sources which include amniotic fluid, Wharton's jelly, placenta, and amniotic membrane. These stem cells are believed to represent an intermediate cell type between embryonic stem cells and adult stem cells. Whether they are truly pluripotent and/or multipotent still needs to be elucidated. Figure prepared by Karlien Kallmeyer. Modified from Figure 1 by Pappa and Anagnou (2009).

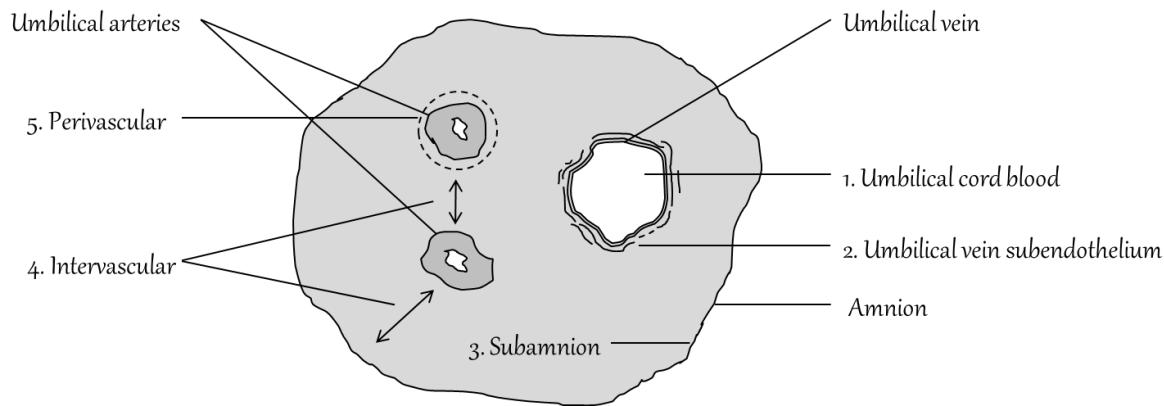
#### 2.2.1.3.1 Umbilical cord blood

The human placenta, from which UCB can be collected, is a fetal maternal organ that separates maternal and fetal circulation. The precursor cells of the placenta, called trophoblasts, appear after day four post-conception as an outer layer of cells of the blastocyst. Once fully developed, the placenta serves as an interface between the mother and the developing fetus where nutrients and waste products can be exchanged (Gude *et al.*, 2004). Fetal circulation enters the placenta via umbilical arteries embedded in the umbilical cord. After nutrients have been absorbed and waste products released, fetal blood returns to the fetus via the umbilical vein in the umbilical cord. Maternal blood enters the placenta via spiral arteries of the uterus

and circulates around the chorionic villa. The chorionic villa has a placental membrane separating the fetal blood from the surrounding maternal blood in the intervillous space. The existence of MSCs in UCB has been shown by Lee and colleagues (Lee *et al.*, 2004a). They reported the possibility of obtaining single cell-derived, clonally expanded MSCs from UCB with remarkable potential to differentiate into multiple lineages of mesodermal and non-mesodermal origin. Conflicting results have been obtained describing the successful isolation of MSCs from UCB; thus further investigation into UCB as a MSC source is needed (Bieback *et al.*, 2008, Kern *et al.*, 2006, Lu *et al.*, 2006).

#### 2.2.1.3.2 Wharton's jelly

The human umbilical cord is formed from the extra-embryonic mesoderm at day 13 of embryonic development (reviewed in Karahuseyinoglu *et al.*, 2007). During pregnancy, the elastic cord links the mother and the fetus. The umbilical cord consists of two umbilical cord arteries and one umbilical vein, encased in a protective mucous connective tissue known as Wharton's jelly (Sobolewski *et al.*, 1997). Wharton's jelly is located between the amniotic epithelium and umbilical vessels. It functions to protect enclosed blood vessels from compression, torsion, and bending, while providing bidirectional blood flow between the fetal and maternal circulations. In 1991 fibroblast-like cells were isolated from Wharton's jelly of human umbilical cord (McElreavey *et al.*, 1991). Wharton's jelly is comprised of three regions, the perivascular zone, the intervascular zone, and the subamnion (Figure 6) (Troyer and Weiss, 2008). Whether MSCs isolated from different compartments in umbilical cord represent different population still needs further investigation (Karahuseyinoglu *et al.*, 2007). Stem cells derived from Wharton's jelly have a mesenchymal phenotype and only express low levels of pluripotent embryonic stem cell markers (Fong *et al.*, 2007, Fong *et al.*, 2011). They also have increased levels of genes associated with the immune system (Fong *et al.*, 2011). They show potential as a source of stem cells for allogeneic transplantation therapy.



**Figure 6. Schematic image showing the structure of the human umbilical cord.**

The umbilical cord consists of two umbilical arteries and one umbilical vein, embedded within the connective tissue known as Wharton's jelly. Wharton's jelly includes the subamnion, intervacular, and perivascular regions (zones 3 to 5). Figure prepared by Karlien Kallmeyer. Modified from Figure 1 by Troyer and Weiss (2008).

### 2.2.2 *In vitro* characterization

Currently there is a lack of standardization when defining MSCs *in vitro*. Mesenchymal stem cells are defined by functional assays using cultured cells. The cells are expanded *in vitro* as two-dimensional monolayers of adherent cells in specialized medium. They cannot yet be identified by a single marker. Generally, a panel of negative and positive markers allows for their phenotypic characterization. A general agreement is that adult human MSCs (hMSCs) lack hematopoietic marker expression (CD45, CD34, CD14, or CD11), and the platelet and endothelial adhesion molecule CD31, but they express CD105 (SH2), CD73 (SH3/4), CD44, CD90 (Thy-1), and stromal antigen 1 (STRO-1) (reviewed in Chamberlain *et al.*, 2007, Kolf *et al.*, 2007). Different marker expression profiles have, however, been noted between MSCs from different sources. Particularly the expression of CD34, which is negative in humans and rats, has been found to be positive in mice (Javazon *et al.*, 2004). Significant phenotypic differences may also exist in MSCs derived from different human tissues (Bianco *et al.*, 2008). In addition to their phenotypic characterization, MSCs must have the capacity to differentiate *in vitro* into adipose tissue, bone, and cartilage (Pittenger *et al.*, 1999, Zuk *et al.*, 2002).

*In vitro* approaches subject the cells to conditions different from those found in their anatomic location. It is believed that adult stem cells exist as heterogeneous

populations *in vivo*. Thus, isolation procedures and culturing conditions used may show bias towards specific subsets of cells or cellular characteristics (da Silva Meirelles *et al.*, 2008).

### 2.2.3 *In vivo* characterization

The *in vivo* location, identification, and activities of MSCs are still poorly understood. The role of MSCs in their multiple anatomical locations is not well understood. Whether they constitute a specific homogenous cell type or a heterogeneous population still needs to be elucidated. In search of the *in vivo* identity of MSCs, it has recently been postulated that MSCs are situated throughout the body as pericytes (Corselli *et al.*, 2010, da Silva Meirelles *et al.*, 2008). Similarities between pericytes and MSCs do exist in their phenotypic profiles and differentiation capacity (Crisan *et al.*, 2008). Fibroblasts also share similar characteristics to MSCs (reviewed in Haniffa *et al.*, 2009). However, in an attempt to remove fibroblast contamination from freshly isolated MSCs, it was suggested that markers such as CD166 and CD9 should be used to distinguish the two cell types from one another (Halfon *et al.*, 2011).

Some of the concerns are that MSCs cannot be reproducibly isolated, especially when identification is based on morphologic or phenotypic characteristics, because of the lack of unique markers. The artificial conditions during culturing may introduce experimental artifacts that do not accurately represent MSC characteristics *in vivo* (Si *et al.*, 2010). As these conditions are not standard and differ between research groups, it is extremely difficult to compare both *in vitro* and *in vivo* results.

When the current *in vitro* approaches (via specific marker identification and differentiation assays) are considered, one of the concerns regarding MSCs is that their origin may determine their ultimate functional characteristics. This is because they appear to exert different functions within their residing tissues, and that these cells are subjected to conditions different from their *in vivo* niche (Abdi *et al.*, 2008). The expression profile of *in vitro* isolated MSCs may be determined by the culture conditions rather than be representative of their *in vivo* characteristics. *In vivo* identification and tracking of MSCs is a difficult task that still needs to be verified before these cells can be translated to the clinic.

## 2.2.4 Therapeutic effects

Mesenchymal stem cells are easily available, can be isolated from various tissues, show low immunogenicity and high proliferative properties *in vitro* while maintaining their undifferentiated multipotent state (da Silva Meirelles *et al.*, 2008). Clinically, MSCs are an attractive cell type for tissue repair, both as immune system modulators and as a source of new tissue following cell engraftment. Adult MSCs show promise therapeutically, due to their differentiation capacity for the repair of damaged tissue, but also for their ability to activate endogenous progenitor cells by secreting various factors (reviewed in Ankrum and Karp, 2010). The mechanisms behind the therapeutic effects exerted by MSCs are each explored in greater detail below. Further research is required to fully understand the therapeutic benefits and mechanism of action exerted by MSCs.

### 2.2.4.1 Differentiation potential

Mesenchymal stem cells are multipotent and thus retain the ability to differentiate into multiple cell types. *In vitro*, differentiation can be directed by the addition of chemical inducers or cytokines (Bunnell *et al.*, 2008). Friedenstein and colleagues were the first to demonstrate the osteogenic potential of BM-derived cells (now referred to as BM-MSCs) (Friedenstein *et al.*, 1966). The multipotency of BM-MSCs has been investigated further and shows differentiation not only into bone, but also into multiple connective tissue types (Alison *et al.*, 1998, Dennis *et al.*, 1999, Pittenger *et al.*, 1999). Heterogeneity of adult MSC differentiation potential has been noticed. Baksh *et al.* (2004) has suggested a model to explain this heterogeneity in differentiation. They suggest that the MSC pool consists of stem cells as well as a subpopulation of cells at different stages of differentiation. This implies the existence of quadra-, tri-, bi-, and uni-potential MSCs. Figure 1 explains the self-renewal ability of stem cells. This figure also depicts the progression of cells from being stem cells to precursor cells and finally fully committed and differentiated mature cells.

Commitment and differentiation of MSCs is a controlled process regulated by transcription factors, cytokines, growth factors, and extracellular matrix (ECM) components. *In vitro*, differentiation is influenced by culturing MSCs in the presence of growth factors (reviewed in Liu *et al.*, 2009). Growth factor-induced signaling has

been shown to regulate MSC differentiation and includes the transforming growth factor-beta (TGF- $\beta$ ), platelet-derived growth factor (PDGF) and fibroblast growth factor (Ng *et al.*, 2008) super families. The gene expression profile of hMSCs during tri-lineage differentiation has been investigated, and a common set of genes, namely *ZNF145* and *FKBP5* were up-regulated during initial commitment (Liu *et al.*, 2007).

The differentiation ability of MSCs shows potential for the biological repair of articular cartilage and bone. The use of MSCs in bone repair was initially applied on the basis of their ability to differentiate into osteoprogenitors and osteoblasts (Jones and Yang, 2011). However, the therapeutic action of MSCs is also exerted through trophic or paracrine effects (refer to section 2.2.4.2). The first *in vivo* bone experiments were performed using diffusion chambers loaded either with whole BM (Friedenstein *et al.*, 1966) or culture expanded cells (Ashton *et al.*, 1980). Another study showed that donor-derived bone could be developed by subcutaneous implantation of a scaffold seeded with hMSCs in a nude mouse model (Muraglia *et al.*, 1998, Pelegrine *et al.*, 2013). The repair of large bone defects in animals (Kon *et al.*, 2000, Pelegrine *et al.*, 2013) and humans (Quarto *et al.*, 2001) has been shown using autologous MSCs. Allogeneic BM transplantation in three children affected by osteogenesis imperfect (OI) improved their bone structure through the differentiation of donor MSCs into functional osteoblasts (Horwitz *et al.*, 1999). The *in vitro* chondrogenic potential of MSCs is well known using the pellet culture system (Johnstone *et al.*, 1998, Sekiya *et al.*, 2002b). Whether the *in vitro* formation of cartilage truly represents stable cartilage, or is just a transient cartilage template which will eventually be replaced with bone by endochondral ossification, still needs further investigation (Augello *et al.*, 2010). The clinical application of MSCs differentiated into the adipose lineage has potential in reconstructive surgery (Zeve *et al.*, 2009) and for cosmetic surgery. The differentiation potential of MSCs renders them as an ideal cell candidate for tissue regeneration strategies.

#### 2.2.4.2 Paracrine effects

Paracrine signaling can be described as a form of communication between cells. Communication is achieved by the production of a signal to induce changes in the surrounding cells. The action occurs locally. Paracrine mechanisms involve (i) stimulation of receptor mediated survival pathways; (ii) the induction of stem cell

homing and differentiation; and (iii) the regulation of anti-inflammatory effects in wounded areas (Burdon *et al.*, 2011). Paracrine effects of adult stem cells (such as MSCs) have been studied in heart repair and in wound healing. Gnechi *et al.* (2008) described the mechanism of repair using adult stem cells in heart. Adult stem cells were depicted to exert paracrine effects on the locally present cardiomyocytes, endothelial cells, smooth muscle cells, fibroblasts, and cardiac stem cells in response to specific environmental stimuli (*i.e.* ischemia). They responded by becoming activated and were involved in tissue protection, repair, and regeneration (Gnechi *et al.*, 2008). A study showed that BM-MSC-conditioned medium (containing high levels of growth factors and chemokines) enhanced wound healing in mice (Chen *et al.*, 2008). In a rat model of myocardial infarction, autologous MSC transplantation exerted paracrine signals leading to vascular regeneration (Tang *et al.*, 2005). Paracrine factors are involved in tissue regeneration by affecting the homing, immunosuppressive properties and differentiation of resident or allogeneic/autologous stem cells (Burdon *et al.*, 2011).

#### 2.2.4.3 Immunological properties

Phenotypically, MSCs are described as non-immunogenic as several immunological surface antigens important for B-cell and T-cell recognition are not expressed on their surface. Human MSCs are major histocompatibility complex class I positive (MHC class I+), MHC class II-, CD40-, CD80- and CD86- (Tse *et al.*, 2003). Mesenchymal stem cell mediated immunoregulation results from the cumulative action displayed by several molecules. The mechanism mediating inhibition of allogeneic T-cell responses by MSCs is not yet fully understood. The expression of indoleamine 2,3-dioxygenase (IDO), which is induced by interferon-gamma (IFN- $\gamma$ ), catalyzes the conversion of tryptophan to kynurenine, and has been identified as a T-cell inhibitory effector pathway (Meisel *et al.*, 2004). The IDO protein is expressed by human MSCs and exhibits functional IDO activity upon stimulation with IFN- $\gamma$ . In the absence of IFN- $\gamma$ , MSC immunosuppressive activity is augmented by Toll-like cell surface receptors through an autocrine IFN- $\beta$  signaling loop (Opitz *et al.*, 2009). Another MSC-secreted factor, interleukin-6 (IL-6), has been reported to decrease the stimulation ability of dendritic cells (DCs) on T-cells by inhibition of monocyte differentiation towards DCs (Djouad *et al.*, 2007). Mesenchymal stem cells derived

from adipose tissue, UCB, and Wharton's jelly have been shown to suppress mitogen-induced T-cell proliferation as efficiently as BM-MSCs (Yoo *et al.*, 2009).

Immunosuppressive effects of MSCs were firstly shown *in vivo* using a baboon model. This study showed that systemic infusion of BM-MSCs prolonged the survival of allogeneic skin grafts in baboons receiving MSCs (Bartholomew *et al.*, 2002). The use of MSCs has been suggested for the treatment and prevention of graft-versus-host disease (GvHD) in allogeneic stem cell transplantation. Successful treatment of severe treatment-resistant GvHD has been shown with MSCs (Le Blanc *et al.*, 2004). Presumably this is due to the ability of MSCs to suppress donor T-cell proliferation. A study showed that BM-derived MSCs were able to suppress the development of GvHD when administered after donor T-cell recognition of the antigen. Furthermore, the addition of the regulatory protein IFN- $\gamma$  was required to initiate the suppressive activity of MSCs (Polchert *et al.*, 2008). Mesenchymal stem cells have been suggested to be precursor cells for BM stroma where they provide a three-dimensional scaffold and enhance proliferation of HSCs. In HSC transplantation, MSCs help to reconstitute BM stroma after chemoradiotherapy and enhance HSC engraftment (Le Blanc and Ringden, 2005). An initial study showed that autologous MSC infusion improved the outcome of HSC transplantation in breast cancer patients (Koc *et al.*, 2000).

#### 2.2.4.4 MSC homing to sites of injury

Mesenchymal stem cell homing has been defined as "the arrest of MSCs within the vasculature of a tissue followed by transmigration across the endothelium" (Karp and Leng Teo, 2009). Stem cells are believed to migrate through the blood or tissue to a specific destination where they can differentiate to replace or regenerate damaged tissues. This migration or mobilization is believed to be directed by cytokines and/or chemokines that are upregulated under conditions of inflammation, released into circulation, and stimulate MSCs to down-regulate the adhesion molecules that keep them in their niche (Liu *et al.*, 2009). The chemokine receptors CCR1, CCR4, CCR7, CXCR5, and CCR10 are expressed on MSCs and may be involved in homing (Honczarenko *et al.*, 2006, Von Luttichau *et al.*, 2005). Chemokines have been reported in the migration of certain progenitor cells. Stromal derived factor-1 (SDF-1) and its receptor CXCR4 are crucial for BM retention, mobilization, and homing of

HSCs (Levesque *et al.*, 2003, Petit *et al.*, 2002). *In vitro* work also showed that MSC migration was regulated by SDF-1/CXCR4 and hepatocyte growth factor/c-Met complexes (Son *et al.*, 2006).

There is evidence that infused MSCs home in response to inflammation or injury. It is unclear whether MSCs actively home to tissues using leukocyte-like cell-adhesion and transmigration mechanisms or whether they become passively entrapped in small-diameter blood vessels. It is suggested that specific MSC-endothelium interactions regulate transmigration; however, further studies are required under conditions of inflammation to test this hypothesis (reviewed in Karp and Leng Teo, 2009, and Ley *et al.*, 2007).

#### 2.2.4.5 Anti-cancer/ tumorigenic properties

The tumour environment resembles that of inflamed tissues. High levels of signaling molecules, growth factors and chemokines present in this environment drive the homing of MSCs to the tumour site (Dvorak, 1986). Pro- versus anti-tumour effects have been observed for MSCs (reviewed in Houthuijzen *et al.*, 2012). A concern is that once localized within the tumour, MSCs could promote cancer progression by modulating the immune system and facilitating tumour growth, angiogenesis and metastasis. However, BM-MSCs exhibited anti-lymphoma activity in two distinct xenograft severe immunodeficient (SCID) mouse models of disseminated non-Hodgkin's lymphoma (NHL) (Secchiero *et al.*, 2010). A study by Ayuzawa and colleagues evaluated the anti-cancer potential of human umbilical cord matrix stem cells (hUCMSC) for malignant breast cancer carcinoma cells (MDA 321 line) (Ayuzawa *et al.*, 2009). Naïve hUCMSCs were administered intravenously and significantly attenuated tumour growth in the lung. The hUCMSCs were found localized in tumour tissues in the lung, but were absent in healthy lung tissue. This showed the homing ability of MSCs to tumours. The differences in tumorigenic properties observed between different groups could be attributed to the source of MSCs, the isolation techniques used and the heterogeneity of the isolated MSCs.

### 2.2.5 Clinical applications of MSCs

There has been a paradigm shift in the use of MSCs for therapy. Initially MSC therapy focused on their multilineage differentiation capacity, especially into bone

and cartilage. However, clinical trials are starting to focus more on tissue repair based on MSC immunomodulatory properties as well as their ability to secrete trophic molecules such as cytokines/growth factors and soluble ECM glycoproteins (Ankrum and Karp, 2010). Trounson *et al.* (2011) reviewed clinical trials (from <http://www.clinicaltrials.gov>) for stem cell therapies up to the third quarter of 2011 and found 123 trials for MSCs. The effect of MSCs on a large number of conditions was investigated in these trials. The top three disease classifications were bone/cartilage disease, immune rejection/autoimmunity and heart disease. The majority of the trials are Phase I (safety studies) and Phase II (proof of concept for efficacy in human patients) studies. The data available on clinical trials where MSCs were utilized seem to portray mixed data on their efficacy. A systematic review and meta-analysis of clinical trials using intravascular delivery of MSCs was conducted and concluded that MSC therapy appeared to be safe (Lalu *et al.*, 2012). To further define the safety profile of MSCs, large scale controlled clinical trials are required with rigorous reporting of adverse events.

The first clinical trial using culture-expanded MSCs was carried out in 1995. It was a Phase I trial to determine the feasibility of collection, *ex vivo* culture-expansion and intravenous infusion of human BM derived progenitor stromal cells (Lazarus *et al.*, 1995). An initial case report in 2004 described the remission of advanced-stage steroid-resistant acute GvHD in a child treated with MSCs (Le Blanc *et al.*, 2004). This report led to further studies. A phase II trial using BM-derived MSCs in 55 patients with severe and steroid-resistant acute GvHD reported that more than half of the patients responded to the treatment (Le Blanc *et al.*, 2008). Whether the cell donor was HLA matched or unmatched did not appear to affect the success of the treatment. However, a Phase III clinical trial (NCT00366145) using a Food and Drug Administration (FDA) approved MSC product (Prochymal; Osiris Therapeutics, Inc. Columbia, MD, USA) for acute GvHD, failed to reach its endpoints (reviewed in Galipeau, 2013, and Ankrum and Karp, 2010). Further trials are attempting to verify the phase I and II findings and are analyzing the problems associated with the Phase III trial. The safety and efficacy of Prochymal has also been studied in a Phase I trial in patients with myocardial infarction (MI) (Hare *et al.*, 2009) and in Phase I to III trials for Crohn's disease (<http://www.clinicaltrials.gov>). Phase I to III Randomized Clinical Trials (RCT) have indicated cell therapy using BM-MSCs or BM-

mononuclear cells (BMMCs) as a safe treatment for the repair of cardiac function (reviewed in Sanz-Ruiz *et al.*, 2010).

Clinical trial results seem to portray mixed data on the safety and efficacy of MSCs. However, application of cell-based therapies utilizing MSCs is a relatively young field. The lack of uniformity in the trial results may be due to (1) a lack of standardization and optimization of cell isolation and delivery protocols; (2) inconsistency in nomenclature and terminology used; and (3) the variability in the type and/or source of MSCs being investigated in different clinical settings (Sanz-Ruiz *et al.*, 2010).

Cells conforming to the minimum criteria to be characterized as MSCs are believed to vary between different sources (Dominici *et al.*, 2006). It is therefore critical to establish the most efficient way to isolate, culture, expand, and characterize a population of cells with potent therapeutic potential in cell-based applications. It is speculated that MSCs derived from different sources are not the same cells but do possess the potential to exert similar therapeutic properties. This study has chosen to compare adipose tissue, UCB, and Wharton's jelly as sources of MSCs to investigate which of these tissues could provide the most effective product for cell-based therapy translatable to the clinic.

## Chapter 3: Isolation of primary mesenchymal stem cells from human tissues

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### 3.1 Introduction

Mesenchymal stem cells can be derived from various tissues while still presenting similar basic biological features. However, there are substantial differences that exist between cells of different origins. These include differences in age-related functional properties (Xin *et al.*, 2010) and in expansion potential when cultured under identical conditions. Mesenchymal stem cells from different tissues may show significant phenotypic differences that reflect their distinct functional properties (Bianco *et al.*, 2008). Bone marrow-derived MSCs are the best-characterized source of MSCs for autologous transplantation. Increasing interest in MSCs has led to further investigations into the ideal source. Alternative MSC sources are currently being investigated. Popular alternatives seem to be MSCs isolated from adipose tissue, UCB, and Wharton's jelly. Adipose tissue, mostly obtained as lipoaspirate, is more abundantly available than BM-aspirates, yielding more stem cells, and is becoming a preferred choice of adult stem cells for future clinical applications (Lin *et al.*, 2008). The umbilical cord, obtained less invasively and usually routinely discarded after birth, harbours MSCs in the UCB as well as in the Wharton's jelly. These cells are considered as the youngest form of adult stem cells.

Although adipose-derived stem cells can be harvested in large quantities, they need to be expanded *in vitro* before transplantation. Expansion protocols need to be optimized in order to provide maximal yields while preserving the desired cell characteristics. Zuk and colleagues showed that human adipose tissue was indeed a source of multipotent stem cells (Zuk *et al.*, 2002, Zuk *et al.*, 2001). The SVF can easily be isolated from adipose tissue, and contains ASCs. It has been shown that the type of surgical procedure used for adipose tissue harvesting can affect the yield as well as the growth characteristics of ASCs. Resection and tumescent (standard) liposuction were found to be preferable over ultrasound-assisted liposuction (Oedayrajsingh-Varma *et al.*, 2006). Isolation of ASCs involves tissue digestion of the SVF, although alternative methods of isolation have been suggested. Mechanical

dissociation is able to yield large quantities of adherent MSCs with similar phenotypic and differentiation potential (Baptista *et al.*, 2009).

It has been widely accepted that UCB is a rich source of HSCs. Less defined is whether UCB can serve as a source of MSCs. Successful isolation of MSCs from cord blood units has been controversial. Mesenchymal-like cells were isolated in only 25% of UCB samples. The other 75% of samples displayed cells with an osteoclast morphology (Erices *et al.*, 2000). A study comparing MSCs from BM to UCB failed to yield MSCs in UCB (Mareschi *et al.*, 2001). Success has however been noted when a different isolation protocol was employed. Lee *et al.* (2004a) used negative immunoselection and limiting dilution assays, contrary to the density gradient centrifugation isolation protocol commonly employed, and confirmed UCB as a potential source for MSCs. Zeddou and colleagues attempted to confirm and compare the criteria and the methods used in literature to isolate MSCs from UCB (Zeddou *et al.*, 2010). Their results showed low yields of MSC from UCB, far too low to be considered as a reliable source for experimental and clinical use. Instead, they confirmed the use of umbilical cord matrix as a rich source of MSCs.

Isolation of fibroblast-like cells from Wharton's jelly in umbilical cord was originally described in 1991 (McElreavey *et al.*, 1991). These cells are classified as MSCs as they can adhere to plastic, express MSC surface markers and have the ability to differentiate into cells of the mesodermal lineage (Reviewed in Troyer and Weiss, 2008). There is no standardized method for isolating MSCs from Wharton's jelly. Enzymatic treatment with collagenase is the most widely used technique for isolating stromal cells (Pereira *et al.*, 2008, Karahuseyinoglu *et al.*, 2007, Lu *et al.*, 2006, Sarugaser *et al.*, 2005). Differences in enzymes used, incubation times and removal of cord vessels have been noted. Another method without enzymatic digestion has been suggested whereby umbilical cord segments were sectioned longitudinally to expose the Wharton's jelly and incubated for a week to allow cells to adhere (De Bruyn *et al.*, 2011).

It is easy to determine whether cells grow well in new culture conditions, but it is harder to note whether these cells have undergone any changes. Culture conditions are believed to influence the genetic stability of cells in culture. Genetic changes can

occur especially during extended passaging. Subtle changes, genetically, and epigenetically are difficult to detect, but can affect the performance of cells. Comparisons between culture media have shown better MSC proliferation when cultured in α-MEM compared to DMEM (Chen *et al.*, 2009, Sotiropoulou *et al.*, 2006). Plating densities may play an important role in the proliferative capacity of the cells. It has been shown that initial plating densities of 5 000 to 10 000 cells/cm<sup>2</sup> resulted in much higher numbers of MSCs during expansion (Sotiropoulou *et al.*, 2006).

The production of MSCs for use in the clinical setting will rely on defining optimal cell culture conditions to efficiently isolate and *ex vivo* expand MSCs while still maintaining their cellular qualities by taking an in depth look at the transcriptome. This will allow us to pin point definitively the impact culturing conditions have on the cells. This chapter focused on determining whether adult and neonatal tissue sources could indeed yield cells, specifically whether they could yield MSCs. Isolation and culturing protocols were defined from the literature and used throughout the study. Although there is no novelty associated with culturing MSCs, the refinement of the methodology currently used in the literature will eliminate the discrepancies between different research groups.

## 3.2 Materials

### 3.2.1 Media, supplements, reagents and plasticware

Dulbecco's Modified Eagle's Medium (DMEM 1X + GlutaMAX™), MEM Alpha Medium (α-MEM, 1X + GlutaMAX™), penicillin (10,000 Units/ml)-streptomycin (10,000 µg/ml) (p/s), and Trypsin- ethylenediaminetetraacetic acid (EDTA) 1X were obtained from GIBCO by Life Technologies™, Grand Island, NY, USA. Phosphate Buffered Saline 1X (PBS, pH 7.4), and Fetal Bovine Serum (FBS) were obtained from GIBCO by Invitrogen™, Grand Island, NY, USA. Culture flasks, 25 cm<sup>2</sup> and 80 cm<sup>2</sup> vented filter cap were from NUNC™, Roskilde site, Kamstrupvej, Denmark. Centrifuge tubes, 50 ml and 15 ml were from Corning, NY, USA. Serological pipettes, 5 ml, 10 ml and 25 ml, and sterile culture dishes (100 x 20 mm, 58 cm<sup>2</sup>, TC treated, vented) were from Greiner bio-one by Lasec, South Africa. Specimen collection containers (120 ml) were from SPL Life Science, Korea. Trypan blue, Toluidine blue O (TBO) and histopaque-1077 were from Sigma-Aldrich, Steinheim, Germany. Falcon cell strainers, 70 µm, were from BD biosciences, Bedford, MA,

USA. Neomedic 50 ml disposable syringes were from Neomedic, Hertfordshire, UK. Cord blood collection bags were from Pall Medical, Port Washington, NY. Versalyse™ lysing solution was from Beckman Coulter, California, US. Collagenase Type I was from GIBCO, Grand Island, NY, USA.

### 3.2.2 Equipment

The following equipment was used: Thermo Forma CO<sub>2</sub> water jacketed incubator (3111TF), ESCO Class II biological safety cabinet (AC2-4Si), and Thermo Scientific centrifuge (SL16R) from Lasec, South Africa; Fluorescence microscope (Zeiss Axiovert 200), Zeiss Axiocam digital camera, and an inverted light microscope (Zeiss Primo Vert) from Carl Zeiss Werke, Göttingen, Germany; Beckman Coulter FC500 MCL as well as the Gallios (10 colour, 3 laser) flow cytometers from Beckman Coulter Miami, USA; Neubauer improved bright-line counting chamber (depth 0.100 mm, 0.0025 mm<sup>2</sup>) from Paul Marienfeld GmbH & Co KG, Lauda-Königshofen, Germany.

## 3.3 Methods

An important objective of this study was to determine whether MSCs could be isolated from adipose tissue, UCB, and Wharton's jelly, cultured under standard conditions, and expanded over at least ten passages in a reproducible manner. The isolated cells were classified as MSCs once they conformed to the minimal criteria proposed by the ISCT.

### 3.3.1 Tissue collection

Adipose tissue, UCB, and Wharton's jelly were collected from volunteers.

#### 3.3.1.1 Adipose tissue

Adipose tissue was obtained from volunteers undergoing liposuction and abdominoplasty procedures, who had given informed consent (refer to Appendix A to view an example of the informed consent form), in collaboration with Prof. Piet Coetze (Head of Plastic Surgery, Steve Biko Academic Hospital) and Dr. Danie Hoffman (private practice in Pretoria).

### 3.3.1.2 Umbilical cord blood

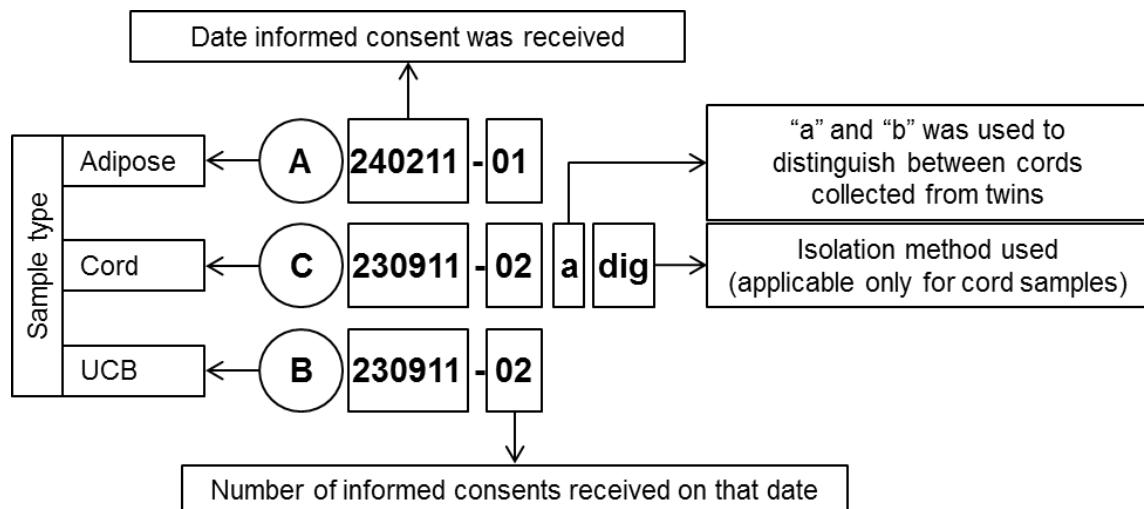
Umbilical cord blood units were obtained from term deliveries of volunteers who had given informed consent (refer to Appendix B to view an example of the informed consent form), in collaboration with Dr. Hennie Lombard (Steve Biko Academic Hospital).

### 3.3.1.3 Wharton's jelly

Umbilical cords ( $\pm 15$  cm) were obtained from term deliveries of volunteers who had given informed consent (refer to Appendix B to view an example of the informed consent form), in collaboration with Dr. Hennie Lombard (Steve Biko Academic Hospital).

### 3.3.1.4 Sample coding

All samples collected were coded to keep the identity of the donor anonymous. The following coding strategy was used:



### 3.3.2 Screening for HIV, Hepatitis B and Hepatitis C

At the first passage<sup>1</sup> of all new isolations (adipose tissue, UCB, and Wharton's jelly), routine Human Immunodeficiency Virus (HIV), Hepatitis B, and Hepatitis C screening tests (nucleic acid tests) were performed by South African National Blood Services (SANBS). None of the samples used in the studies described herein tested positive

<sup>1</sup> "passage" refers to the following procedure: isolated cells were grown and maintained in culture flasks, once the surface of the flasks were 80-100% covered with cells, the cells were detached from the surface via enzymatic digestion and re-plated into new flasks at a specific density. Every time this procedure was performed it was referred to as a new passage, starting with P0 as the initial seeding.

for HIV, Hepatitis B, or Hepatitis C. This data forms part of Chapter 7 of the MSc dissertation of Ms Fiona van Vollenstee.

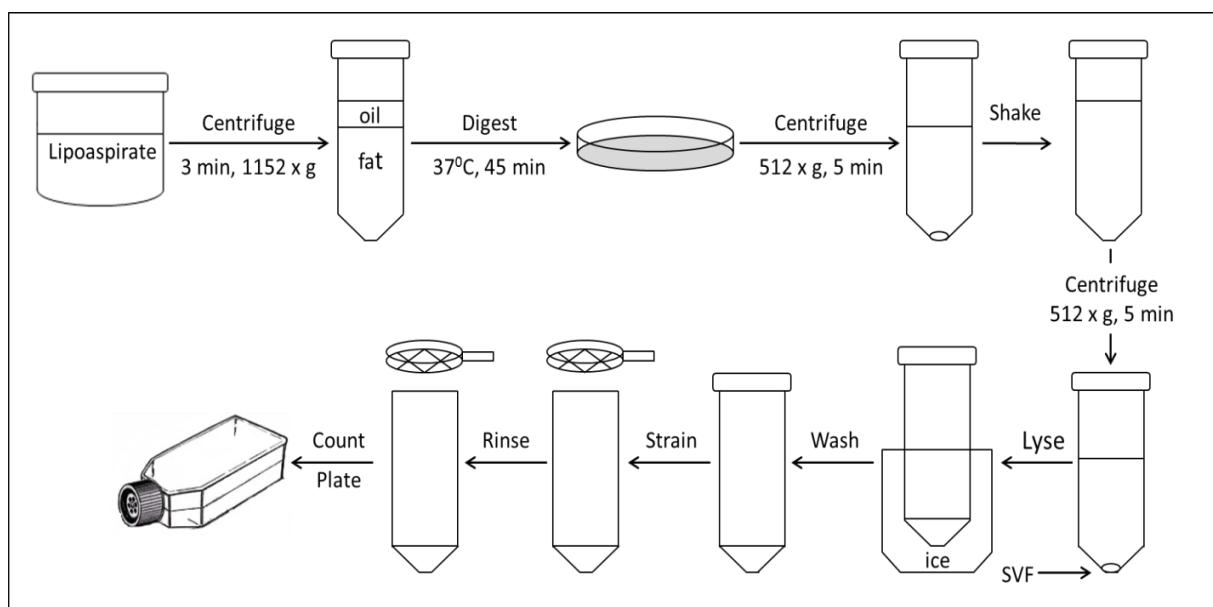
### **3.3.3 Isolation of Primary Mesenchymal Stem Cells**

Mesenchymal stem cells were isolated from adipose tissue, UCB, and Wharton's jelly according to the following protocols.

#### **3.3.3.1 Isolation from lipoaspirate/ adipose tissue**

Mesenchymal stem cells were isolated from adipose tissue as previously described by Zuk *et al.*, (2001) and Bunnel *et al.*, (2008) with minor modifications. Briefly, adipose tissue was collected in sterile specimen collection containers, containing PBS. The tissue was washed in PBS and further processed to obtain the SVF. The adipose tissue was initially processed following the Coleman method (Pu *et al.*, 2008) by transferring the samples into 50 ml tubes and centrifuging for 3 min at 1 152 x g in sterile PBS (5% (v/v) (p/s) twice to remove any excess oil before tissue digestion. The tissue was transferred to a sterile culture dish and digested at 37°C, 5% CO<sub>2</sub> for 45 min with 0.1% (w/v) Collagenase Type I (volume required was half that of the washed adipose tissue volume) prepared in PBS (2% (v/v) p/s). The sample suspension was agitated every 15 min during the incubation period. Following digestion, the samples were transferred into 50 ml tubes and centrifuged (512 x g, 5 min) to obtain the SVF that contained the ASCs. The samples were then shaken vigorously to thoroughly disrupt the pellet and to mix the cells; this step completed the separation of the stromal cells from the primary adipocytes. The centrifugation step was repeated. The pellet was re-suspended in 2 ml α-MEM containing 10% (v/v) FBS and 1% (v/v) p/s, to neutralize the collagenase activity, and centrifuged at 184 x g for 5 min to remove the enzyme. The red blood cells (RBCs) in the pellet were lysed using 1 ml Versalyse™ lysing solution, for 10 min at RT. The SVF was collected by centrifugation (184 x g, 5 min after addition of 4 ml PBS (2% (v/v) p/s)), washed with 2 ml PBS (2% (v/v) p/s) and the cell pellet re-suspended in 2 ml complete growth medium (CGM consisting of α-MEM, 10% (w/v FBS, 1% (v/v) p/s). The cells were filtered through a 70 µm cell strainer to remove cellular debris. The cell strainer was washed with an additional 1 ml CGM to obtain any additional cells. Cells were counted and seeded at a density of 5 x 10<sup>5</sup> cells per cm<sup>2</sup> in 25 cm<sup>2</sup> flasks overnight at 37°C, 5% CO<sub>2</sub> in CGM. Following 24 hour

incubation, the plates were washed with PBS (1% (v/v) p/s) to remove non-adherent cells, resulting in a heterogeneous cell population containing MSCs.



**Figure 7. Scheme for processing of adipose tissue and isolation of adipose-derived stem cells.**

Lipoaspirate samples were processed by enzymatic digestion and the stromal vascular fraction (SVF) collected. Adipose derived stem cells from the SVF were allowed to adhere to the plastic culture dish, and non-adherent cells were washed away after 24 hours. Figure prepared by Karlien Kallmeyer.

### 3.3.3.2 Isolation from Umbilical cord blood

The protocol described by Bieback *et al.*, (2004) was used for isolating MSCs from UCB with minor adjustments. A cord blood collection bag was used for collection of UCB units from term deliveries of mothers who had given informed consent. The sample was processed within two hours of delivery.

The UCB was drawn from the collection bag using a syringe and transferred to 50 ml tubes. To isolate mononuclear cells (MNC) each UCB unit was carefully loaded onto Histopaque-1077 solution (1 part Histopaque and 2 parts UCB). After density gradient centrifugation at 400 x g for 30 min at RT, MNCs were collected from the interface and 3 ml PBS (1% (v/v) p/s) was added. Red blood cells were lysed by adding 1 ml Versalyse to the cell pellet for 10 min. The cells were then washed twice

in 1 ml PBS (1% (v/v) p/s). The whole pellet was seeded in a 25 cm<sup>2</sup> flask in 3 ml CGM. Medium was added after 12 hours and every 24 to 48 hours thereafter. After five days the cells were washed once with PBS (2% (v/v) p/s) to remove the non-adherent cells and fresh CGM was added.

### 3.3.3.3 Isolation from Wharton's Jelly

A 15 cm piece of cord was collected from term deliveries from volunteer mothers who had given informed consent. Cords were collected in PBS containing 5% (v/v) p/s, and transported to the lab at RT. Cells were isolated within two hours of delivery.

The cord was extensively washed with PBS (5% (v/v) p/s) to remove excessive blood. Two different isolation methods were used for isolating MSCs from Wharton's jelly with minor modifications: (1) the isolation protocol described by Pereira *et al.*, (2008) referred to as the "digested" method, and (2) the protocol described by de Bruyn *et al.*, (2011) referred to as the "pieces" method. The cord was divided into two, and further processed accordingly.

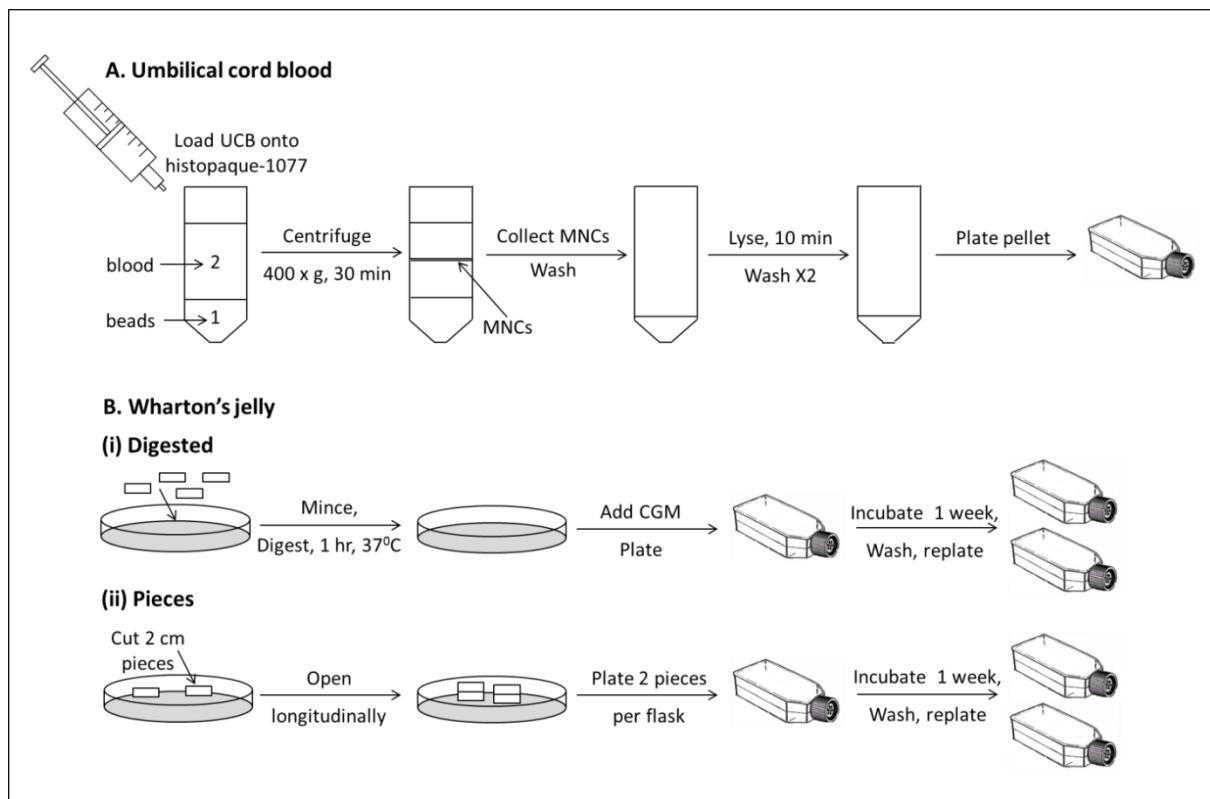
#### 3.3.3.3.1 Pereira: "Digested" protocol

The cord piece was placed in a sterile culture dish, and cut into 5 to 10 mm pieces. Collagenase Type I at 2 mg/ml (5-10 ml, depending on the cord size) prepared in PBS containing 2% (v/v) p/s solution was added, and the cord was finely minced. Minced material was incubated in collagenase for 1 hour at 37°C, 5% CO<sub>2</sub>. The collagenase activity was stopped with an equal volume of CGM, and the suspension transferred into two 80 cm<sup>2</sup> flasks and incubated for one week before the cultures were washed once with PBS (2% (v/v) p/s), and the medium replaced with fresh CGM.

#### 3.3.3.3.2 de Bruyn: "Pieces" protocol

The cord piece was placed in a sterile culture dish, and cut into 2 cm segments, and washed with PBS (5% (v/v) p/s). The cord was opened longitudinally with a sterile scalpel, and washed again (to expose a wider area of the Wharton's Jelly to have contact with the plastic surface). As much as possible of the arteries, and vein were removed; however not all was removed as Wharton's jelly is lost through this process. The sections were transferred to 25 cm<sup>2</sup> flasks - two pieces of cord per flask

in 3 ml of CGM. Fresh CGM was added to the segments daily. Cultures were maintained at 5% CO<sub>2</sub> and 37°C. After 7 days the tissue segments were removed and the cultures were washed once with PBS (2% (v/v) p/s) and medium was replaced. The tissue pieces that were removed from the flasks were transferred to a new 80 cm<sup>2</sup> flask containing 10 ml CGM.



**Figure 8. Scheme for processing neonatal tissue-derived MSCs.**

(A) Umbilical cord blood was processed using density gradient centrifugation. Two parts blood was loaded on top of one part beads, centrifuged and mononuclear cells (MNCs) collected. MNCs were treated with lysing solution to remove any contaminating red blood cells and plated in complete growth medium. (B) Umbilical cord was processed by two methods to obtain Wharton's jelly derived MSCs; (i) by enzymatic digestion following with the digested tissue being kept in culture for 1 week to allow for the MSCs to adhere to the culture dish, and (ii) by plating pieces of cord that had been opened longitudinally to allow for migratory cells to exit the cord and adhere to the plastic culture dish. Figure prepared by Karlien Kallmeyer.

### 3.3.4 Culturing and expansion conditions of MSCs

The isolated cells were maintained at 37°C, 5% CO<sub>2</sub> in CGM. When cells were 80-90% confluent, they were passaged by the addition of 0.25% trypsin-EDTA 1X for 15 min at 37°C. Trypsin activity was stopped by the addition of an equal volume of CGM. The suspension was centrifuged at 184 x g for 5 min and re-suspended in fresh CGM. Cells were counted using the trypan blue dye exclusion assay (Tennant, 1964) and re-plated at a density of 5 000 cells per cm<sup>2</sup>.

### 3.3.5 Determination of proliferation kinetics

The number of doublings in cell populations (PD) and the time period (in hours) in which they occur (PDT) were calculated according to the following formulae (Isaikina *et al.*, 2006, Christodoulou *et al.*, 2013):

$$PD = \frac{\log(N/N_0)}{\log 2}$$

$$or \quad PD = \frac{(1/\log 2) \times \log(N/N_0)}{}$$

$$PDT = \frac{\text{culture time } (t)}{PD}$$

$$or \quad PDT = t \times \frac{(\log 2 / (\log N - \log N_0))}{}$$

N<sub>0</sub> is the number of viable cells seeded, and N is the number of cells harvested, and t is the time (in hours) between seeding and harvesting. A proliferative index (PI) was also calculated to serve as a standardized measure of the proliferative capacity of the cells. A PI was defined as the ratio of the number of population doublings (PD) in a specific passage over the respective time period (PDT) in which the former took place.

### 3.3.6 Microscopy

A single adipose-derived MSC sample and a single Wharton's jelly-derived MSC sample were seeded in sterile culture dishes, and grown to low and high confluency. The cells were seeded from a low passage and a high passage. Cells were fixed

with 4% (v/v) formaldehyde (1 hour) and stained with 1% (w/v) TBO (1% (w/v) Na<sub>2</sub>CO<sub>3</sub>). Images were captured with an inverted fluorescence microscope.

### 3.3.7 Statistical analysis

A non-parametric, related-sample Wilcoxon Signed Rank Test comparing the median was performed between the digested cords and cord pieces groups to determine whether statistically significant differences exist across categories of P0r-P4, P1-P4, and P4f-P9f (designation of “r” and “f” described below). A non-parametric, independent-samples median test across category of P1-P4 between groups of sample source (adipose, digested, and pieces) was also performed. A Friedman non-parametric ANOVA for related samples was performed to determine whether statistically significant differences exist for distribution across categories of P1-P5, P6-P10, P11-P15, and P16-P23 for the adipose group. Significance was set at p < 0.05.

## 3.4 Results

Cells were isolated from three different sources, adipose tissue, UCB, and Wharton’s jelly. These cells were grown *in vitro* and further characterized to determine whether they could be classified as MSCs (see Chapter 4 and 5). This chapter only looked at whether cells could be harvested and cultured *in vitro* from each tissue source. Cells were routinely plated at 5 000 cells/cm<sup>2</sup>, and harvested at approximately 80% confluence. The number of cells harvested at each passage was noted, and was used to determine the proliferation rate of the cells.

### 3.4.1 Adipose-derived cells

A total of 14 adipose samples (Table 2) were collected and processed. The majority (79%) of the samples were collected from the private sector. The age of volunteers varied with a minimum age of 18 years and a maximum age of 68 years. The majority of samples came from volunteers in the young adult (18-24, 38%) and adult (25-49, 31%) groups (Figure 9). Variability in cell yield was found between samples (Figure 10). On average 9.4 x 10<sup>6</sup> cells were isolated from 1 gram of fat. Cells with a fibroblastic morphology were obtained from adipose samples (Figure 15). Figure 11 (A-C) depicts the proliferation dynamics of the ASCs during *in vitro* sub cultivation.

Sufficient data was collected from nine adipose samples up to passage 12, and was used to determine the PDT for ASCs. A mean PDT of 193.4 hours was calculated (Table 5). The PDT varied greatly between samples. The minimum PDT - 92.35 hours - was at passage 9, and the maximum PDT - 421.9 hours - was at passage 1 (Figure 11 A). This represents a 4.5 fold difference. Peak proliferation rate (PI) was observed at passage 9 (Figure 11 B). Cells have undergone approximately 17 PDs from passage 1 to passage 12 (Figure 11 C).

### 3.4.2 Wharton's jelly-derived cells

Ten umbilical cords (Table 3) were processed. Seven cords produced adherent cells in culture. One cord sample, C090311-02 dig, did produce adherent cells with fibroblastic morphology. These cells were large and flattened, with a high PDT of 414.4 hours. The cells were cultured with difficulty up to passage 10. A reduction rather than an increase in cell number was found during culturing. Each of the other six cords was processed by two protocols, either as “digested cords” or “cord pieces”. After cord samples were processed, the digested tissue suspension was not further processed but plated in culture flasks, allowing the cells in suspension to adhere to the flasks. Thus, an initial cell yield could not be determined. In the same manner, an initial cell yield for cord pieces could not be determined. An interesting observation for both cord protocols was that in the initial culture, denoted passage 0, cells adhered and proliferated to form confluent patches. In an attempt to avoid cells becoming contact inhibited, these cultures were trypsinized (before 80% confluent), and all the cells evenly spread across the flask. These cultures were still termed passage 0 cells, and were denoted as passage 0r, where r is for replated. Technical difficulties with incubators were encountered during the study that led to cultures being lost. The cord samples were affected by this, leading to the need to thaw samples from frozen down stocks to complete the study. Early cord passages were from freshly isolated cultures, while later passages were from frozen stocks, and are denoted by an “f” after the passage number. Cords from fresh and thawed samples were regarded separately when determining the proliferation rate of the cells. Figure 12 (A-C) depicts the proliferation dynamics of digested cords and cord pieces. Less variability was seen in PDT values between passage 0r and passage 4 (Figure 12 A). The average PDT was 57.6 hours in digested cords and 77.8 hours in cord pieces (Table 5). The different isolation protocols did not influence the proliferation of

the cells as small differences in PDT were found (when considering passage 0r to passage 4 only). Samples from passage 4f to passage 9f showed greater variability in PDT values between samples, and isolation protocol used (Figure 12 A). Freezing of cells seemed to influence the proliferation of the cells. The average PDT for samples from passage 4f to passage 9f was 162.7 hours for digested cords, and 296.2 hours for cord pieces (Table 5). Peak proliferation rate (when considering PI and PDT values) was at passage 0r for passage 0r to passage 4 cells, and was at passage 4f for passage 4f to passage 9f cells for both cord groups. Cells had undergone 12 PDs for digested cords and 11 PDs for cord pieces after four passages (Figure 12 C). Both the digested cords and cord pieces produced cells with a fibroblastic morphology resembling MSCs (Figure 15).

### 3.4.3 Umbilical cord blood-derived cells

Six UCB samples were processed (Table 4). Only a single UCB sample yielded adherent cells with a fibroblastic morphology. On average 62.5 ml UCB was collected per sample. Figure 16 shows the appearance of UCB derived adherent cells. Cells displayed a rounded morphology (Figure 16 A, B) in initial cultures. Trypsinization was unable to lift all of these cells during passaging, and these cultures were terminated. Only a single UCB sample produced cells resembling MSCs (Figure 16 C). These cells were large and flat in morphology, resembling senescent cells. This sample was kept in culture for 7 passages, and used for phenotypic characterization (Chapter 4).

**Table 2. Sample information: Adipose-derived stem cells**

Patient ID	Collection Date & Place	Patient details	Amount Collected
A240211-01	24 Feb '11 Private practice	19 yr old female Caucasian	Not recorded Lipoaspirate
A090311-01	9 March '11 Private Practice	Not recorded	2 x bottles Lipoaspirate
A230311-01	23 March '11 Private Practice	46 yr old female Race not recorded	2 x bottles Lipoaspirate
A040411-01	4 April '11 Steve Biko	18 yr old male Caucasian	100 ml Lipoaspirate, foot
A130411-01	13 April '11 Private Practice	60 yr old female Caucasian	41.872 g Lipoaspirate
A130411-02	13 April '11 Private Practice	68 yr old male Caucasian	18.385 g Lipoaspirate
A200411-01	20 April '11 Private Practice	25 yr old female Caucasian	46.9 g Lipoaspirate
A050511-01	5 May '11 Private Practice	19 yr old female Caucasian	36.8 g Lipoaspirate
A100511-01*	10 May '11 Private Practice	19 yr old female Caucasian	62.6 g Lipoaspirate
A220611-01	22 June '11 Private Practice	33 yr old female Black	52.3 g Lipoaspirate
A270611-01	27 June '11 Private Practice	20 yr old female Caucasian	25.1 g Lipoaspirate
A270611-02	27 June '11 Private Practice	41 yr old female Caucasian	33.7 g Lipoaspirate
A050711-01	5 July '11 Steve Biko	50 yr old female Race not recorded	14.3 g Lipoaspirate
A180811-01	19 Aug '11 Steve Biko	39 yr old male Black	55.6 g Lipoaspirate

\*Patient A100511-01 was the same patient as A240211-01 who donated adipose tissue on different occasions

**Table 3. Sample information: Wharton's jelly derived stem cells**

Patient ID	Collection Date & Time	Isolation Method		Amount processed	Culture
C010311-01	11 Mar '11 3 hours	Pieces	Y	Not recorded	Terminated
		Digested	N	n/a	n/a
C090311-02	30 Mar '11 1 hour	Pieces	Y	10 cm	Terminated
		Digested	Y	10 cm	P0 – P10
C160211-01	1 May '11 7 hours	Pieces	N	n/a	n/a
		Digested	Y	20 cm	Terminated
C160311-01	17 May '11 2 hours	Pieces	N	n/a	n/a
		Digested	Y	10 cm	Terminated
C220211-02	8 Jun '11 > 2 hours	Pieces	Y	5 cm	P0r – P13
		Digested	Y	5 cm	P0r – P13
C190511-06	10 Jun '11 2 hours	Pieces	Y	15 cm	P0r – P13
		Digested	Y	15 cm	P0r – P8
C210911-06	22 Sept '11 30 min	Pieces	Y	12 cm	P0r – P2, P3f – P10f*
		Digested	Y	10.5 cm	P0r – P2, P3f – P8f*
C230911-01	23 Sept '11 30 min	Pieces	Y	12 cm	P0r – P4, P4f – P10f*
		Digested	Y	6 cm	P0r – P3, P4f – P10f*
C230911-02a	23 Sept '11 Twin 1, 30 min	Pieces	Y	8 cm	P0r- P4, P4f – P10f*
		Digested	Y	4 cm	P0r – P4, P4f – P10f*
C230911-02b	23 Sept '11 Twin 2, 30 min	Pieces	Y	8 cm	P0 – P3, P3f – P8f*
		Digested	Y	4 cm	P0r – P4, P4f – P10f*

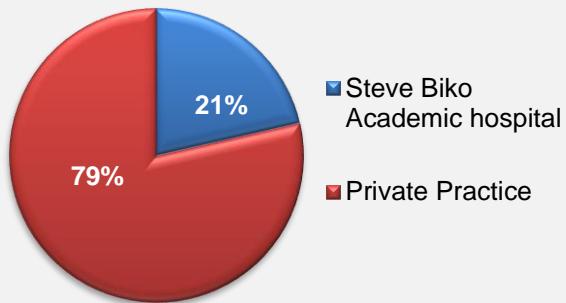
\*Note: Due to technical problems with incubators, cord samples were lost and had to be thawed from frozen stocks to continue with experiments. The "f" after a passage number indicates that this was a frozen sample.

**Table 4. Sample information: Umbilical cord blood derived stem cells**

Patient ID	Collection Date & Time	Amount processed	Culture
B090311-02	30 Mar '11 1 hour	60 ml	P0 – P7
B160211-01	1 May '11 7 hours	65 ml	P0 Terminated
B160311-01	17 May '11 2 hours	50 ml	P0 - P1 Terminated
B220211-02	8 Jun '11 > 2 hours	60 ml	Terminated
B190511-06	10 Jun '11 2 hours	75 ml	Terminated
B230911-02b	23 Sept '11 Twin 2, 30 min	65 ml	Terminated

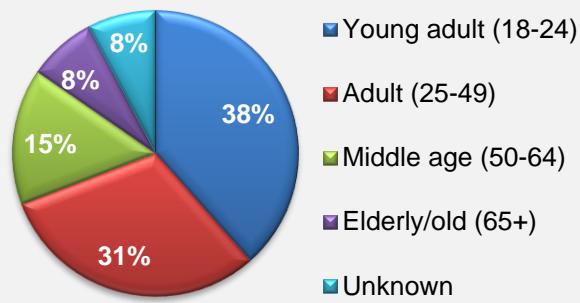
A.

Sample collection sites



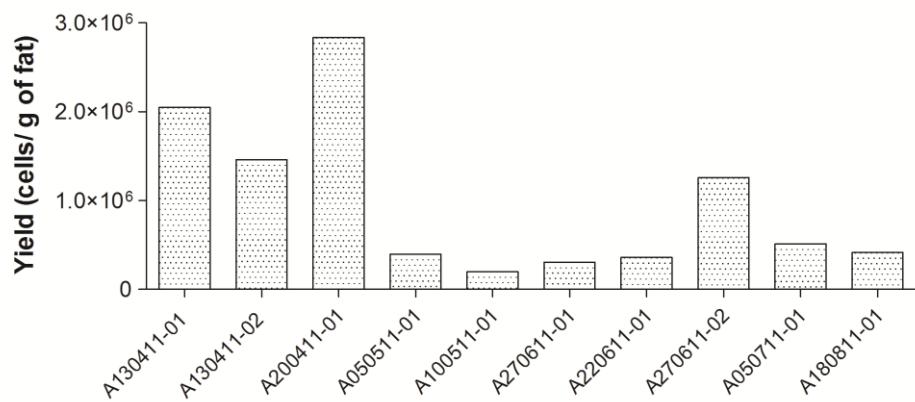
B.

Patient age



**Figure 9. Adipose sample information.**

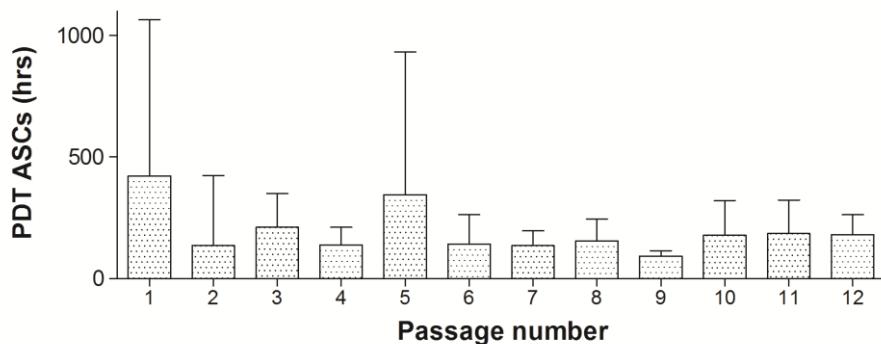
(A) Sites of sample collection. (B) The age of patients from whom the samples were collected.



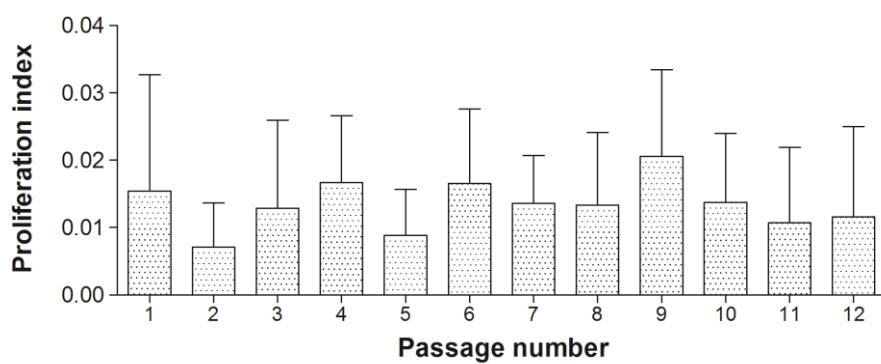
**Figure 10. Cell yield per gram of fat collected for Adipose samples.**

After the adipose tissue was processed, the total cell yield per gram of fat collected was calculated.

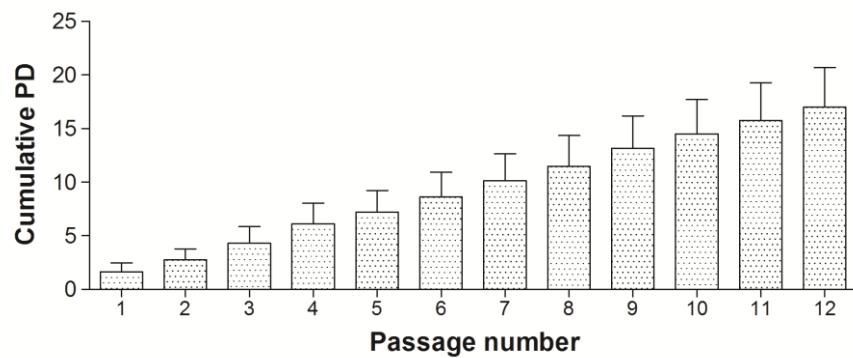
A.



B.



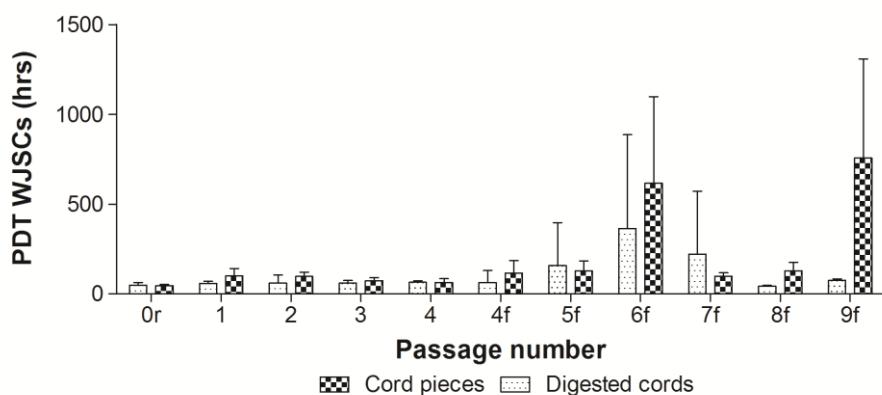
C.



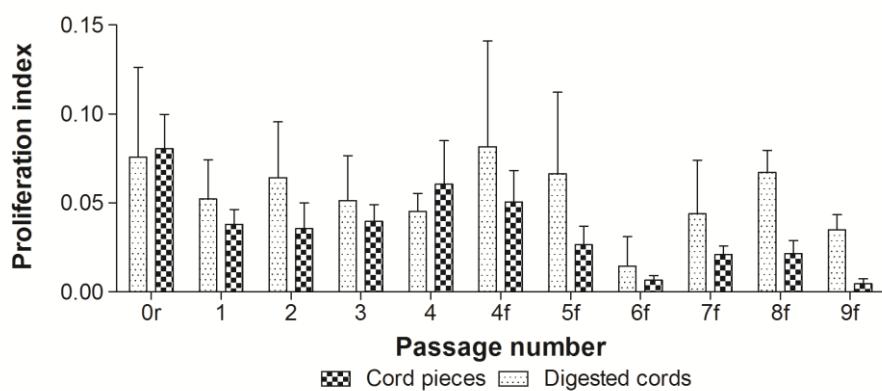
**Figure 11. Proliferation kinetics of adipose-derived MSCs over 12 passages.**

(A) Cell population doubling times (PDT, hours) at respective passages. (B) Proliferation index (PI; ratio of PD over respective PDT). (C) Number of cumulative population doublings (PD) over 12 passages. The results represent the average of 9 adipose samples ( $n = 9$ ) with SD.

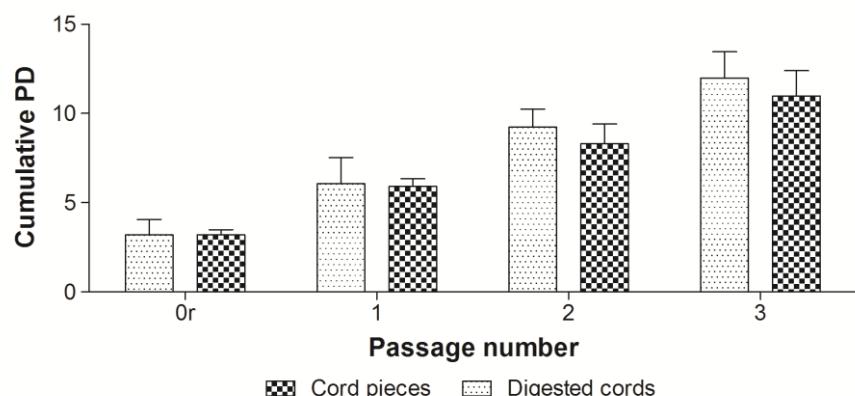
**A.**



**B.**



**C.**



**Figure 12. Proliferation kinetics of Wharton's jelly-derived MSCs.**

(A) Cell population doubling times (PDT, hours) at respective passages. (B) Proliferation index (PI; ration of PD over respective PDT). (C) Number of cumulative population doublings (PD) over passages. The results represent the average of at least 6 cord samples ( $n = 6$ ) per group with SD.

**Table 5. Proliferation kinetic data of adipose- and Wharton's jelly-derived MSCs**

	Mean PDT (hours)		
	Digested cords	Cord pieces	ASCs
P1 – P4*/***	69.4 ± 24.6	85.7 ± 64.6	226.8 ± 365.1
P0r – P4*	57.6 ± 23.2	77.8 ± 60.5	Data not available
P4f – P9f**	162.7 ± 280.4	296.2 ± 545.8	Data not available
P1 – P12***	Data not available	Data not available	193.4 ± 280.5

Cords: \*n = 6, \*\*n = 4; Adipose: \*\*\*n = 9. Data are shown with SD.

**Table 6. Proliferation kinetic data of adipose-derived MSCs at low and high passages**

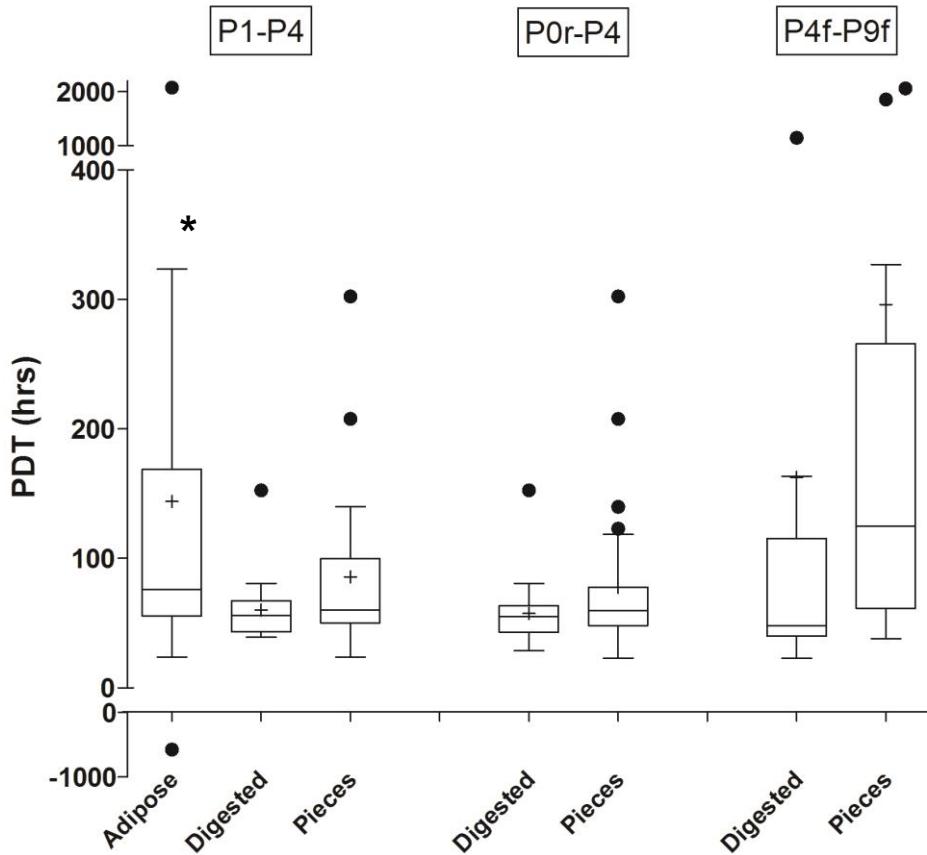
ASCs	n	Mean PDT (hours)
P1 – P5	62	247.4 ± 381.1
P6 – P10	65	163.8 ± 127.9
P11 – P15	53	196.9 ± 204.6
P16 – P23	42	192.9 ± 207.3

Data are shown with SD. "n" is the total number of PDT values used in that category and it considered all the data generated for all the cultured adipose samples.

### 3.4.4 Comparison between tissue sources

During culturing, MSCs from Wharton's jelly appeared to be passaged more frequently than the MSCs from adipose tissue. This was confirmed when comparing the average PDT values from passage 1 to passage 4 between sources (Table 5). Both digested cords and cord pieces had a mean PDT approximately three-fold lower than for ASCs. The cells maintain their morphology at low and high passages. Figure 13 depicts the distribution of PDT values between MSCs from adipose tissue, digested cords, and cord pieces in categories of P1-P4, and from digested cords, and cord pieces in categories P0r-P4, and P4f-P9f. Due to the non-parametric distribution of the data, median values better represent the data sets. The distribution of values within adipose, digested cords, or cord pieces showed great variability, especially in the adipose group and the cords for the P4f-P9f category. Comparison of median values between sources showed that the median PDT for adipose (76.04 hours) was statistically significantly higher than for digested cords (56 hours) or cord pieces (60 hours). No statistically significant differences were found between cords in P0r-P4 or P4f-P9f categories. A few adipose samples were grown beyond passage 12. Differences in mean PDT in adipose samples of low and high passage

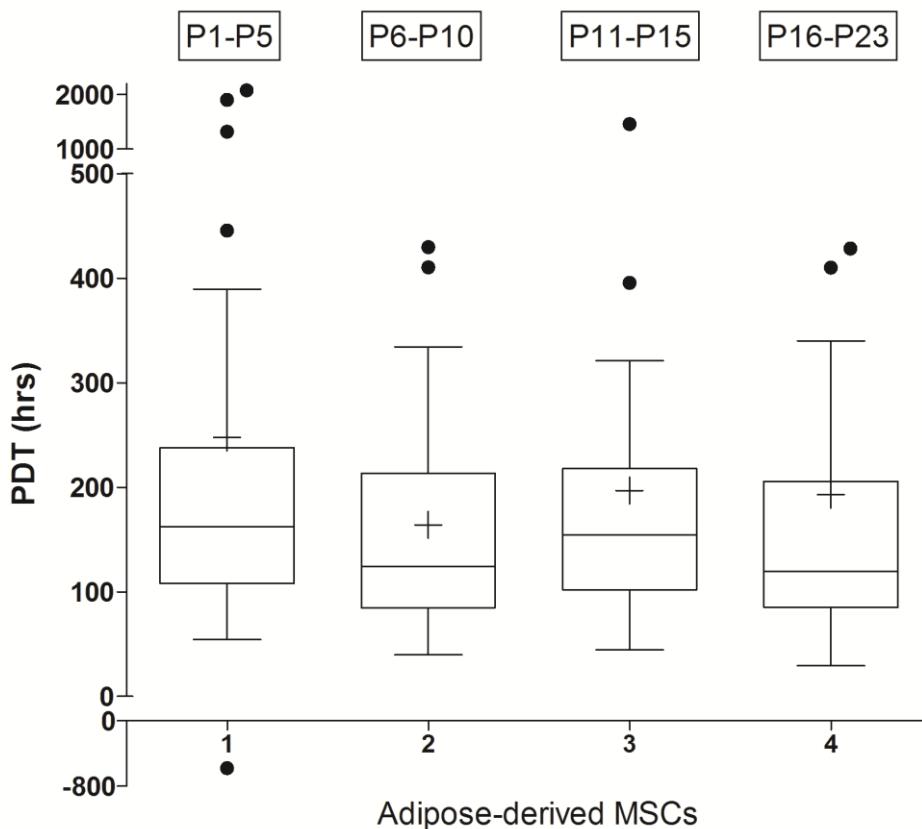
were compared (Table 6). A decrease in PDT was found with increasing passages. Due to the non-parametric distribution of the data, median values better represent the data sets. The distribution of values within adipose was compared at low and high passage (Figure 14). No significant differences in the median values between adipose passages were found (Figure 14). Mesenchymal stem cells from cord and adipose tissue showed a fibroblastic morphology (Figure 15).



	P1-P4			P0r-P4		P4f-P9f	
	Adipose	Digested	Pieces	Digested	Pieces	Digested	Pieces
n	78	21	21	27	26	22	22
Minimum	-578.3	39.06	23.95	28.6	23.07	22.87	38.1
25% Percentile	55.76	43.17	50.38	43.1	48.22	39.9	61.5
Median	76.04	56	60	55.03	59.91	47.93	125.1
75% Percentile	168.8	67.34	99.93	63.37	77.9	115.6	266
Maximum	2075	152.7	302.4	152.7	302.4	1146	2063
Mean	144	60.38	85.65	57.6	77.75	162.7	296.2
Std. deviation	260.5	24.57	64.6	23.2	60.52	280.4	545.8
Std. error	29.5	5.361	14.1	4.464	11.87	59.78	116.4

**Figure 13. Box and whisker plot showing the PDT values for adipose- and Wharton's jelly-derived MSCs.**

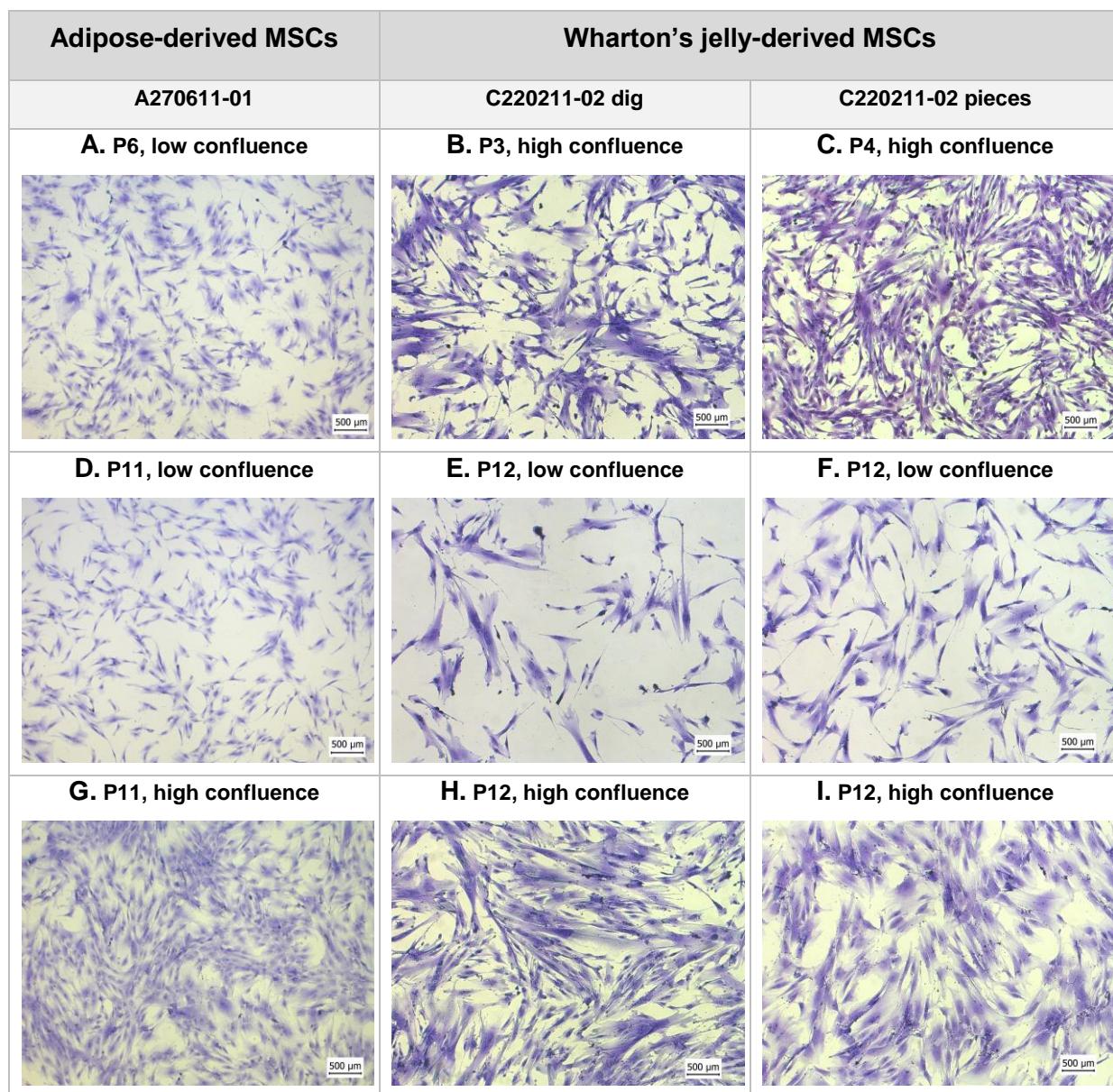
The minimum, 25<sup>th</sup> percentile, median, 75<sup>th</sup> percentile, and maximum values are plotted. Mean values are shown as "+" and outliers are shown as "•". Samples from adipose tissue, digested cords, and cord pieces were compared in category P1-P4. Digested cords and cord pieces were compared in categories P0r-P4, and P4f-P9f. "n" represent the number of values used in each category. Significance is shown when \*p < 0.05.



	P1-P5	P6-P10	P11-P15	P16-P23
n	62	65	53	42
Minimum	-578.3	39.75	44.72	29.31
25% Percentile	108.1	84.72	101.9	84.91
Median	162.4	124.2	154.5	119.4
75% Percentile	237.8	213.5	218	205.5
Maximum	2075	844.5	1460	985.2
Mean	247.4	163.8	196.9	192.9
Std. deviation	381.1	127.9	204.6	207.3
Std. error	48.39	15.87	28.1	31.99

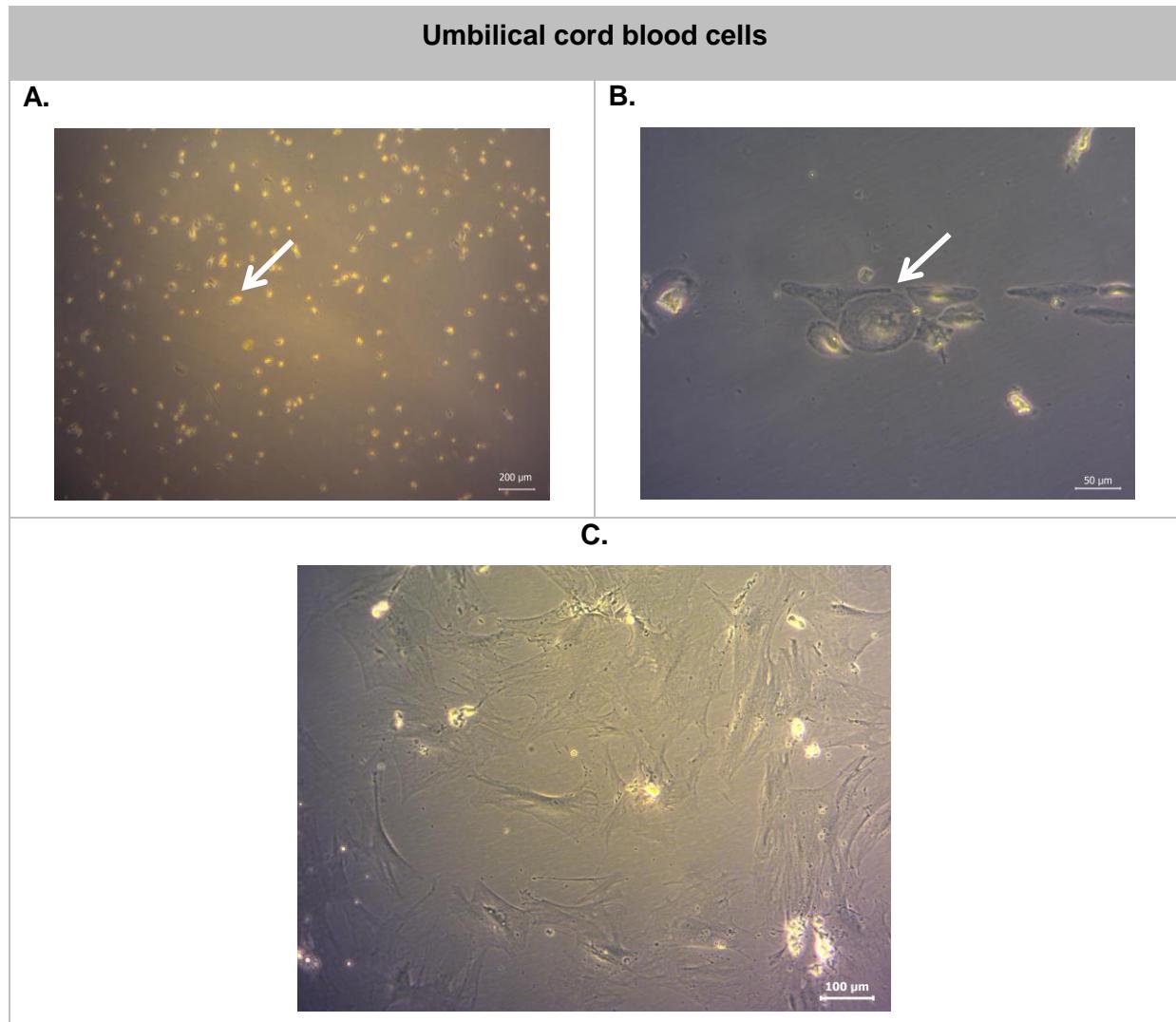
**Figure 14. Box and whisker plot showing the PDT values for adipose- derived MSCs at high and low passage.**

The minimum, 25<sup>th</sup> percentile, median, 75<sup>th</sup> percentile, and maximum values are plotted. Mean values are shown as “+” and outliers are shown as “•”. Samples from adipose tissues were compared in category P1-P5, P6-P10, P11-P15, and P16-P23. “n” represent the number of values in each category. Significance is shown when \*p < 0.05.



**Figure 15. Morphology of adipose- and Wharton's jelly-derived MSCs.**

Cultured MSCs from sample A270611-01 at low passage (A) and high passage (D, G), from sample C220211-02 dig at low passage (B) and high passage (E, H), and from sample C220211-02 pieces at low passage (C) and high passage (F, I). (A-C, G-H) Samples at high confluence and (D-F) at low confluence. Cells were stained with toluidine blue O for better visualization.



**Figure 16. Morphology of umbilical cord blood-derived cells.**

(A-B) Cells with a rounded morphology (white arrow) were found in UCB cultures. (C) Although large cells adhered to the culture dish, they died in culture.

### 3.4 Discussion

The work conducted by Caplan and Friedenstein identified BM as a rich source of MSCs (Caplan, 1991, Friedenstein *et al.*, 1976). To date, BM-MSCs are the best described, especially in the clinical setting. They serve as the gold standard to which other sources of MSCs are compared (Kern *et al.*, 2006). This study used isolation techniques commonly used in the literature to attempt to standardize MSC isolation protocols for adipose tissue, UCB, and Wharton's jelly.

Culture conditions could enhance or change properties of MSCs if protocols are not standardized. Classical basal culturing medium for MSCs consists of DMEM or α-

MEM supplemented with 10 to 20% FBS (Bieback *et al.*, 2011). Alternatives to using FBS as a serum supplement for *in vitro* culturing are being investigated. Xenogeneic serum bears the risk of transferring xenogeneic proteins that could be infectious or immunogenic, especially if used in the clinical setting. Autologous serum as a substitute rapidly expanded human MSCs with stable gene expression, whereas FBS induced more differentiated and less stable transcriptional profiles (Shahdadfar *et al.*, 2005). This study used the same culturing medium for all MSC sources.

Adipose tissue is commonly processed using enzymatic digestion and density centrifugation to isolate the SVF, which contains MSCs (Zuk *et al.*, 2002, Zuk *et al.*, 2001). An extra processing step based on the Coleman technique was introduced during MSC isolation from adipose tissue in an attempt to refine the current protocols used (Pu *et al.*, 2008). This involved an extra centrifugation step to remove the oil layer from the collected fat graft. Umbilical cord, specifically Wharton's jelly, has been processed using two different methods, either using enzymatic digestion or explant cultures (De Bruyn *et al.*, 2011, Pereira *et al.*, 2008). Both techniques were utilized in this study with success, yielding MSCs. Three cords did not yield MSCs; this was due to contamination of the cultures when plated in culture dishes rather than culture flasks (Table 3). Several studies have demonstrated that 100% of umbilical cords processed contained MSCs (Lu *et al.*, 2006, Zeddou *et al.*, 2010, Wang *et al.*, 2004). Umbilical cord blood has been suggested as another source of MSCs. However, MSCs are only present at low frequencies. Kern *et al.* (2006) had a success rate of only 29% from processed UCB units. It has been suggested that the rate could be enhanced to 63% by pre-coating culture flasks with fetal calf serum (Bieback *et al.*, 2004, Kern *et al.*, 2006). This was attempted for the present study, but with no success. The success of isolating MSCs from UCB is likely to be influenced by the quality of the unit (Bieback *et al.*, 2004). Quality can be affected by the time of processing, volume of blood collected, and the mononuclear cell count. Processed UCB units did not produce fibroblastic progenitors. Adherent cells in cultures consisted of single cells distributed throughout the flask with a rounded morphology, which resisted trypsinization (Figure 16 A, B). A previous study noted that these cells were macrophage-like with limited proliferation capacity, and were not detachable by trypsin (Zeddou *et al.*, 2010). Only a single UCB unit produced

adherent cells. These cells displayed a large flattened morphology (Figure 16 C), and had difficulty proliferating.

Adipose tissue and Wharton's jelly from umbilical cord successfully yielded MSCs with a fibroblastic morphology (Figure 15). A study by Majore *et al.* (2009) identified two cell populations within cells isolated from umbilical cords. A population of small-sized cells and another with larger-sized cells were found. Mesenchymal stem cell markers were expressed on both populations, but expression was at a higher level in the small-sized population. The small-sized population exhibited higher proliferation capacity. During culturing in this study, different sized cells were seen in the umbilical cord cultures. After the umbilical cord cultures underwent stress when the incubators ran out of CO<sub>2</sub>, cells became larger with little proliferation capacity. These cultures were maintained, and interestingly, after a week or two, populations of smaller cells were visible and proliferated. The cultures seemed to have recovered. These cells were however not further evaluated, and were not used for phenotypic and differentiation experiments as they may have been affected by the adverse culture conditions. A study proposing the use of umbilical cord explants as an alternative to enzymatic digestion for isolating MSCs showed no significant differences in terms of proliferation capacities between the isolation techniques (De Bruyn *et al.*, 2011). Table 5 summarized the proliferation kinetics of the isolated MSCs. Both cords, either from digested cords or cord pieces had a mean PDT from passage 1 to 4 that was approximately 3 fold lower than for the ASCs. Christodoulou *et al.* (2013) found that Wharton's jelly stem cells had a mean PDT of 31.9 compared to 402.3 for ASCs. The PDT of Wharton's jelly stem cells was much lower than determined in this study as they only used the samples with the lowest PDT for comparisons. All cord samples with complete growth data were included in determining the mean PDT. It can still be seen that Wharton's jelly MSCs has a greater proliferation capacity compared with ASCs. Limitations to this study included the amount of comparable data available between the different sources. Only passages 1 to 4 from the digested cords, and cord pieces could be compared to the isolated ASCs. As no data was generated from frozen adipose-derived cells, comparisons to passages 4f to 9f from the cords could only be compared between digested cords and cord pieces. A closer look at the adipose samples compared the median PDT values from low to high passage cells, however although a decrease in

PDT was seen with increasing passage number, the difference was not statistically significant. A larger population size should be compared to further test this decrease in PDT.

In conclusion, MSCs were isolated from adipose tissue and Wharton's jelly but not from UCB. Both isolation techniques for the umbilical cords produced MSCs. Cells from Wharton's jelly displayed greater proliferation capacity than from adipose tissue. One must ask whether cells of different origin are the same, or whether the application of similar isolation and culturing techniques make them appear the same *in vitro*. Understanding how MSCs act *in vivo* is a difficult task as the precise nature and *in vivo* location of MSCs is a topic of intense debate.

## Chapter 4: Phenotypic characterization of mesenchymal stem cells

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### 4.1 Introduction

Mesenchymal stem cells are plastic adherent cells with multilineage differentiation capacity that can be isolated from various adult and fetal tissues (da Silva Meirelles *et al.*, 2006). After isolation, this constitutes a heterogeneous population with a mixture of cells with varying proliferation and differentiation potentials. The identity of MSCs *in vivo* remains poorly characterized (da Silva Meirelles *et al.*, 2008). Functional assays using cultured cells are commonly used to identify MSCs *in vitro*. These include phenotypic characterization and differentiation assays. Currently no single surface marker has been identified as being unique to MSCs. Thus MSCs are characterized on the basis of morphological characteristics and surface antigen expression.

The first definitive markers for MSCs isolated from BM were proposed by Pittenger *et al.* (1999). These BM-derived MSCs were characterized by their positive expression for SH2 and SH3. The markers SH2 and SH3 were later shown to correspond to CD105 and CD73 respectively (Barry *et al.*, 1999, Barry *et al.*, 2001). These two markers along with CD90 are positively expressed *in vitro* on isolated MSCs and to date remain the primary molecules used to identify MSCs. In an attempt to standardize the markers used for identification of MSCs, a panel of markers has been proposed. As a minimal prerequisite, MSCs must express CD105, CD73, CD90, and must lack hematopoietic lineage markers CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR (Dominici *et al.*, 2006). The antigen CD105, also referred to as endoglin, is a transforming growth factor beta receptor III (TGF- $\beta$ 3) (Barry *et al.*, 1999). This receptor plays a role in TGF- $\beta$  signaling during MSC chondrogenic differentiation, and has been found to mediate interactions between MSCs and HSCs in the BM microenvironment. Expression of CD105 is not exclusive to MSCs, since it is also expressed on endothelial cells, syncytiotrophoblasts, macrophages, and connective tissue stromal cells. The antigen CD73 is an ecto-5'-nucleotidase involved in regulating BM stromal interactions, MSC migration and also modulates the adaptive immune system (reviewed in Boxall and Jones, 2012). The

function of CD90 (Thy-1) is ill defined. This is partly due to its expression on various cells, namely on thymocytes, lymphocytes, fibroblasts, neural cells, HSCs, MSCs, and ovarian follicular cells (Barker and Hagood, 2009). It has been proposed as a mediator of cell-cell interactions including the adhesion of monocytes and leukocytes to endothelial cells and fibroblasts (reviewed in Boxall and Jones, 2012). Another marker, STRO-1 has been described as the best known MSC marker; however it has not been proposed as part of the MSC phenotype. It is used for the enrichment of immature cells from BM-MSCs (Simmons and Torok-Storb, 1991). The problem with using STRO-1 as an MSC marker is that its expression is down-regulated during culturing. Successful use of STRO-1 will be in combination with other markers. The use of STRO-1 together with CD106 has been proposed (Gronthos *et al.*, 2003). Sorting of double positive cells (STRO-1+ CD106+) has been shown to yield a highly enriched population of BM-MSCs. However, as for STRO-1, CD106 expression also declines during culturing.

Flow cytometry is a powerful and relative easy approach which can be used to determine the phenotype of cells (Lindner *et al.*, 2010). It involves immunophenotyping of cells using fluorescently labeled monoclonal antibodies against specific surface antigens. Of particular importance is whether MSCs isolated from different sources have an identical phenotypic profile. The proposed MSC phenotypic markers are also expressed on other cell types. Thus antibody-based isolation of MSCs is valuable as a pre-enrichment step, but cannot be used to determine the purity of *in vitro* expanded MSCs (Lindner *et al.*, 2010). This chapter investigated whether adipose-, UCB-, and Wharton's jelly-derived MSCs exhibited the general MSC phenotype during culturing.

## 4.2 Materials

### 4.2.1 Primary cell cultures

Mesenchymal stem cells isolated from adipose tissue, UCB, and Wharton's jelly (refer to Chapter 3 for isolation procedures) were tested for their phenotypic marker expression over several passages.

### 4.2.2 Media, supplements, reagents, and plasticware

The monoclonal antibodies used for flow cytometry are described in Table 7. Additional flow cytometry consumables were obtained from Beckman Coulter (Miami, USA).

**Table 7. Conjugated monoclonal antibodies**

Monoclonal antibodies	Fluorochrome	Manufacturer
Mouse anti-human CD73	APC	BioLegend, San Diego, USA
	FITC	eBioscience, San Diego, USA
Mouse anti-human CD105	PE	Beckman Coulter, Miami, USA
Mouse anti-human CD90	FITC	
	PC5	
Mouse anti-human CD34	PC7	
	ECD	
	PE	
	FITC	
Mouse anti-human CD45	PC5	Beckman Coulter, Miami, USA
	PC7	
	ECD	

### 4.2.3 Equipment

The FC500 MCL and Gallios (10 colour, 3 laser) flow cytometers from Beckman Coulter (Miami, USA) were used during the study.

## 4.3 Methods

### 4.3.1 Immunophenotype analysis

In order to analyze surface marker expression profiles of MSCs, flow cytometric analysis was performed using specific fluorochrome-conjugated monoclonal antibodies. Immunophenotyping was performed on cells at each passage.

#### 4.3.1.1 Sample preparation

After trypsinization, cells were washed and re-suspended in PBS. For each sample, a 100 µl cell aliquot was incubated for 15 min at 37°C in the dark after addition of a panel of monoclonal antibodies (Table 8). Following incubation, cells were washed three times with PBS containing 2% (v/v) FBS, re-suspended in 1 ml PBS and then analyzed for antigen expression. The washing steps were performed to remove excess monoclonal antibodies in order to reduce non-specific staining. Based on the availability of monoclonal antibodies, three different 5-colour monoclonal antibody panels were used during the study (refer to Table 8). Antibody panel 2 was predominantly used for phenotypic characterization. A single tube containing unstained cells was prepared on each experimental day to verify protocol settings and to serve as a negative control. This was necessary to ensure that results were comparable from day-to-day. Separate unstained cells were prepared for each sample type (adipose-, UCB-, and Wharton's jelly).

**Table 8. Monoclonal antibody combinations used**

5-colour monoclonal antibody panel				
CD marker	Volume (µl)	Fluorochrome		
		Panel 1	Panel 2	Panel 3
CD73*	5	APC**	FITC	FITC
CD105	10	PE	PE	PE
CD90*	5	FITC**	PC5	PC5
CD34*	10	PC7	ECD	PC7
CD45	10	PC5	PC7	ECD

\*Note: on occasion, CD34-PE, CD34-FITC, CD73-APC, and, CD90-FITC were run in a separate tube when the complete antibody panel was not available; \*\* 10 µl of antibody was used for these

#### 4.3.1.1 Flow cytometer setup, data acquisition and analysis

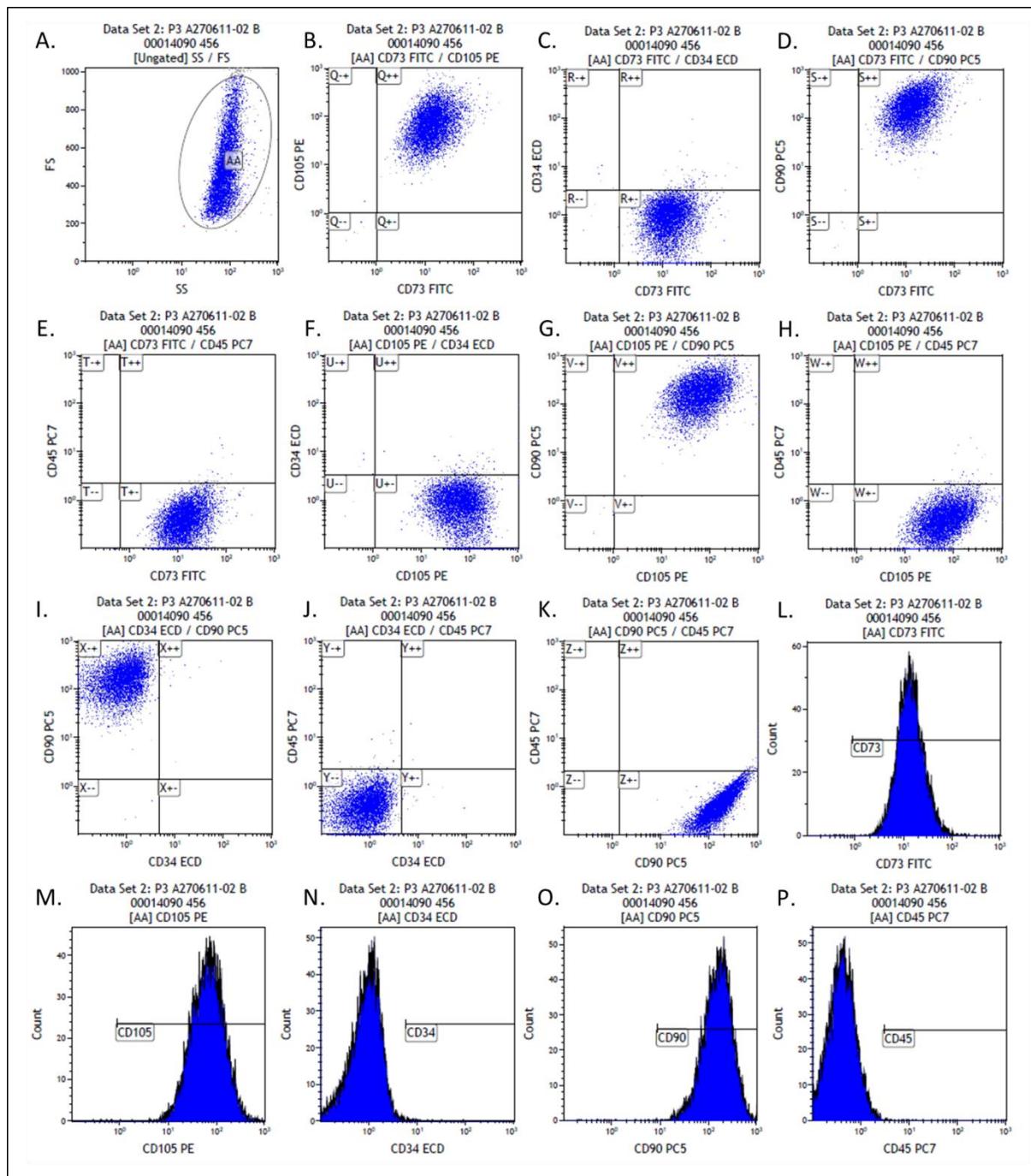
The same analysis strategy was followed throughout the study. In summary, cells were gated on forward scatter (proportional to size of cell) and side scatter (proportional to granularity and structural complexity) to exclude debris and cell aggregates (Figure 17 A). The analysis protocol consisted of two-parameter plots (Figure 17 B-K) as well as one-parameter plots (Figure 17 L-P). Unstained cells were analyzed prior to the labeled samples and the regions were set accordingly. A minimum of 5 000 events per sample were analyzed during data acquisition. All data sets were analyzed using Kaluza Flow Cytometry analysis software 1.2 (Beckman Coulter, Miami, US).

Instrument voltage settings were set so that the unstained population was visible in the first decade (Figure 18 A) and lower left quadrant (Figure 18 B). Cell populations appearing in the first decade were viewed as negatively stained. Any population appearing in decade two to four were viewed as positively stained. Two-parameter plots show four distinct populations. The upper left quadrant represents the population that is negative for the x-axis fluorochrome and positive for the y-axis fluorochrome. The upper right quadrant represents the population positive for both fluorochromes. The lower left quadrant represents the population negative for both fluorochromes. The lower right quadrant represents the population positive for the x-axis fluorochrome and negative for the y-axis fluorochrome. One-parameter plots represent the frequency distribution of the number of events per channel. Tree plots are used to summarize one-parameter plot data (Figure 19). This approach compared the phenotype of the events included in the positive region (> first decade). The branches are used to categorize cell populations based on whether they have a negative or positive result for a specific phenotypic data set.

#### 4.3.2 Statistical analysis

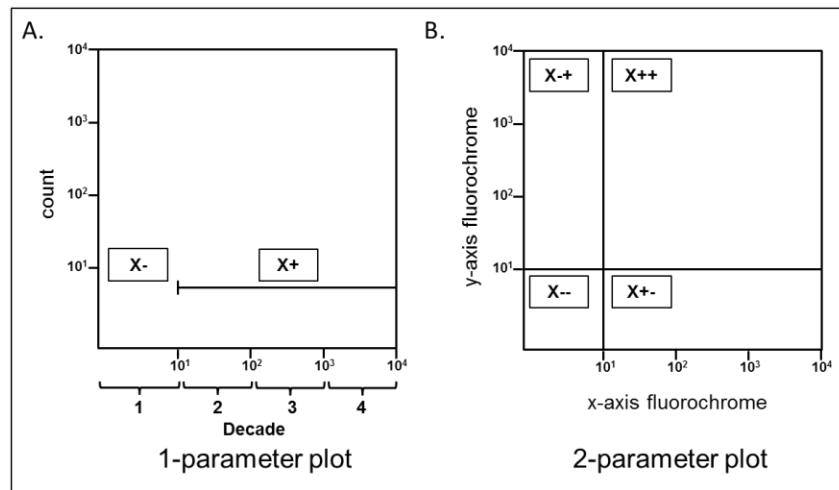
A non-parametric Kruskal-Wallis ANOVA was done within the adipose and cord groups to determine whether statistically significant differences were found across categories of passage number. If significance was noted, this was followed by a non-parametric multiple comparison (pairwise) test to determine where significance occurred. A non-parametric Kruskal-Wallis pairwise comparison of median and

distribution across categories of sample source were performed for each passage. Significance was set at  $p < 0.05$ .



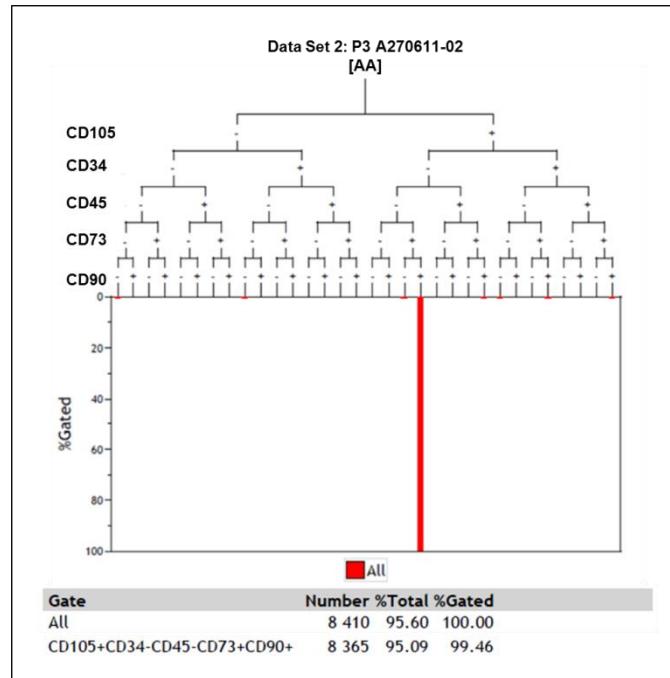
**Figure 17. Analysis strategy used for determining the phenotypic profile of MSCs.**

Flow cytometric analysis of sample A270611-02 at passage 3 showing positive expression for CD73, CD105, CD90 and lack CD34 and CD45 expression. (A) Dot plot showing the cell population, AA. (B-K) Two-parameter plots. (L-P) One-parameter plots. Plots (B-P) were gated for the cell population, AA.



**Figure 18. Flow cytometry plots.**

(A) One-parameter plot: the first decade represents the negative population and decades two to four represent the positive population. (B) Two-parameter plot: shows four distinct populations ( $X-+$ ,  $X++$ ,  $X--$ ,  $X+-$ ).

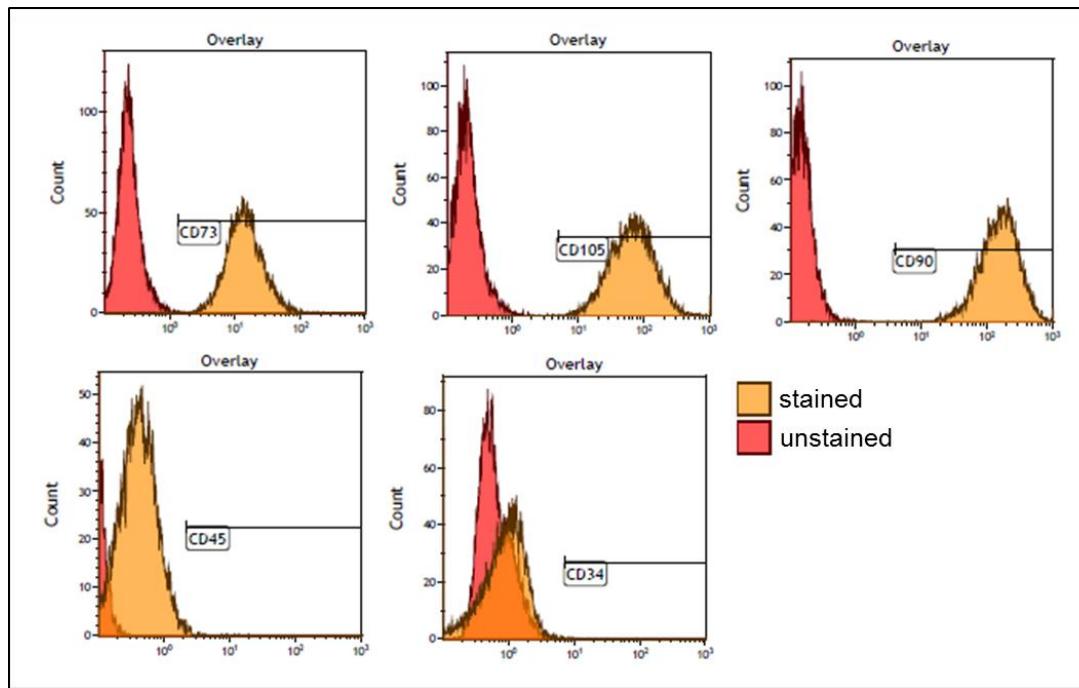


**Figure 19. Tree plot generated for determining the combined phenotypic profile of the MSC population.**

Flow cytometric analysis of sample A270611-02 at passage 3 showing an overall MSC phenotype. A total of 99.46% of the cell population expressed the MSC phenotype  $CD105+CD34-CD45-CD73+CD90+$ .

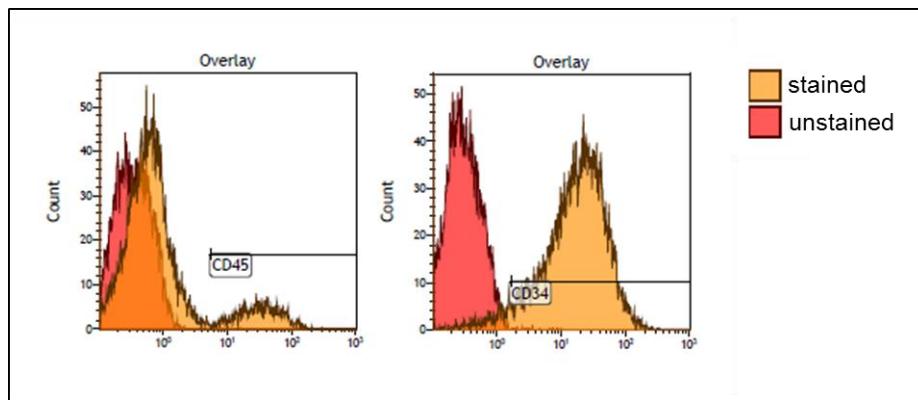
## 4.4 Results

The phenotypic profile of MSCs isolated from adipose tissue, UCB, and Wharton's jelly was determined over at least 10 passages. To better visualize the differences between unstained and stained samples, overlay plots were created for each antigen (CD marker). The data obtained from the stained sample was overlaid with the corresponding data obtained for the unstained sample. It is acknowledged that an unstained sample does not adequately control for non-specific staining. Therefore, results were carefully inspected by taking into account all available information, including the 2-parameter plots. Two-parameter plots show better resolution between positive and negative populations, thus distinguishing subtle staining differences from one another. Figure 20 shows an example of overlays created for sample A270611-02 at passage 3. It can clearly be seen that this sample is staining positive for the CD markers, CD73, CD105, and CD90, and staining negative for CD45 and CD34. The stained CD45 histogram is distinct from the unstained histogram. However, the shift still falls in the first decade, and inspection of the 2-parameter plots confirmed the CD45 peak to be negative. Figure 21 shows an example of CD45 and CD34 positive cell populations. Some low passage cultures did display CD45 expression. This was seen as a smaller second population implying weak expression. Strong CD34 expression (seen as a strong population shift into decade three to four) was mostly seen in low passage cultures, becoming weaker with increased passaging. It was shown that the phenotypic profile of fresh and thawed samples at low and high passages did not vary significantly; only small differences were found at the two decimal positions (Table 9). Thus, thawed and fresh samples at the same passage were used interchangeably. Table 10 summarizes which thawed samples were used.



**Figure 20. Overlays of 1-parameter plot histograms depicting the difference between the unstained and stained sample.**

The data for the A270611-02 P3 stained sample (orange histogram) was overlaid with the corresponding data obtained from the unstained sample (red histogram). The negative population is depicted in the first decade and the positive population is depicted in decades 2 to 4. The expression of CD73, CD105, CD90, CD45, and CD34 was determined.



**Figure 21. Overlays of 1-parameter plot histograms depicting positive CD45 and CD34 staining.**

Samples that were weakly CD45 positive showed a second distinct positive population in decades 2 to 4. Samples that were CD34 positive showed a positive population shift into decades 2 to 4.

**Table 9. Comparison of MSC marker expression between fresh and thawed samples**

Passage	MSC marker expression (%)	
	Fresh	Thawed
P3	97.41	97.83
P5	94.58	94.76
P9	95.77	95.89

**Table 10. Samples from frozen stocks**

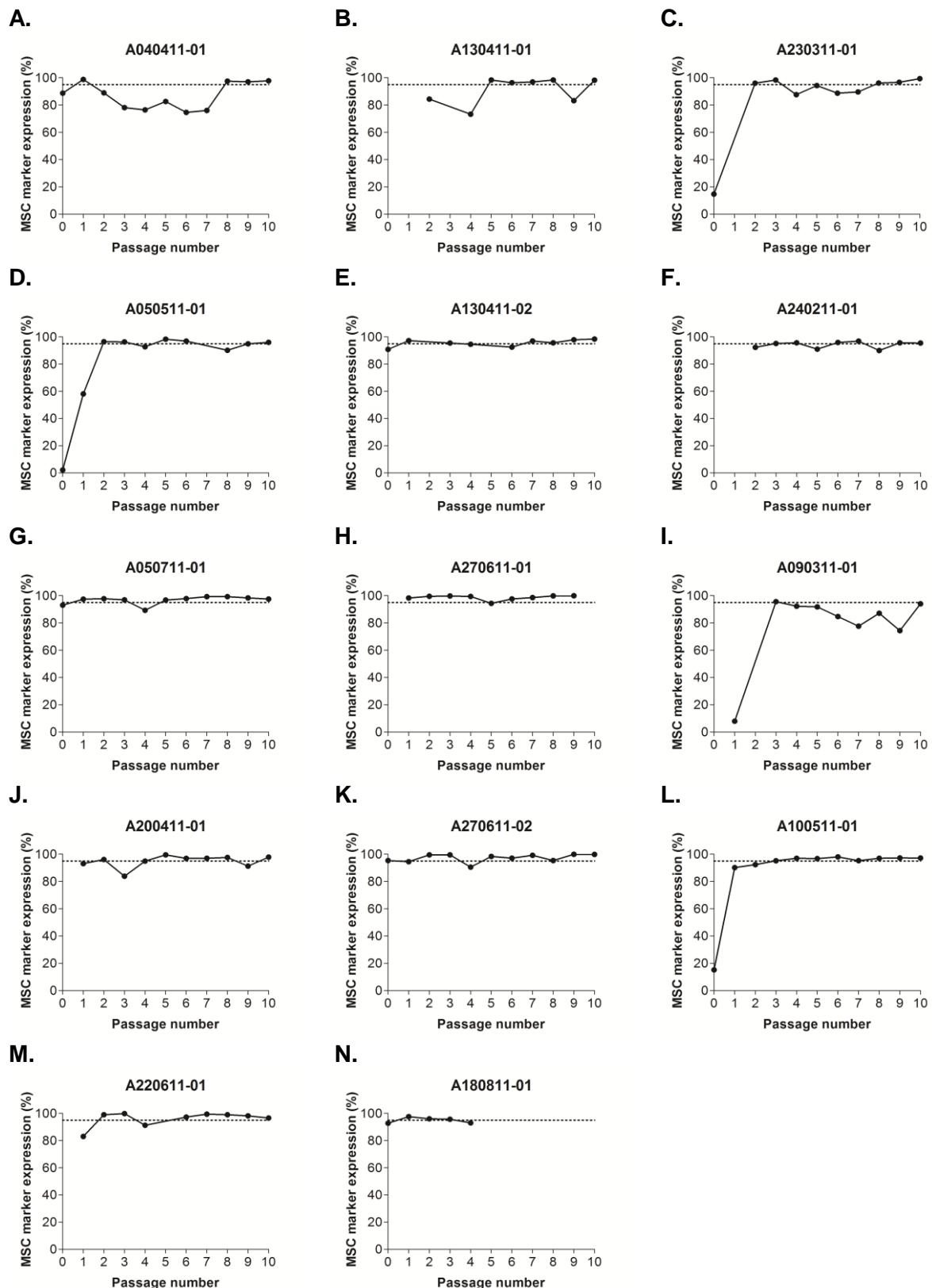
Sample	Passage
C210911-06 dig	P2 to P10
C210911-06 pieces	P2 to P10
C230911-01 dig	P3 to P10
C230911-01 pieces	P3 to P10
C190511-06 pieces	P6 to P10

#### 4.4.1 Phenotypic profile of adipose-derived stem cells

Fourteen adipose samples were processed to obtain adipose-derived stem cells (Figure 22). Their phenotypic profiles were determined over ten passages in an attempt to verify whether these stem cells could indeed be classified as MSCs. For a cell population to be classified as MSCs, they need to be positive ( $\geq 95\%$ ) for cell surface antigens CD73, CD90, and CD105, and negative ( $\leq 2\%$ ) for CD34 and CD45. Only the individual marker expression is usually reported in the literature. In this study the individual marker expression as well as the combined MSC marker expression profile (referred to as an MSC phenotype) was determined. An MSC marker profile of CD73+, CD90+, CD105+, CD34-, and CD45- in at least 95% (referred to as the 95% threshold) of the cell population was required for the cells to be classified as MSCs. In four adipose samples (A050511-01, A230311-01, A090311-01, and A100511-01) less than 20% of the cell population had an MSC phenotype at passage 0 (Figure 22 C, D, I, L). High CD34 and CD45 expression contributed to this phenotype (Figure 23 F, G). These cultures did acquire an MSC phenotype after passage 2 or 3. The other ten samples showed a relatively consistent phenotype in close proximity to the 95% threshold from passage 2 onwards. Samples A040411-01, A130411-01, and A090311-01 displayed more variability in their phenotypes across passages. Phenotypic characterization was not

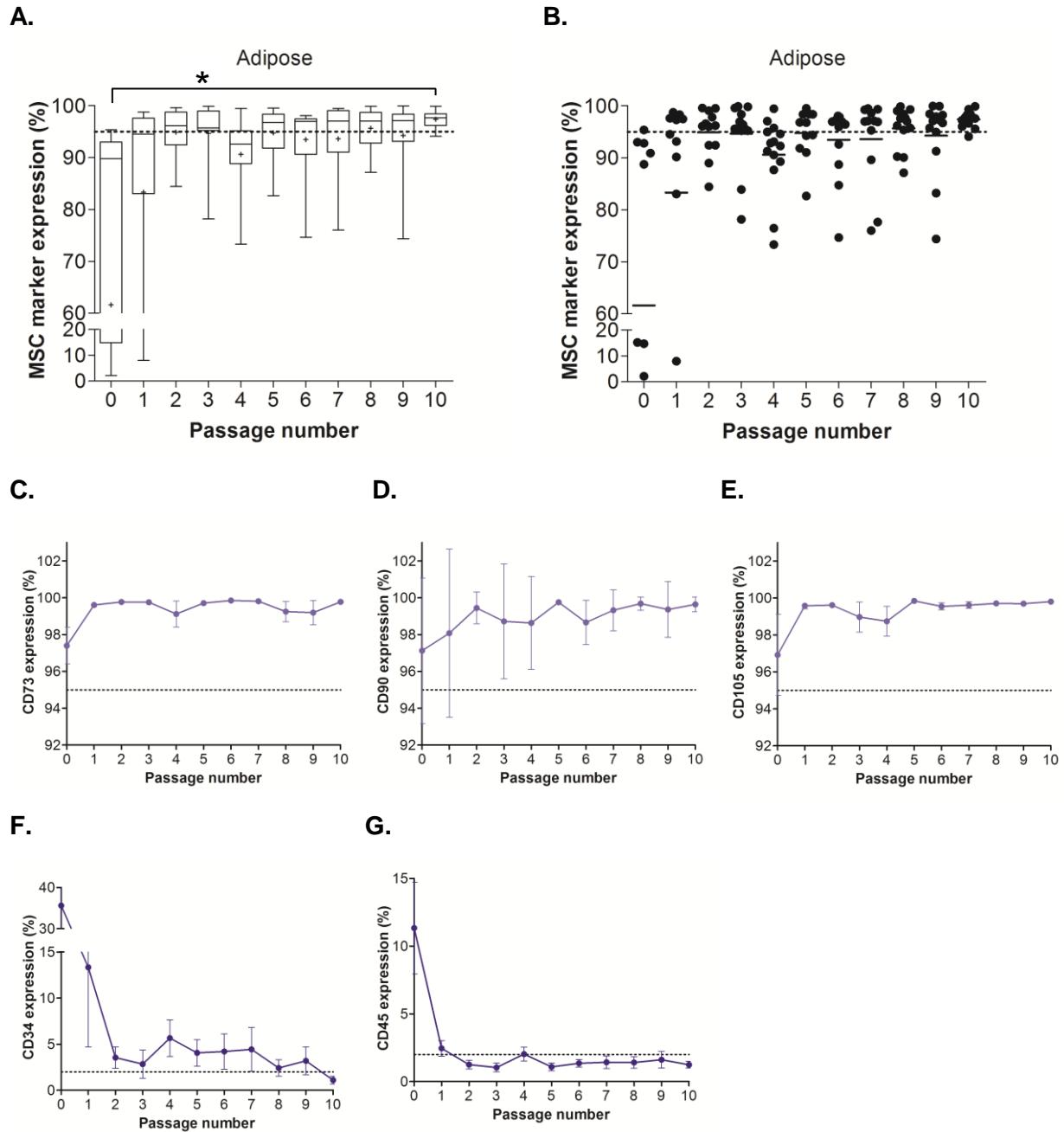
always possible at low passage numbers due to low cell yields. The cells in sample A180811-01 did not grow well and were only cultured until passage 4.

Descriptive statistics was used to summarize the distribution of MSC marker expression for all the adipose samples as a group at each passage (Figure 23). Distribution variability was found amongst the different passages. However a significant difference in distribution was only observed between passage 0 (most dispersed distribution) and passage 10 (most compacted distribution) (Figure 23 A). A scatter plot (Figure 23 B) showed that this difference was due to the samples being more dispersed along the y-axis for passage 0, whereas passage 10 showed clustering of samples in close proximity to each other. Very low MSC marker expression profiles, below 20% in a third of the samples, were found in early passages, namely passage 0 and 1, whereas from passage 2, the minimum values were above the 70% mark. Inter-patient variability resulted in long whiskers, especially below the 25<sup>th</sup> percentile. Mean values were much lower than the median values as a result of low MSC marker expression in a few samples pulling down the average marker expression profile of the group. Outliers were not excluded due to the small sample size. Across passages 1 to 10, two thirds of the samples clustered around the 95% threshold. Samples at passage 4 displayed a spread out distribution above and below the 95% threshold. The individual marker expression for CD73, CD90, and CD105 from passage 0 to passage 10 was above 95% (Figure 23 C-E). The individual markers followed a similar trend, lower expression at passage 0, and then seem to reach a plateau at about passage 1 to 2. Small standard deviations were found for CD73 and CD105, implying that these markers were consistently expressed at the same levels in the different samples. Samples showed greater variability in CD90 expression during culturing. Regarding CD34 expression, high expression was seen at low passages and decreased from passage 2 (Figure 23 F). However, across passages, adipose-derived stem cells maintained low levels of CD34 expression above the 2% threshold during culturing. The expression of CD45 was high in early passages, but was maintained at low levels below the 2% threshold from passage 2 onwards (Figure 23 G). Three samples were cultured beyond passage 10 (Figure 24). The MSC marker expression seemed to stabilize after passage 10 and maintained MSC phenotypes up to passage 20 (sample A200411-01) and passage 26 (sample A240211-01 and A040411-01).



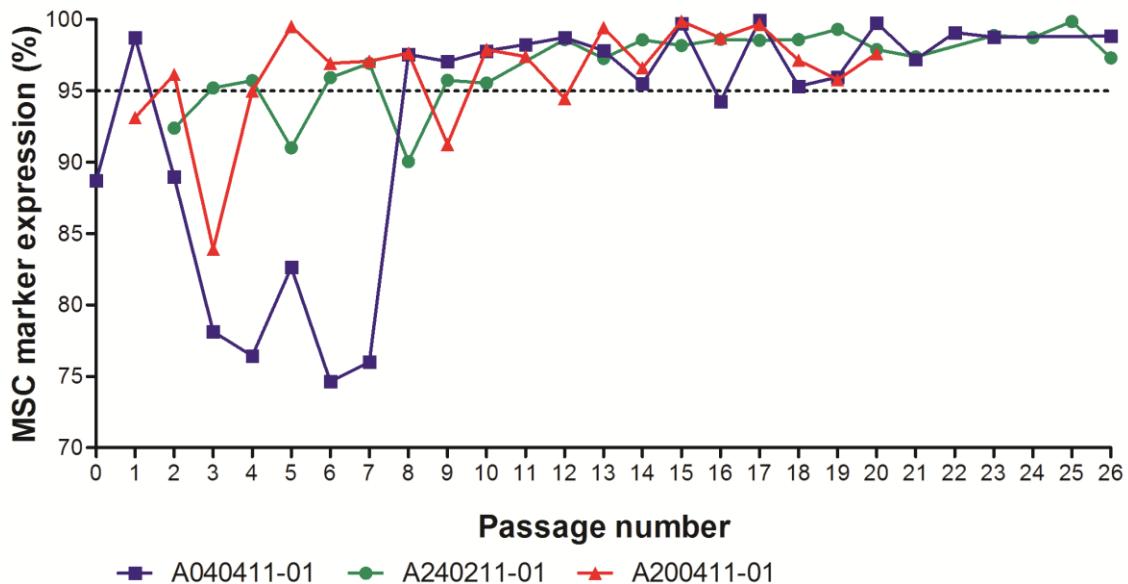
**Figure 22. The percentage of adipose-derived stem cells with an MSC phenotype.**

(A-N) Fourteen adipose samples were processed and phenotypic analysis performed over 10 passages. The dotted line indicates the 95% threshold.



**Figure 23. Phenotypic characterization of adipose-derived stem cells over 10 passages.**

(A) Box and whisker plot showing the percentage of the cell population expressing an MSC phenotype (CD73+, CD90+, CD105+, CD34-, and CD45-) for adipose-derived stem cells. The minimum, 25<sup>th</sup> percentile, median, 75<sup>th</sup> percentile, and maximum values are plotted. Mean values are shown as “+”. (B) Scatter plot showing sample distribution. Mean values are shown as a line. (C-E) Represents CD73, CD90, and CD105 marker expression, and (F-G) represents CD34 and CD45 marker expression. The dotted line indicates the 95% or 2% threshold.



**Figure 24. An MSC phenotype was maintained with extended culturing.**

Three adipose samples were cultured beyond passage 10. This was done to determine whether their phenotypic profile changed with extended culturing. The phenotype stabilized after passage 10 and remained above 95%, maintaining an MSC phenotype. The dotted line represents the 95% threshold.

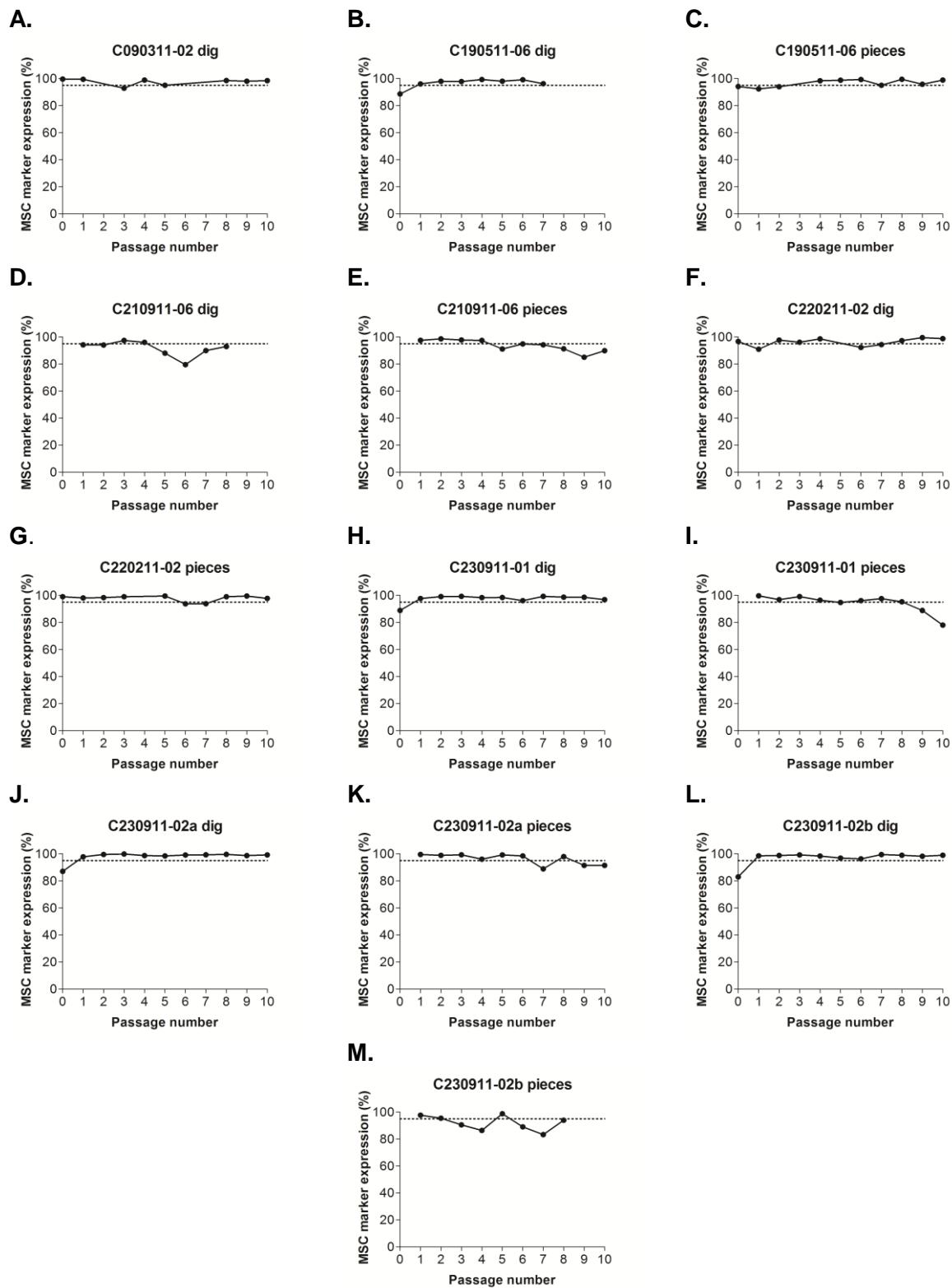
#### 4.4.2 Phenotypic profile of Wharton's jelly-derived stem cells

In an attempt to determine the best technique for isolating MSCs from Wharton's jelly, it was necessary to determine differences in phenotype between the cells from the different isolation procedures. Digested cords ( $n = 7$ ) and cord pieces ( $n = 6$ ) were kept separately as two different groups of samples. Seven cord samples were successfully cultured by enzymatic digestion and cord explant pieces. An MSC phenotype was attained by passage 1 or 2 (Figure 25). The samples showed a relatively consistent phenotype in close proximity to the 95% threshold from passage 1 onwards. Only two samples, C210911-06 dig and C230911-02b pieces, displayed variability in their phenotype across passages.

Descriptive statistics was used to summarize the distribution of MSC marker expression between the digested cords and cord pieces. Variability in marker expression was noticed between passages for the digested cords (Figure 26 A). Passage 0 for the digested cords showed the greatest variability in MSC marker expression between samples, and displayed a mean MSC phenotype below the 95%

threshold. An MSC phenotype was obtained in passages 1 to 5, and passages 7 to 10. Passage 6 had a mean MSC marker expression less than 95%. This was due to a single sample, C210911-06 dig, showing low marker expression pulling the mean down. Cells from this sample did not grow well and displayed larger cells with a more flattened morphology. Due to the low sample number, sample outliers were not considered and all values were included. The cord pieces (Figure 26 B) showed greater variability in distribution than the digested cords. Passage 0 for the cord pieces consisted of only two cords, which was too small a sample size from which to derive any significant conclusions. An MSC phenotype was observed in passages 1 to 6, and in passage 8. The samples at passage 7, 9, and 10 showed greater variability which pulled the mean values down to less than 95% MSC marker expression. The mean values for both groups are close to the median values, and the whiskers did not extend too far below the 25<sup>th</sup> percentile. Thus the cord groups showed less inter-patient variability. The individual marker expression for CD73, CD90, and CD105 from passage 0 to passage 10 was above 95% (Figure 26 C-E). These markers showed similar trends between passages for both groups from passage 0 to passage 8. From passage 8, the cord pieces displayed a slight trend downwards in marker expression, whereas the digested cords displayed a slight increase. All three markers showed a dip in marker expression at passage 7, suggesting the possibility of experimental error being responsible for this result. Error bars are compact, except from passage 7 where larger standard deviations were observed, especially in the cord pieces. Expression of the CD34 marker (Figure 26 F) was very low for both cord groups, with samples showing less than 2% CD34 positive staining. Great variability with large standard deviations was observed at passage 6 for the digested samples, leading to a mean CD34 expression greater than 2%. This expression was lost to almost negligible levels in passages 7 to 10. Expression of the CD45 marker (Figure 26 G) differed between digested cords and cord pieces. The digested samples showed approximately 6% CD45 expression at passage 0, but this expression was lost and stayed below 2% positive until passage 10. The pieces samples displayed greater variability in CD45 marker expression between passages with a large standard deviation from passage 3 to 10 above the 2% threshold.

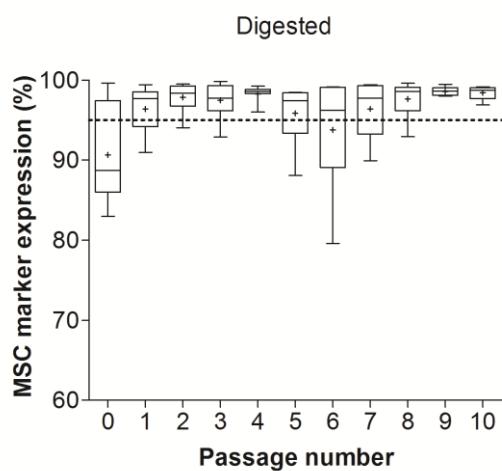
A single cord sample's explanted pieces were cultured for one week (labeled I, also referred to as the initial cord pieces) and then sequentially transferred to another flask to produce a new explant culture (labeled II). Five explant cultures were produced, labeled I to V. The phenotypic profile of these cord explants was determined for five passages, and Figure 27 show that these cord explants maintained an MSC phenotype. Cord explant C190511-06 I showed the greatest variability in its phenotype.



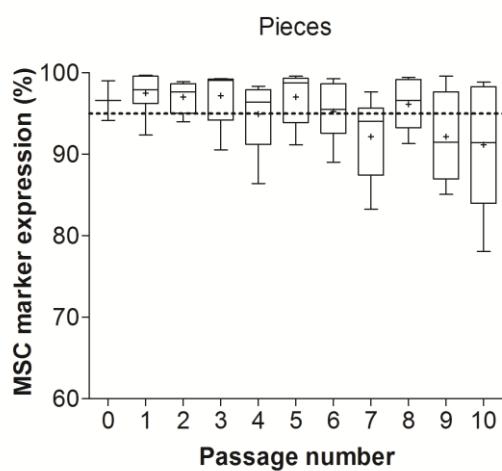
**Figure 25. The percentage of Wharton's jelly-derived stem cells with an MSC phenotype.**

(A-M) Thirteen cord samples were processed and phenotypic analysis was performed over 10 passages. The dotted line indicates the 95% threshold.

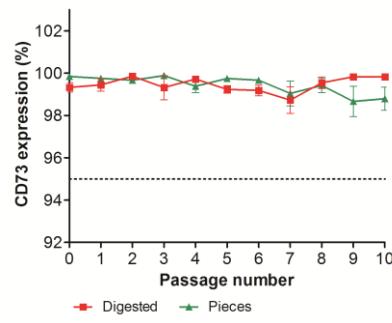
A.



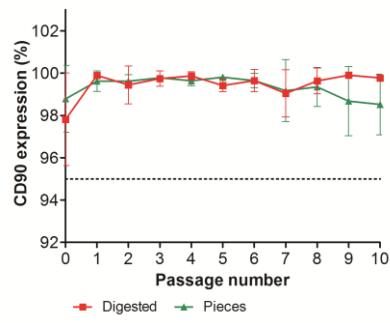
B.



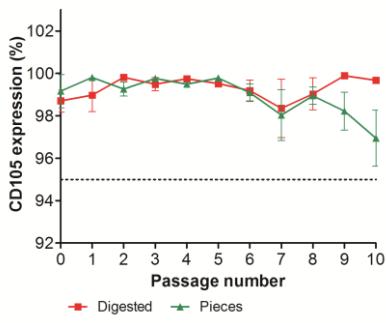
C.



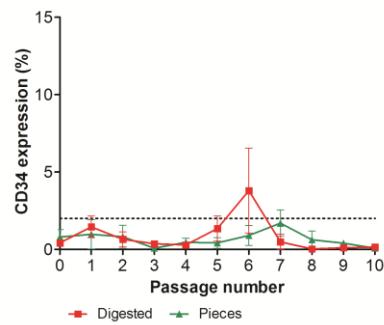
D.



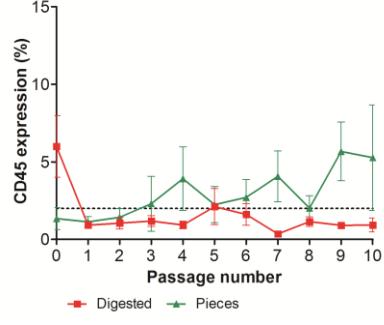
E.



F.

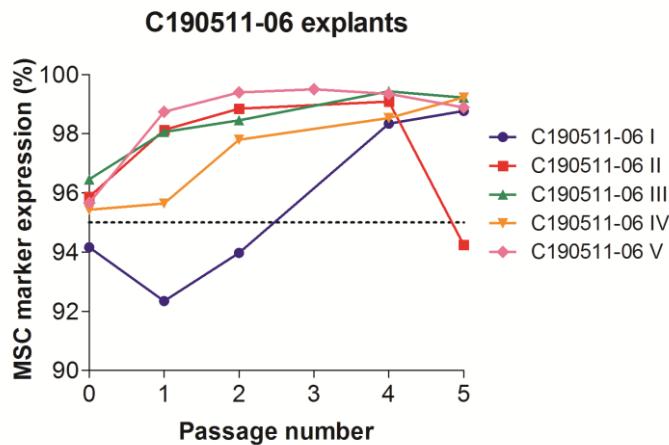


G.



**Figure 26. Phenotypic characterization of Wharton's jelly-derived stem cells over 10 passages.**

Box and whisker plot showing the percentage of the cell population expressing an MSC phenotype for (A) digested cords, and (B) cord pieces (CD73+, CD90+, CD105+, CD34-, and CD45-). The minimum, 25<sup>th</sup> percentile, median, 75<sup>th</sup> percentile, and maximum values are plotted. Mean values are shown as “+”. (C-G) Represents CD73, CD90, and CD105 marker expression, and (E-F) represents CD34 and CD45 marker expression for digested cords (red line) and cord pieces (green line). The dotted line indicates the 95% or 2% threshold.



**Figure 27. Umbilical cord explants continued to produce cells with an MSC phenotype.**

A single cord sample's explanted pieces were cultured for one week (labeled I) and then transferred to another flask to produce a new explant culture (labeled II). This process was repeated until five explant cultures were produced. The phenotype of each new explant culture was determined over at least 5 passages.

#### 4.4.3 Adipose- and Wharton's jelly-derived stem cells exhibit similar phenotypic profiles

Both adipose- and Wharton's jelly tissue were sources of adherent cells of fibroblastic morphology (refer to Chapter 1) expressing an MSC phenotype. Table 11 summarizes all of the descriptive statistics generated for the adipose and cord samples. Comparisons of median and distribution across categories of sample source showed that a statistically significant difference was only observed at passage 4. Inspection at passage 4 revealed that this significance was due to the digested cords displaying a very uniform marker expression profile compared to cord pieces and adipose samples. Adipose-derived stem cells showed greater variability in marker expression at early passages compared to the cords. High CD34 and CD45 marker expression was observed at passage 0 to 2. The ASCs seem to maintain CD34 expression above the 2% threshold during culturing, whereas the cords had low CD34 expression below the 2% threshold. The markers CD73, CD90, and CD105 were strongly expressed in the adipose and cord samples; CD90 expression was however variable in adipose samples between passages. Adipose samples showed greater variability in marker expression as observed by larger

standard deviations than for the cords, especially in early passages. The mean and median values for the cords were similar. Both cord groups had a less dispersed distribution than the adipose samples. The ASCs had a mean MSC marker expression below the 95% required to be classified as MSCs when the overall phenotype was considered. The digested cords, when compared to the cord pieces, better maintained their MSC phenotype. Less variability in marker expression was observed, especially with CD34 and CD45 expression in the digested cords.

**Table 11. Descriptive statistics for adipose- and Wharton's jelly-derived stem cells**

Passage	n	Min	25% Percentile	Median	75% Percentile	Max	Mean	SD
<b>ADIPOSE</b>								
0	8	2.14	14.81	89.81	92.96	95.35	61.6	42.4
1	11	7.95	83.03	94.52	97.59	98.75	83.3	27.65
2	12	84.4	92.39	96.12	98.74	99.57	94.91	4.609
3	13	78.14	95.21	95.71	98.97	99.87	94.64	6.403
4*	14	73.29	88.86	92.54	95.17	99.44	90.6	7.368
5	11	82.64	91.82	96.76	98.37	99.51	94.75	4.897
6	13	74.65	90.63	96.93	97.43	98.04	93.44	6.937
7	12	76.01	91.02	97.02	99.06	99.47	93.6	8.274
8	13	87.11	92.8	97.02	98.71	99.85	95.65	4.012
9	13	74.36	93.09	97.06	98.32	99.95	94.22	7.435
10	12	94.07	96.17	97.68	98.41	99.88	97.39	1.631
<b>Cord PIECES</b>								
0	2	94.16	94.16	96.59	99.02	99.02	96.59	3.437
1	6	92.34	96.26	97.89	99.57	99.67	97.48	2.678
2	6	93.97	95.07	97.63	98.66	98.88	97.02	1.984
3	5	90.52	94.18	99.08	99.2	99.27	97.17	3.76
4*	5	86.4	91.2	96.42	97.91	98.34	94.93	4.856
5	6	91.16	93.86	98.76	99.32	99.57	97.04	3.373
6	6	89.01	92.57	95.53	98.66	99.26	95.26	3.702
7	6	83.26	87.45	94.05	95.66	97.64	92.14	5.205
8	6	91.3	93.27	96.6	99.17	99.45	96.16	3.222
9	5	85.07	86.96	91.48	97.68	99.58	92.15	5.697
10	5	78.04	83.96	91.44	98.3	98.84	91.19	8.312
<b>DIGESTED cords</b>								
0	6	82.99	86	88.74	97.44	99.61	90.63	6.267
1	7	90.97	94.2	97.72	98.53	99.44	96.38	2.942
2	6	94.05	96.79	98.38	99.25	99.53	97.86	1.994
3	7	92.9	96.15	97.75	99.3	99.86	97.52	2.41
4*	7	96	98.35	98.59	98.85	99.27	98.3	1.063
5	6	88.08	93.33	97.46	98.45	98.47	95.83	4.011
6	6	79.58	89.11	96.22	99.12	99.16	93.76	7.389
7	6	89.93	93.24	97.75	99.34	99.44	96.42	3.8
8	6	92.93	96.19	98.61	99.1	99.62	97.66	2.441
9	5	98	98.11	98.62	99.09	99.5	98.6	0.5744
10	5	96.91	97.69	98.76	99.08	99.19	98.46	0.9063

\*Note: non-parametric Kruskal-Wallis pairwise comparison of median and distribution across categories of sample source showed significance (\* p < 0.05) at passage 4 between adipose, cord pieces, and digested cords

**Table 12. Phenotypic characterization of UCB-derived cells**

Sample	Passage	Percentage expression	Phenotype				
			CD73	CD90	CD105	CD34	CD45
B160211-01	P0	95.41%	-	-	-	+	+
B160311-01	P0	78.24%	-	-	-	+	+
		16.30%	+	-	-	+	+
		57.79%	-	-	+	+	+
B090311-02	P1	38.19%	+	-	+	+	+
		P0	98.91%	+	+	+	-
	P2	95.41%	+	+	+	-	-

#### 4.4.4 Phenotypic characterization of UCB-derived cells

Phenotypic characterization of UCB-derived cells was performed on at least passage 0 of three UCB samples (Table 12). As discussed in Chapter 1, only three UCB samples produced adherent cells after isolation. Both B160211-01 and B160311-01 showed strong CD34 and CD45 marker expression, but lacked CD90 expression. Expression of CD105 and CD73 was virtually absent at P0, and increased at P1. Sample B090311-02 was the only UCB sample that seemed to contain MSCs. Although this sample displayed an MSC phenotype, the cells grew slowly and stopped growing at passage 7 (see Chapter 1). Unfortunately, phenotypic characterization was only performed for passage 0 and 2 due to a full antibody panel not being available during the culturing of this sample, as well as too few cells having been harvested at each passage.

#### 4.5 Discussion

The phenotypic profiles of MSCs from three sources, namely, adipose tissue, UCB, and Wharton's jelly were compared. Mesenchymal stem cells from adipose tissue and Wharton's jelly expressed the classical MSC phenotype (CD73+, CD90+, CD105+, CD34-, and CD45-). Difficulty was experienced in isolating MSCs from UCB (described in Chapter 3), and only three UCB samples yielded adherent cells. These cells grew slowly and were terminated after passage 1, except for B090311-02 which survived culturing up to passage 7. Only B090311-02 expressed an MSC phenotype. This sample highlights the fact that the expression of the MSC phenotype does not necessarily imply the successful isolation of functional MSCs.

The unsuccessful UCB samples exhibited great variation in CD105 and CD73 expression. High expression of CD34 and CD45 implied that contaminating hematopoietic cells are likely to have constituted a significant part of the cell population.

There is a need for comprehensive analysis of the immunophenotype of expanded MSCs from different sources. One study has performed comprehensive phenotypic characterization of human ASCs. Donor-specific differences in marker expression and high variability in the expression of CD34 was observed (Baer *et al.*, 2013). Isolated ASCs expressed characteristic MSC markers, and also showed donor-specific variability in the expression of 49 other markers. It was also found that in primary ASCs, early passages did not uniformly express MSC markers. At passage 2 or 3 a morphologically homogenous population of fibroblastic cells expressing the MSC phenotype was observed. The same variability at early passages for ASCs was observed in this study (Figure 23). The ASCs expressed MSC markers in slightly less than 95% of the cell population during passaging (Figure 23 A). High marker expression was obtained in greater than 95% of the cell population for CD90, CD105, and CD73 individually (Figure 23 C-F); however, variability in CD34 and CD45 expression (Figure 23 G-H) influenced their overall phenotype. Strong CD45 expression was only found in early passages. Expression of CD34 on the other hand was highly expressed in early passages, and expression was reduced with culturing. Expression of CD34 was however increased above the accepted 2% threshold. This variability in CD34 expression has previously been noted in ASCs (Baer *et al.*, 2013, De Ugarte *et al.*, 2003, Gronthos *et al.*, 2001, Lin *et al.*, 2008). The variability in the phenotypic profile of ASCs has been reported to be due to donor-dependent differences (Baer *et al.*, 2013). Differences exist in the liposuction site, collection, isolation, and culturing techniques. Although this study kept the isolation procedure and culturing techniques standardized, the location of the lipoaspirate could not be controlled. The CD34 antigen is a hematopoietic stem cell-associated surface marker. The expression of CD34 in ASCs has been suggested to correlate with the ability of the stem cells to replicate and differentiate, and the loss of CD34 expression may be due to the commitment and/or differentiation status of the cells into specific lineages (Suga *et al.*, 2009).

Wharton's jelly-derived MSCs have been shown to share most of their immunophenotype with BM-MSCs (reviewed in Anzalone *et al.*, 2010) and ASCs (Christodoulou *et al.*, 2013). They express CD73, CD90, CD105, and HLA class I. These markers represent the MSC phenotype and are all expressed on BM-MSCs and ASCs. The presence of pluripotency stem cell markers (POU1F1, NANOG, SOX2, and LIN28) has been shown on WJ-MSCs. However, detailed DNA microarray analysis has shown that these markers were actually only present at very low levels (Fong *et al.*, 2011). Both isolation techniques used in this study produced MSCs from Wharton's jelly with an MSC phenotype (Figure 26). Higher MSC marker expression was obtained for the MSCs from Wharton's jelly as compared to adipose tissue. Regarding the MSC phenotype, no differences in phenotype were observed between digested cords and cord pieces. The cord pieces were however re-cultured weekly, and the MSCs that were derived therefrom maintained a stable phenotype in culture (Figure 27). Thus, the potential of the cord pieces to generate greater cell numbers from a single cord may be therapeutically beneficial, especially if high cell numbers are required for transplantation. Other studies have reported that phenotypically, MSCs from enzymatic digestion and cord pieces showed no difference in marker expression profiles (Azandeh, 2012, Tong *et al.*, 2011). However, a study by Salehinejad *et al.* (2012) showed that although the MSC phenotype was observed in all WJ-MSCs regardless of the isolation technique used, the levels of expression differed. The different isolation techniques also resulted in differences in cell number and proliferation capacity of isolated cells (Salehinejad *et al.*, 2012).

In summary, MSCs from Wharton's jelly did seem to show a more uniform MSC profile over passages, higher levels of marker expression when compared to ASCs. A lot more variability in phenotype was observed in early ASC passages, whereas WJ-MSCs seemed to attain the MSC phenotype as early as passage 0. Further studies are therefore required to confirm whether the intensity of marker expression differs in MSCs from these sources. Some limitations to this study include the small sample size in the cord samples and the experimental procedures used. For instance, to be able to make definite comparisons between samples and passages, a more standardized phenotypic analysis is required. Due to fluorochrome availability constraints, the fluorochrome antibody combinations varied during the study and

may have had an influence on the observed results. The differences between sources of MSCs should be extended to studies of the transcriptome to identify any similarities and/or differences. Another concern is that the current standard MSC phenotype, CD73+, CD90+, CD105+, CD34-, and CD45-, may not be adequate to isolate a pure population of MSCs.

## Chapter 5: Differentiation into multiple lineages of mesenchymal origin

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### 5.1 Introduction

Mesenchymal stem cells are a heterogeneous population of non-hematopoietic adult stem cells present in nearly all post-natal organs and tissues (da Silva Meirelles *et al.*, 2006). They have the ability to self-renew and exhibit multi-lineage differentiation potential into mesenchymal tissues (Pittenger *et al.*, 1999). The minimum criteria defined by ISCT state that MSCs must have the ability to differentiate into adipose, bone, and cartilage *in vitro*. However, they have also been shown to differentiate into other mesenchymal tissues such as muscle (Beier *et al.*, 2011). Stem cell plasticity describes the ability of these cells to form tissues from different germ layers. Under appropriate culture conditions, MSCs have plasticity by differentiating into non-mesodermal tissues such as neurons (Ning *et al.*, 2006) and epithelial cells (Brzoska *et al.*, 2005). The differentiation capacity of MSCs suggests a role in the turnover and maintenance of adult mesenchymal tissues (Caplan, 2009). Effective clinical application of MSCs will rely on understanding the biological effectors that are responsible for maintaining a specific differentiation state. Also, the ability to modulate differentiation into specific lineages would prevent spurious differentiation.

Adipose tissue consists, amongst other things, of adipocytes, vascular endothelial cells, fibroblasts, and macrophages. Adipocytes can be found in both brown and white fat. Brown fat expends energy and is mitochondria rich, while white fat mainly stores energy and is lipid rich (Cook and Cowan, 2008). Mesenchymal stem cells originate from the embryonic mesoderm and neurectoderm, and are believed to be the precursor cells for adipocytes. High calorie intake without energy expenditure promotes adipocyte hyperplasia. Signaling factors are responsible for this rise in adipocytes by inducing the conversion of MSCs to preadipocytes and finally differentiation into mature adipocytes (Tang and Lane, 2012). Mesenchymal stem cells become committed to preadipocytes by bone morphogenetic protein (BMP) -2 and -4 (Huang *et al.*, 2009) and Wnt signaling (Bowers and Lane, 2008), causing them to go into growth arrest. Differentiation inducers such as hormones and mitogens (insulin, glucocorticoids and cAMP inducers) then initiate mitotic clonal

expansion where the cells' fibroblastic morphology is lost and the accumulation of cytoplasmic triglycerides occurs, eventually forming mature adipocytes (Tang *et al.*, 2004). Peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) along with CAAT/enhancer binding proteins (CEBP) are key regulators of adipogenesis (Kang *et al.*, 2007).

The physical consequences of aging to the skin, specifically to the face, have attracted a lot of attention in the past decade. Age-related facial lipoatrophy has a minimal adverse impact on the health of the individual, thus it is not considered to be a target for scientific research or medical treatment. Yet in today's society, an entire industry has developed to improve the outward appearance of individuals. A problem with using adipose tissue for soft-tissue fillers and breast augmentation is maintaining long-term survival of transplanted autologous fatty tissue. Attention is being focused on enhancing autologous fat grafting through the potential utilization of adipose derived-stem cells to improve graft survival.

The relationship between obesity and bone is complex. Both adipocytes and osteoblasts are derived from a common multipotent MSC, thus leading one to believe that the two pathways are interconnected (refer to Figure 28). Obesity increases adipocyte differentiation and fat accumulation, and is believed to decrease osteoblast differentiation and bone formation. Cao (2011) reviewed the effects of obesity on bone metabolism. This review concluded that an increase in pro-inflammatory cytokines due to obesity may promote osteoclast activity and bone resorption.

Bone is composed of cells and ECM which becomes mineralized by deposition of calcium hydroxylapatite. It consists of three cell types, osteoblasts, osteoclasts, and osteocytes. Osteoblasts are bone-forming cells responsible for synthesizing the organic components of the bone matrix. During matrix synthesis they become cuboidal to columnar in shape, and become flattened again once activity declines. Osteocytes are osteoblasts that become entrapped within lacunae (small cavity within the bone matrix) (Caetano-Lopes *et al.*, 2007). Osteoclasts are very large, multinucleated motile cells responsible for bone resorption. Bone homeostasis is maintained by balancing bone formation and resorption. Osteoblasts regulate bone

resorption through the receptor activator of nuclear factor- $\kappa$ B (RANK) ligand (RANKL) which links to its receptor, RANK, found on the surface of preosteoclast cells, stimulating the differentiation to osteoclasts in the presence of macrophage colony stimulation factor (M-CSF). However, they also secrete a soluble decoy receptor (osteoprotegerin, OPG) that blocks the RANK/RANKL interaction by binding to RANKL which prevents osteoclast differentiation (Caetano-Lopes *et al.*, 2007). The runt-related transcription factor 2 (RUNX2) acts as a differentiation factor for the osteoblast lineage (Lian *et al.*, 2004), resulting in bone formation.

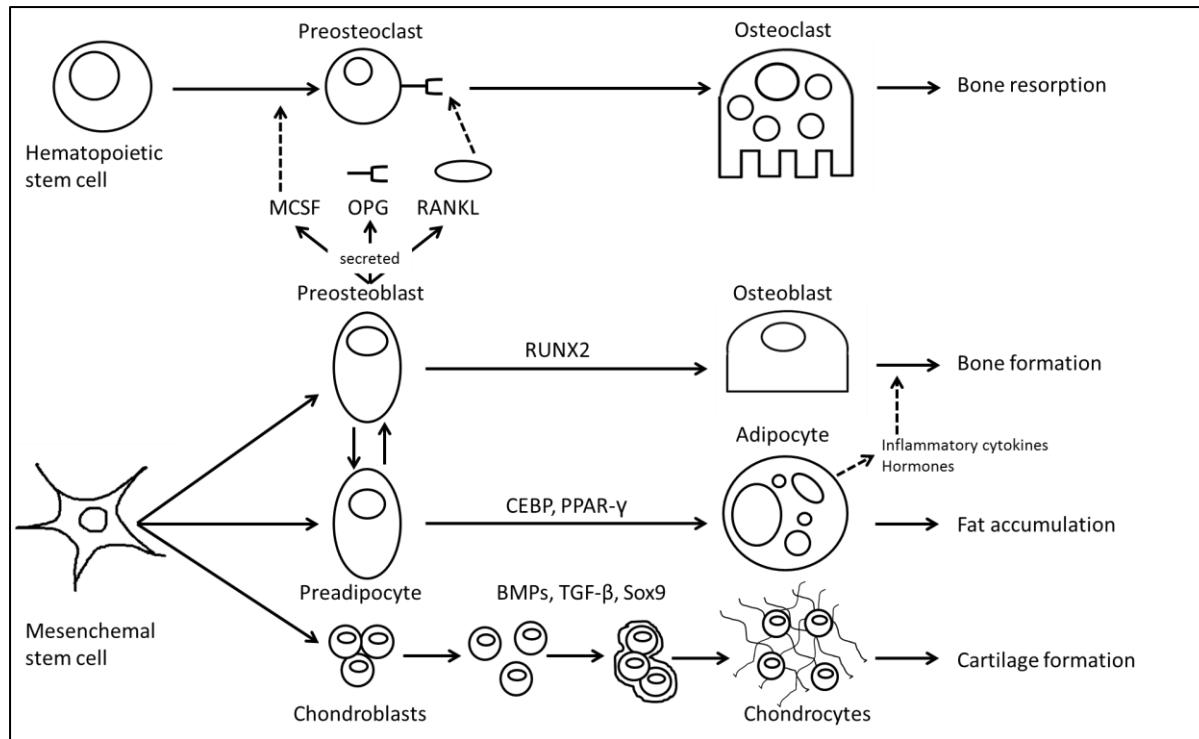
Large bone defects caused by extensive injury, congenital malformations or disease require reconstruction with tissue grafting. Tissue engineered bone grafts have the potential to alleviate the demand for suitable autograft (self) and allograft (non-self) materials for augmenting bone healing (Marolt *et al.*, 2010). Osteoporosis is a systemic disease due to an imbalance in bone homeostasis (Benisch *et al.*, 2012). It is characterized by a reduction in bone quantity and quality leading to an increase in skeletal fragility. In South Africa, The National Osteoporosis Foundation (<http://www.osteoporosis.org.za>) believes that 4-6 million South Africans have the potential to develop the disease. Understanding the mechanism of how MSCs differentiate into bone could provide insight into effectively treating bone defects as well as fractures related to bone disease.

Cartilage is a specialized connective tissue which consists of chondrocytes surrounded by an ECM. Chondrocytes produce and maintain an ECM which is rich in glycosaminoglycans and proteoglycans, which are responsible for the interaction with collagen and elastic fibers. The cartilage matrix is composed mainly of Type II collagen, providing tensile strength, and aggrecan, which provides compressive strength. Cartilage is classified according to its composition. Hyaline cartilage consists mainly of Type II collagen fibers, elastic cartilage of Type II collagen and elastic fibers, and fibrocartilage consists of Type I collagen fibers (Naumann *et al.*, 2002).

Chondrogenesis involves the recruitment and condensation of MSCs followed by differentiation into chondrocytes, chondrocyte maturation, and ECM production. Mesenchymal stem cells undergo mitotic proliferation forming condensations of

rounded cells, known as cellular condensation, and undergo chondrogenesis following exposure to BMPs, TGF- $\beta$  and Sox9 (Keller *et al.*, 2011). The chondroblasts start to separate from each other by the production of matrix components to form the ECM. As the cells start to multiply, they form isogenous aggregates, each surrounded by a condensation of territorial matrix (Zuscik *et al.*, 2008).

Due to its avascular and aneural properties and its small volume of resident chondrocytes, cartilage displays little intrinsic repair capacity in response to injury or disease (Estes *et al.*, 2010). Problems associated with cartilage repair include but are not limited to the need for an abundant and easily accessible source of cells, the harvesting of autologous cartilage tissue, the disease state of harvested cells, and the availability of autologous tissue. To avoid the issues associated with autologous grafts and/or cells in cartilage repair strategies, researchers are investigating the promising potential of adult stem cells (Estes *et al.*, 2010).



**Figure 28. Regulation of mesenchymal stem cell differentiation.**

Fat accumulation, cartilage formation, bone formation, and bone resorption are closely related. Adipocytes, osteoblasts, and chondrocytes have a common origin, the mesenchymal stem cell, while osteoclasts originate from the hematopoietic stem cell. Osteoblasts regulate bone resorption through the RANKL/RANK/OPG pathway. Osteoblast differentiation is regulated by RUNX2. Key regulators of adipogenesis are PPAR- $\gamma$  and CEBP. Adipocytes secrete inflammatory cytokines and hormones which are capable of modulating bone formation. Chondrogenesis is regulated mainly by BMPs, TGF- $\beta$  and Sox9. Figure prepared by Karlien Kallmeyer. Modified from Figure 1 in Cao (2011), and Rosen and Bouxsein (2006). RUNX2, runt-related transcription factor 2; RANK/RANKL, receptor activator of nuclear factor- $\kappa$ B ligand; OPG, osteoprotegerin; PPAR- $\gamma$ , Peroxisome proliferator-activated receptor- $\gamma$ ; CEBP, CAAT/enhancer binding proteins; BMP, bone morphogenetic protein; TGF, transforming growth factor; Sox, sex-determining region Y-type high motility group box.

In this chapter, the *in vitro* differentiation potential of MSCs isolated from two sources namely adipose tissue and umbilical cord, was investigated. Furthermore this study focused on using chemical agents and biological growth factors to induce MSC differentiation rather than genetic engineering approaches, such as looking at gene expression of specific markers related to a specific differentiation lineage. The aims were (1) to determine whether both sources of MSCs are able to differentiate into

adipocytes, osteoblasts, and chondrocytes; and (2) to assess which source of adult stem cells has a greater differentiation capacity.

## 5.2 Materials

### 5.2.1 Primary cell cultures

Mesenchymal stem cells isolated from adipose tissue or Wharton's jelly (from umbilical cord) as described in Chapter 3 was used to determine differentiation capacity. Each sample's phenotypic profile was determined prior to being induced to differentiate as described in Chapter 4. Umbilical cord samples were processed either by enzymatic digestion, referred to as "digested" cultures, or by using explants of cord tissue, referred to as "pieces" cultures. Together the digested and pieces samples make up the cord sample group.

### 5.2.2 Media, supplements, reagents and plasticware

Dulbecco's Modified Eagle's Medium (DMEM 1X + GlutaMAX™), MEM Alpha Medium ( $\alpha$ -MEM, 1X + GlutaMAX™), penicillin-streptomycin, insulin (human recombinant Zinc), and trypsin-EDTA 1X were obtained from GIBCO by Life Technologies™, Grand Island, NY, USA. Phosphate Buffered Saline 1X (PBS, pH 7.4), Fetal Bovine Serum (FBS), and recombinant human Transformed Growth Factor- $\beta$ 3 (TGF- $\beta$ 3), was obtained from GIBCO by Invitrogen™, Grand Island, NY, USA. Dexamethasone, ascorbate-2-phosphate, indomethacin,  $\beta$ -glycerophosphate, cetylpyridinium chloride, 3-isobutyl-1-methylxanthine, formaldehyde, Oil red O (ORO), Alizarin red S (ARS), Toluidine blue O (TBO), and 4',6-diamidino-2-phenylindole (DAPI) were from Sigma-Aldrich Chemie, Steinheim, Germany. L-proline, pyruvic acid sodium salt (pyruvate), absolute ethanol, and 1.5 ml and 2 ml eppendorfs were from Merck, Darmstadt, Germany. Culture flasks, 25 cm<sup>2</sup> and 80 cm<sup>2</sup> vented filter cap, 6-well plates and 96-well untreated plates (without lid) were from NUNC™, Roskilde site, Kamstrupvej, Denmark. Falcon™ cell scrapers, and ITS™ premix universal culture supplement were from BD Biosciences, Bedford, MA, USA. Centrifuge tubes, 50 ml and 15 ml were from Corning, NY, USA. Serological pipettes, 5 ml, 10 ml, and 25 ml were from Greiner bio-one by Lasec, South Africa. QIAmp® DNA FFPE tissue kit was from QIAGEN, Hilden. Isopropanol was from Unilab, Mumbai, India. LR white medium grade acrylic resin was from SPI supplies,

West Chester, USA. Seakem™ LE Agarose was from Lonza, Rockland, ME, USA. GelRed nucleic acid stain was from San Francisco Bay area, US. TBE 10X buffer was from Promega Corporation, Madison, USA. Whatman filter paper was from Whatman International, Maidstone, USA.

### 5.2.3 Equipment

The following equipment was used: Thermo Forma CO<sub>2</sub> water jacketed incubator (3111TF), ESCO Class II biological safety cabinet (AC2-4Si), and Thermo Scientific centrifuge (SL16R) from Lasec, South Africa; Fluorescence microscope (Zeiss Axiovert 200), and Zeiss Axiocam digital camera from Carl Zeiss Werke, Göttingen, Germany; Ultramicrotome (Reichert Ultracut E) from Vienna, Austria; Nanodrop (ND-100); Power Wave X Spectrophotometer from Bio-tek instruments, incorporated; Accublock digital dry bath from Labnet; and a Series 2000 Scientific oven.

## 5.3 Methods

### 5.3.1 Differentiation into lineages of mesenchymal origin

Cells that displayed the MSC phenotype were induced to differentiate along three lineages of mesenchymal origin (adipocytes, osteoblasts, and chondrocytes) using specific induction media (refer to Table 13). Mesenchymal stem cell cultures were trypsinized, counted, phenotypically characterized (refer to Chapter 4) and re-plated into 6-well plates at 5 000 cells per cm<sup>2</sup> (48 000 cells per well or 288 000 cells per plate). Differentiation was monitored using histologic stains specific for each lineage (refer to Table 14). Appendix D is a comprehensive list of the induction media used in literature. The most commonly used media was employed for this study.

**Table 13. Lineage-specific differentiation induced by media supplementation**

Medium	Media	Serum	Supplementation
Control (C)	DMEM	10% (v/v) FBS	1% (v/v) p/s
Adipogenic (A)	DMEM	10% (v/v) FBS	1 µM dexamethasone, 0.5 mM 3-isobutyl-methylxanthine, 200 µM indomethacin, 10 µg/ml insulin, 1% (v/v) p/s
Osteogenic (B)	DMEM	10% (v/v) FBS	0.1 µM dexamethasone, 50 µM ascorbate-2-phosphate, 10 mM β-glycerophosphate, 1% (v/v) p/s
Chondrogenic (Cart)	DMEM	none	0.1 µM dexamethasone, 50 µg/ml ascorbate-2-phosphate, 10 ng/ml TGF-β3, 40 µg/ml proline, 100 µg/ml pyruvate, 1% (v/v) ITS premix

**Table 14. Differentiation markers and assays of lineage-specific differentiation**

Lineage	Lineage-specific determinant	Histologic assay
Adipose	Lipid accumulation	Oil red O stain
Bone	Mineralization	Alizarin red-S stain
Cartilage	Pellet culture system	Toluidine blue-O stain

### 5.3.1.1 Adipogenic differentiation

Once the cells were 70 to 80% confluent, they were induced to differentiate. Briefly, the complete growth medium was replaced with adipogenic-inducing medium and maintained for 21 days with medium top-up and medium changes every second and fourth day respectively. After 21 days of differentiation, the cells were fixed using 4% (v/v) formaldehyde solution (60 min) and stored at 4°C in PBS until use.

The accumulation of lipid droplets was detected by staining the cells with a 0.3 g/100 ml ORO working solution. Briefly, the fixed cultures were washed once with 1 ml PBS (pH 4.2) prior to staining. Each well was stained for 10 min at RT with 1 ml ORO working solution. Both the adipose differentiated wells and the control wells were stained. The ORO stain was removed and cells extensively washed with distilled water to remove any excess unincorporated dye. Cells were visualized by counter staining with 1 ml 0.01% (w/v) TBO (0.01% (w/v) Na<sub>2</sub>CO<sub>3</sub>) for 5 min, followed by three washes with distilled water to remove excess unincorporated dye. Images

were acquired using a fluorescence microscope. A total of five images were captured per well using a 10x magnification objective lens.

The ORO working solution was prepared by adding 6 ml ORO stock solution to 4 ml distilled water to obtain a 0.3 g/100 ml ORO solution. The ORO stock solution was prepared by dissolving 0.5 g of ORO in absolute isopropanol and stirring overnight at RT. The solution was filtered through 2 layers of Whatman filter paper to obtain a 0.5 g/100 ml ORO stock solution.

Differentiation capacity was quantified using (1) a colorimetric assay (quantifying the amount of ORO dye) and (2) Image J imaging software. Refer to section 5.3.2 for details.

#### 5.3.1.2 Osteogenic differentiation

Once the MSCs were 80 to 90% confluent, they were induced to differentiate. Briefly, complete growth medium was replaced with osteogenic-inducing medium and maintained for 21 days with medium top-up and medium changes every second and fourth day respectively. After 21 days of differentiation, the cells were fixed using 4%(v/v) formaldehyde solution (60 min) and stored at 4°C in PBS until use.

Mineralization was detected by staining the cells with 2 g/100 ml ARS (pH 4.1). Briefly, the fixed cells were washed once with 1 ml PBS (pH 4.2) for 5 min. Each well was stained for 10 min at RT with 1 ml ARS. Both the bone differentiated wells and the control wells were stained. The stain was removed and the cells extensively washed with distilled water to remove any excess unincorporated dye. Images were acquired using a fluorescence microscope. A single image was captured per well using a 10x magnification objective lens.

Differentiation capacity was quantified using a colorimetric assay (quantifying the amount of ARS dye). Refer to section 5.3.2 for details. Bone differentiated cells lay down a calcium matrix making it difficult to quantify differentiation by using imaging software. Yes/No answers for differentiation were concluded from the images taken.

### 5.3.1.3 Chondrogenic differentiation

For chondrogenic differentiation, the ‘pellet culture system’ was used with minor modifications (Sekiya *et al.*, 2001). Cell pellets (approximately  $2.5 - 5 \times 10^5$  cells) were cultured in chondrogenic differentiation medium for 21 days. Briefly, a single 25 cm<sup>2</sup> flask containing MSCs (80% confluent) was trypsinized, transferred into a 15 ml tube and centrifuged at 184 x g for 5 min. The pellet was re-suspended in 1 ml chondrogenic medium and centrifuged at 400 x g for 10 min. The undisturbed pellet was kept in the incubator for 21 days (with a loosened cap). The medium was replaced every two days. After 21 days the pellets were fixed in 4% (v/v) formaldehyde and stored in PBS until use. Pellets were removed from the PBS and serially dehydrated (for 15 min per ethanol change) in 30%, 50%, 70%, and 90% ethanol, followed by three changes of absolute ethanol. The sample was then infiltrated with a 50% (v/v) LR White Resin in absolute ethanol solution for one hour, followed by infiltration with 100% LR White Resin for a minimum of four hours. Thereafter, each sample was embedded in 100% LR White Resin. Polymerization of the resin was achieved by thermal curing. Curing occurred at 60°C for 24 hrs. Sections of 1 µm were prepared using an ultramicrotome with a glass knife. Sections were collected onto droplets of water on glass slides and dried on a slide warmer, stained with 1% (w/v) TBO (1% (w/v) Na<sub>2</sub>CO<sub>3</sub>) and images captured with a fluorescence microscope using a 10x magnification objective lens. Yes/No answers for differentiation were concluded from the images captured.

### 5.3.2 Quantification of adipose and bone conversion

Quantitative analysis experiments of MSC differentiation were performed in triplicate using a colorimetric assay and the values normalized to the relative cell number or to the DNA content from the same culture dish. Imaging software was also used to quantify the amount of lipid droplets within the adipose differentiated cultures.

#### 5.3.2.1 Colorimetric assay: for adipose

In order to determine the level of adipose differentiation, the method by Ramírez-Zacarías *et al.*, (1992) was used with minor modifications. Briefly, 1 ml of absolute isopropanol was added to the stained culture dish and incubated for 10 min with gentle shaking at RT. The isopropanol dye solution was pipetted up and down several times to ensure that all the dye was in solution and was then transferred to a

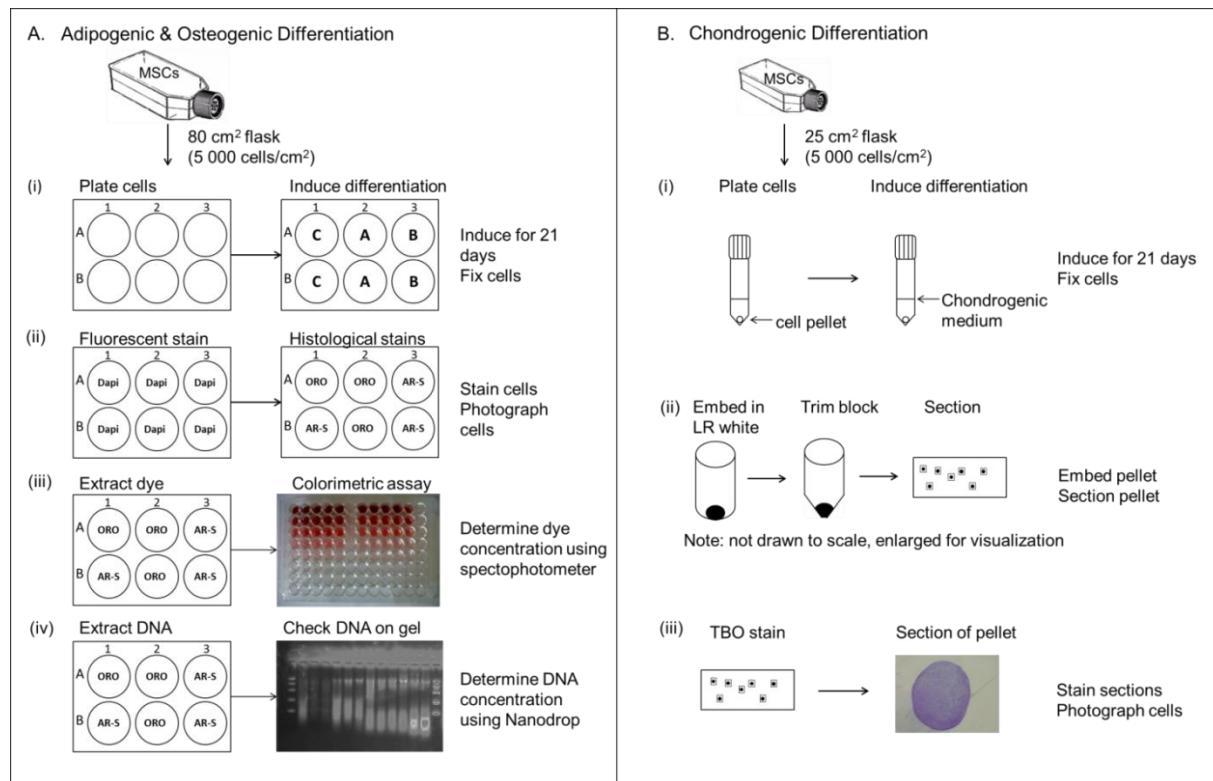
2 ml tube. A further 1 ml of isopropanol was added and the steps repeated. The solutions were pooled and their absorbance measured spectrophotometrically at 359 nm. For adipogenic differentiation, the amount of ORO dye extracted was determined from an ORO standard titration curve.

The ORO standard titration curve was prepared using serial dilutions of the ORO stock solution. Absorbance of each dilution was determined spectrophotometrically at 359 nm and absorbance versus dye concentration plotted. The following dye concentrations were used: 0.5, 0.3, 0.15, 0.075, 0.0375, 0.01875, 0.009375, 0.0046875, 0.00234375, 0.001171875, and 0 g/100 ml.

### 5.3.2.2 Colorimetric assay: for bone

In order to determine the level of bone differentiation, the method of Gregory *et al.*, (2004) was used with minor modifications. Briefly, 1 ml of 10% cetylpyridinium chloride ( $C_{21}H_{38}ClN \cdot H_2O$ , also called hexadecylpyridinium chloride/ CPC) in PBS (without  $Ca^{2+}$ ,  $Mg^{2+}$ ) was added to the stained culture dish ( $9.6\text{ cm}^2$ ) and incubated overnight at RT. The CPC dye solution was pipetted up and down several times to ensure that all the dye was in solution, and then transferred to a 2 ml tube. The absorbance of the solution was measured spectrophotometrically at 562 nm. Osteogenic differentiation was quantified by measuring the intensity of the stained calcium deposition using an ARS standard titration curve.

The ARS standard titration curve was prepared using serial dilutions of the ARS stock solution (2 g/100 ml ARS solution). Absorbance of each dilution was determined spectrophotometrically at 562 nm and absorbance versus dye concentration plotted. The following dye concentrations were used: 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625, 0.0078125, 0.00390625, and 0 g/100 ml.



**Figure 29. Differentiation of Mesenchymal stem cells (MSCs) into three lineages of mesenchymal origin: post-differentiation procedure.**

(A) MSCs were induced to differentiate into the adipogenic and osteogenic lineage. (i) Duplicate wells for each treatment were prepared (C = control, A = adipose induced, B = bone induced), induced for 21 days, and fixed with 4% (v/v) formaldehyde for 60 min. (ii) For the total cell count in each field, unstained cells were incubated with DAPI, and imaged before staining with either oil red O (ORO) or alizarin red S (ARS) for adipose and bone differentiated cultures. Five photos for the adipose-differentiated cultures and a single photo for the bone-differentiated cultures were taken. (iii) Differentiation capacity was quantified using a colorimetric assay (quantifying the amount of ORO and ARS dye). (iv) Finally DNA content (ng/ $\mu\text{l}$ ) was determined per well. (B) MSCs were induced to differentiate into the chondrogenic lineage. (i) MSCs were induced for 21 days, a single pellet per culture, and fixed with 4% formaldehyde for 60 min. (ii) The samples were embedded in LR white resin and sectioned using an ultramicrotome. (iii) The sections were stained with 1% (w/v) toluidine blue O (1% (w/v)  $\text{Na}_2\text{CO}_3$ ) and imaged. Qualitative and quantitative analysis was performed. Figure prepared by Karlien Kallmeyer.

### 5.3.2.3 Determining cell number

The cell number per well was determined by two methods: (1) total DNA content per well, and (2) the cell number by DAPI staining.

#### 5.3.2.3.1 DNA content

A QIAamp® DNA FFPE tissue kit was used for extracting DNA from fixed adherent cells. Briefly, the fixed adherent cells were scraped off the plastic surface using a cell scraper after adding the lysing buffer (supplied with the kit). All sample wells were visually checked for intact monolayers before proceeding with the DNA extraction. The DNA was extracted as per manufacturer's specifications. DNA concentration ( $\text{ng}/\mu\text{l}$ ) was determined spectrophotometrically using a nanodrop. A 1% agarose gel was run at 100V for 20 min to confirm the presence of DNA post extraction. The amount of dye extracted from the colorimetric assays was normalized against the DNA content from the same culture dish.

#### 5.3.2.3.2 DAPI staining

Prior to histological staining, samples were stained with the fluorescent nuclear stain, DAPI. Briefly, samples were washed with PBS prior to staining. Samples were incubated with DAPI (0.02  $\mu\text{g}/\text{ml}$  in  $\text{H}_2\text{O}$ ) for 10 min before imaging. The stain was left in the wells while photos were taken. Samples were imaged with a fluorescence microscope at 10x objective lens magnification (0.8 saturation and an exposure time of 25) using the blue filter. Number of nuclei per image was determined by manual counting. A relative cell number per treatment group was calculated as the average number of nuclei. Five images per treatment group were captured.

#### 5.3.2.4 Image analysis

The software analysis program, Image J 1.46r was downloaded from <http://imagej.nih.gov/ij> and used for all image analysis. An Image J macro for quantification of the area occupied by lipid droplets was prepared by Dr. Arvind Pathak (The Russell H. Morgan Department of Radiology and Radiological Science, Johns Hopkins University School of Medicine). Briefly, the images underwent a series of processing steps to remove 'debris' from the images, followed by changing the threshold to select for the lipid droplets. A binarized (*i.e.* black and white) image showing only the lipid droplets was produced and the area (in pixels) occupied by

these lipid droplets determined. The area was converted to a percentage by dividing the area occupied by lipid droplets by the total area of the image.

### 5.3.3 Statistical analysis

The data generated from the colorimetric assays and image analysis for each experiment is presented as the mean, and standard deviations were displayed as error bars in graphs. A random-effects generalized best square (GSL) regression was used for multiple comparisons between control and test samples. A significance level of  $p < 0.05$  was used.

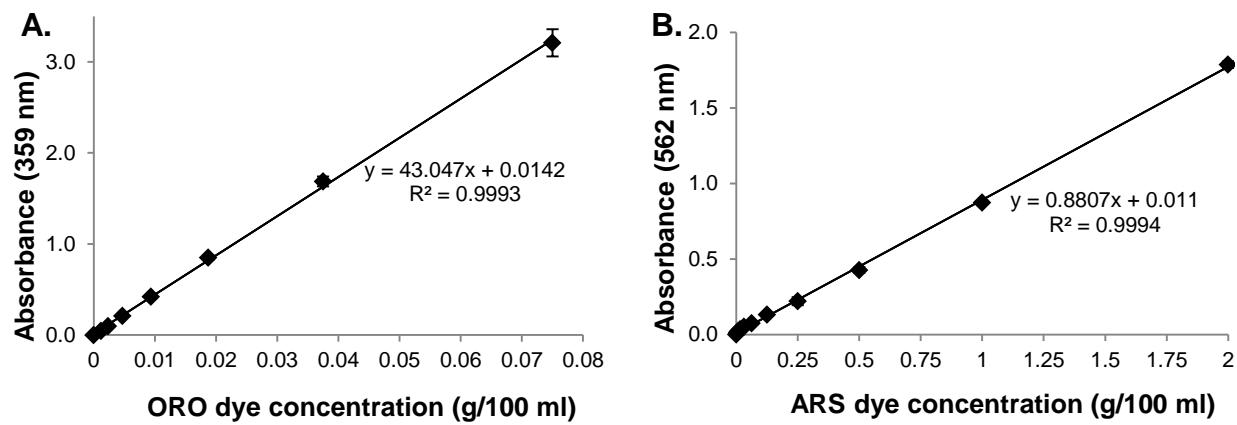
## 5.4 Results

To study their multilineage differentiation capacity, MSCs were differentiated toward the adipogenic, osteogenic, and chondrogenic lineages using lineage-specific induction factors (Table 13). Differentiation capacity of MSCs from umbilical cord and adipose tissue were compared. Ten umbilical cord samples, consisting of five digested samples, and five pieces were analyzed. Twelve adipose samples were analyzed. Incomplete data sets were not included in the data analysis. Following induction, differentiation was assessed using histology (Table 14) and quantified using a colorimetric assay. Standard titration curves (Figure 30) were plotted for both histological stains, ORO and ARS. The first three ORO concentrations, 0.5, 0.3, and 0.15 g/100 ml were too concentrated to be read on the spectrophotometer and gave “OUT” readings, and were excluded. “OUT” indicated an out of range value which could therefore not be read. Linear regression lines were used to determine concentration of dye extracted for each sample. The lower absorbance maximum for ORO at 359 nm instead of 518 nm was used to reduce interference from the TBO counter stain ( $\lambda_{max} 626$  nm).

### 5.4.1 Quantification of lipid droplet formation in differentiating hMSCs

Lipid droplet formation was quantified by two different methods. Firstly histology quantified the percentage of lipid droplets present in the differentiated sample by image analysis and secondly a colorimetric assay related differentiation capacity to the amount of ORO dye taken up by the cells. The data generated were normalized

either to relative cell number or to DNA concentration. For DNA concentration, only three samples per group, three cords and three adipose samples were compared.



**Figure 30. Standard titration curves for oil red O and alizarin red S histological dyes.**

(A) Plot of oil red O concentration versus absorbance at 359 nm. (B) Plot of alizarin red S concentration versus absorbance at 562 nm. Data are expressed as the mean of five readings. Error bars represent standard deviations (SD). A linear trend-line was generated for each plot and the equation used in determining dye concentrations from sample absorbance values.

The colorimetric data for the umbilical cord samples from Figure 31 A, showed background staining in all the controls. Approximately 63% of the positive staining observed in the differentiated cultures was due to background staining from the controls (Figure 31 A). Only two cords showed no differentiation. In contrast to the colorimetric data, the imaging results from Figure 31 C, which show the percentage of lipid droplets per image, revealed that only two cords and a control sample had differentiated. This was confirmed with the raw images, and it was found that none of the cord samples showed lipid droplet accumulation. The three samples that displayed lipid droplet staining were false positives; no true lipid droplets were noticeable. Figure 32 shows the images for the three samples from Figure 31 C that seemingly displayed ORO staining. In these images, no ORO stain was visible. The intensity of the stained cells resulted in false lipid droplet identification by the imaging software.

The colorimetric data from Figure 31 B showed background staining for all the adipose controls. However this background staining was significantly less than for the differentiated samples. Approximately 25% of the positive staining observed in the differentiated cultures from the colorimetric assays was due to background staining from the controls (Figure 31 B). For the image analysis, only 2% background staining was observed (Figure 31 D). All samples showed differentiation into the adipogenic lineage. The imaging results from Figure 31 D showed that only one adipose sample did not differentiate. However, for this sample, contradictory results were obtained, the dye extraction data showed that it differentiated the most, whereas the imaging data showed no differentiation at all. This observation could have been due to experimental error during the analysis process. As selected images were taken, differentiated cells could have been missed, leading to no differentiation being identified. However this sample was still included in the analysis.

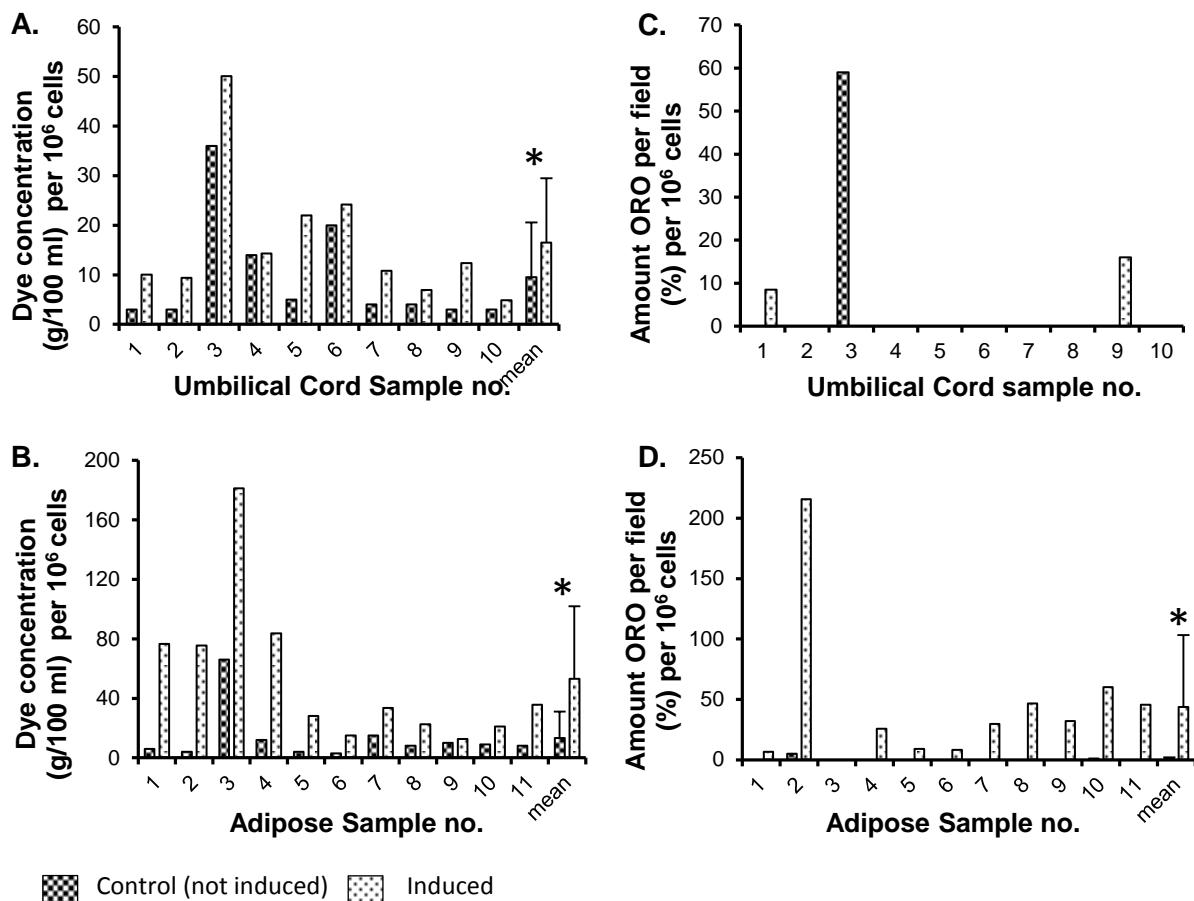
Differentiation capacities between adipose and cord samples were compared. Mesenchymal stem cells from the adipose sample group showed statistically significantly greater differentiation capacity into adipocytes compared with the cord pieces (Figure 33 A) or the cord sample group (Figure 33 A, B). The image analysis showed similar differentiation capacity into adipocytes between the adipose and cord samples; however the difference was not significant (Figure 33 C, D).

#### **5.4.2 Quantification of mineralization in differentiating MSCs**

Calcium deposition was quantified using an alizarin red-based colorimetric assay. The colorimetric assay related differentiation capacity to the amount of ARS dye taken up by the cells. The data generated were normalized either to relative cell number or to DNA concentration. For DNA concentration, three cord- and three adipose samples were compared.

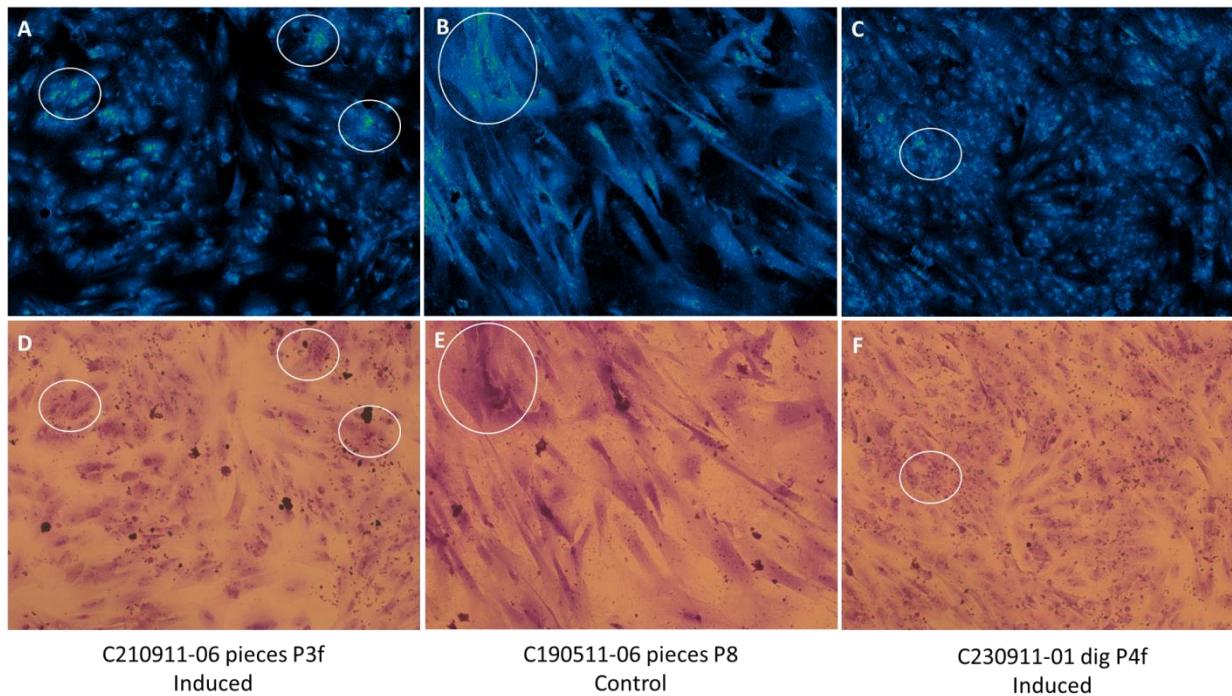
The colorimetric data for the bone-differentiated samples showed that all cord (Figure 34 A) and adipose (Figure 34 B) samples had background staining in the controls. Approximately 55% of the positive staining observed in the differentiated cultures for the cord- and 38% for adipose samples was due to background staining (Figure 34 A, B). Only one cord sample and one adipose sample did not differentiate into the bone lineage. Comparisons between the cord and adipose group revealed

that the digested groups' differentiation was significantly higher than that observed for the pieces; however, no significant differences were apparent between adipose and cord groups (Figure 35 A). The dye extraction data that was normalized to DNA concentration (Figure 35 B) also showed no significant differences between adipose and cord samples.



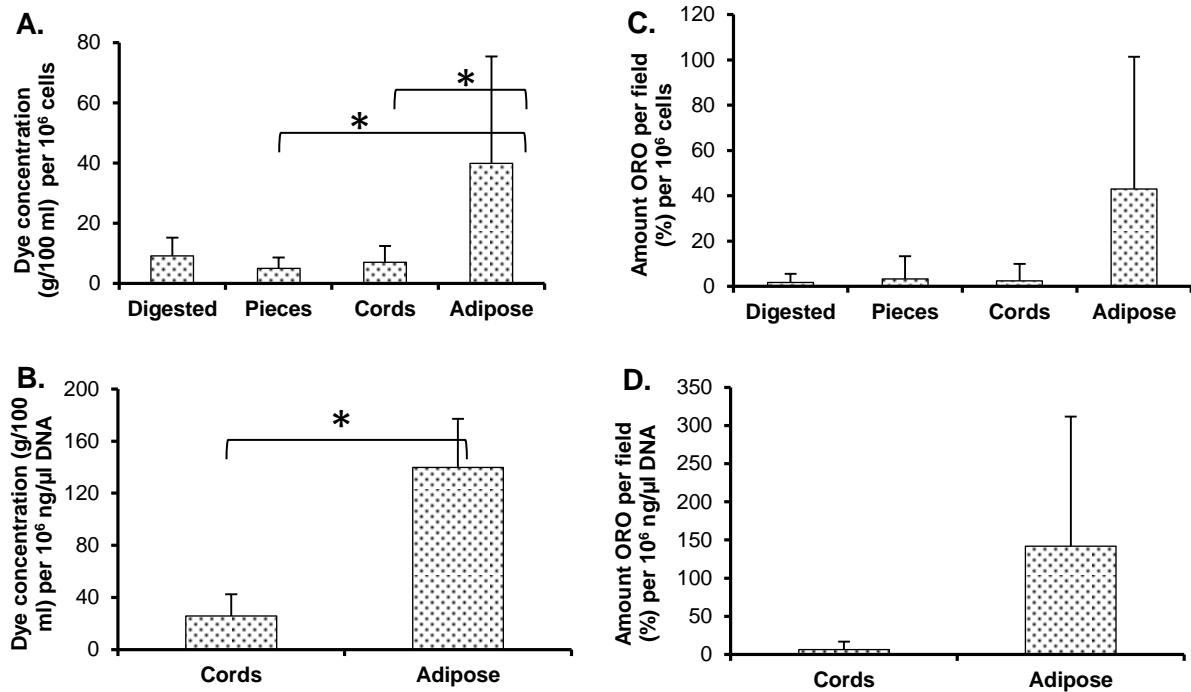
**Figure 31. Mesenchymal stem cells from adipose tissue and Wharton's jelly were differentiated along the adipose lineage.**

Each differentiated sample was compared to its control. (A, B) Colorimetric assays were performed to quantify the amount of oil red O dye extracted from umbilical cord and adipose tissue samples. (C, D) Image analysis using imaging software was performed by quantifying the percentage of each image occupied by oil droplets from umbilical cord and adipose tissue samples. All sample values were normalized to the relative cell number. Error bars represent standard deviations (SD), and significance as compared to the control (only for mean values) is shown when  $*p < 0.05$ . For (A) and (C), the umbilical cord sample numbers represent: 1. C230911-01 dig P4f; 2. C230911-01 pieces P4f; 3. C190511-06 dig P7; 4. C190511-06 pieces P8; 5. C220211-02 dig P8; 6. C220211-02 pieces P6; 7. C230911-02a dig P4f; 8. C230911-02a pieces P4f; 9. C210911-06 pieces P3f; 10. C230911-02b dig P5f. For (B) and (D), the adipose tissue sample numbers represent: 1. A200411-01 P4; 2. A270611-01 P8; 3. A130311-02 10cc P6; 4. A270611-02 P7; 5. A100511-01 P10f; 6. A040411-01 P8f; 7. A050511-01 P11f; 8. A050711-01 P6f; 9. A130411-01 P9f; 10. A240211-01 P2f; 11. A220611-01 P7f.



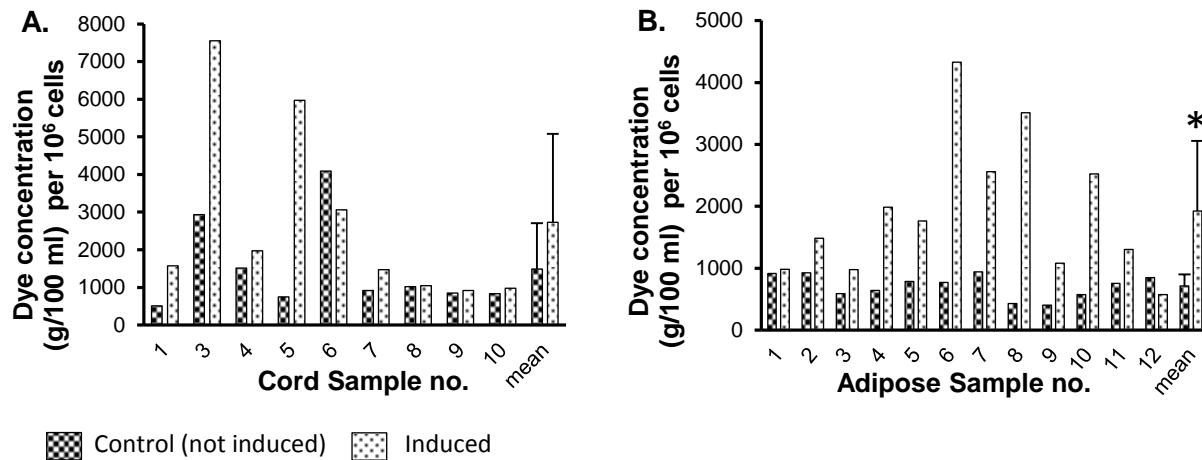
**Figure 32. Image analyses to confirm adipogenesis shows false lipid droplet identification for umbilical cord samples.**

Differentiation into the adipogenic lineage was determined by quantification of lipid droplet staining using imageJ analysis software. The software converted the cells to look blue and any areas containing lipid droplets to green. (A, D) C210911-06 pieces P3f induced, (B, E) C190511-06 P8 control and (C, F) C230911-01 dig P4f induced. Induced: induced to differentiate, control: not induced. (A-C) Pre-analysis images generated showing the cells in blue and the lipid droplets in green. Circles indicate green areas. (D, E, F) Light microscopy images taken of the stained cells. Cell morphology was visualized using toluidine blue O. Oil red O staining was used for lipid droplet identification. Circles indicate the same areas as for (A) (B) and (C) respectively, and show no oil red O staining.



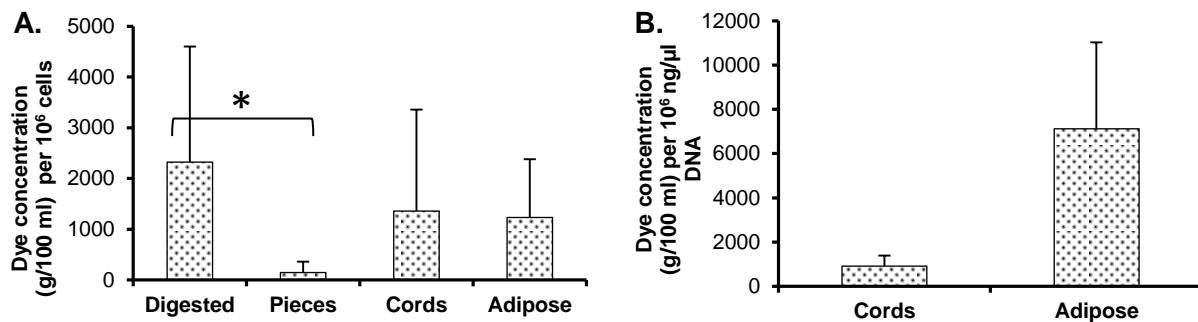
**Figure 33. The extent of adipose differentiation between mesenchymal stem cells from adipose tissue and Wharton's jelly were compared.**

Each differentiated sample was corrected for background staining by subtracting control well staining. (A, B) Colorimetric assays were performed to quantify the amount of oil red O dye extracted from umbilical cord and adipose tissue samples. (C, D) Image analysis using imaging software was performed by quantifying the percentage of each image occupied by lipid droplets from umbilical cord and adipose tissue samples. Umbilical cord samples were divided into digested and pieces. All samples were normalized relative to cell number ( $n = 5$  for digested,  $n = 5$  for pieces, and  $n = 11$  for adipose) as well as to DNA concentration ( $n = 3$  for cords, and  $n = 3$  for adipose). Error bars represent standard deviations (SD), and significance is shown when  $*p < 0.05$ .



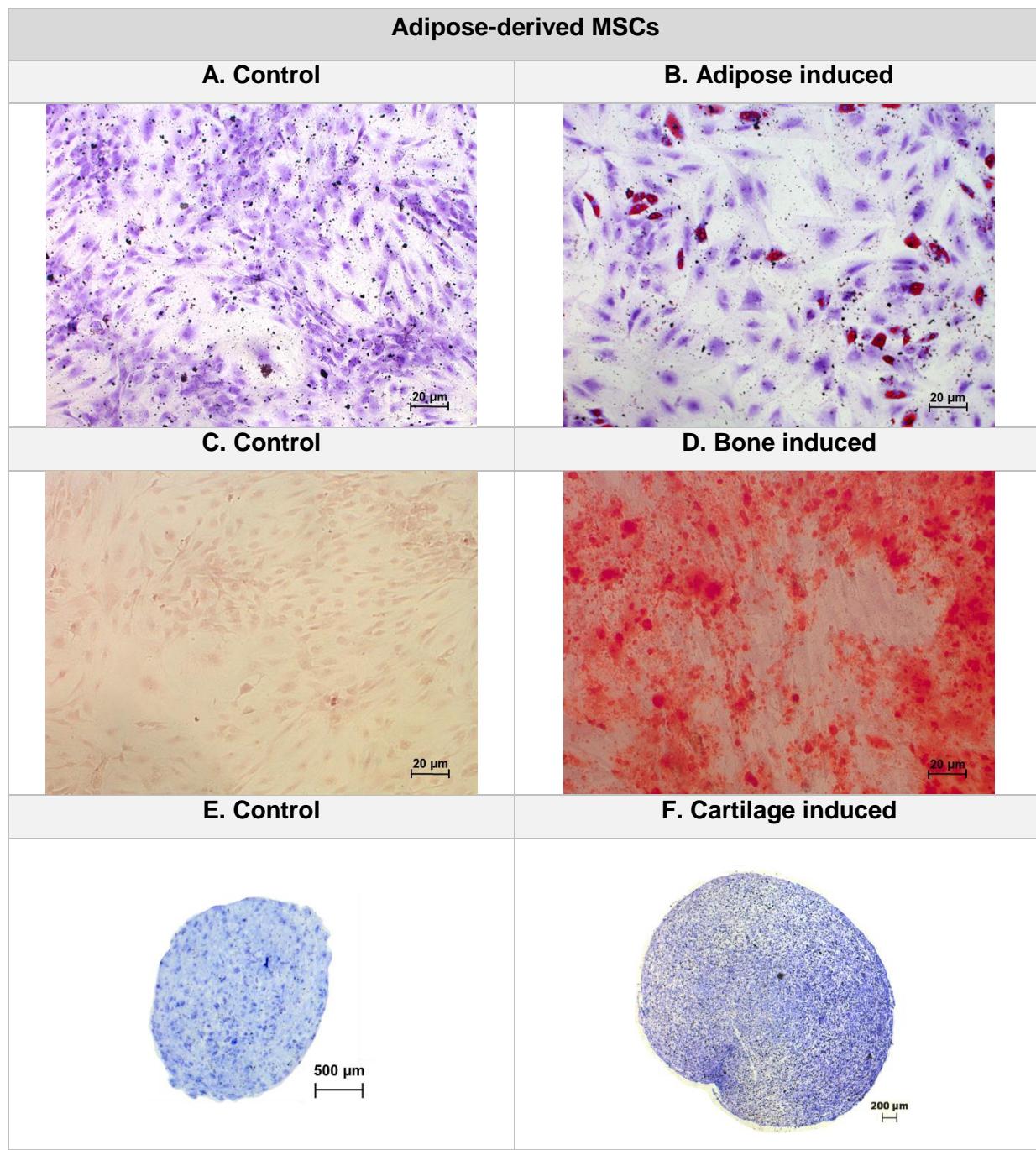
**Figure 34. Mesenchymal stem cells from adipose tissue and Wharton's jelly were differentiated along the bone lineage.**

Each differentiated sample was compared to its control. (A, B) Colorimetric assay was performed to quantify the amount of alizarin red S dye extracted from umbilical cord and adipose tissue samples. All sample values were normalized to relative cell number. Error bars represent standard deviations (SD), and significance as compared to the control (only for mean values) is shown when \* $p < 0.05$ . For (A), the umbilical cord sample numbers represent: 1. C230911-01 dig P4f; 2. C230911-01 pieces P4f; 3. C190511-06 dig P7; 4. C190511-06 pieces P8; 5. C220211-02 dig P8; 6. C220211-02 pieces P6; 7. C230911-02a dig P4f; 8. C230911-02a pieces P4f; 9. C210911-06 pieces P3f; 10. C230911-02b dig P5f. For (B), the adipose tissue sample numbers represent: 1. A200411-01 P4; 2. A270611-01 P8; 3. A130311-02 10cc P6; 4. A270611-02 P7; 5. A100511-01 P10f; 6. A040411-01 P8f; 7. A050511-01 P11f; 8. A050711-01 P6f; 9. A130411-01 P9f; 10. A240211-01 P2f; 11. A220611-01 P7f; 12. A230311-01 P7.



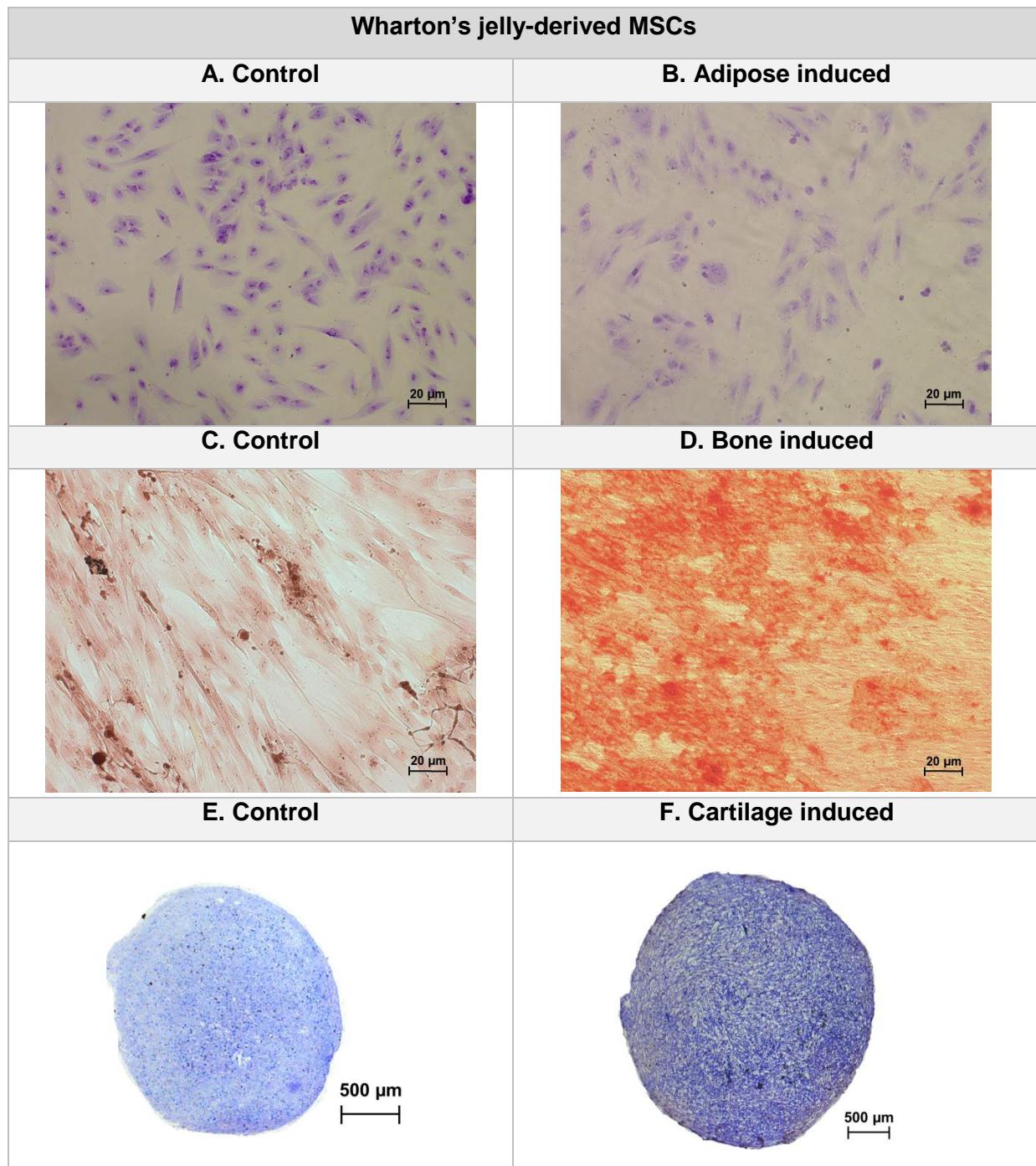
**Figure 35. The extent of bone differentiation between mesenchymal stem cells from adipose tissue and Wharton's jelly were compared.**

Each differentiated sample was corrected for background staining by subtracting control well staining. (A, B) Colorimetric assay was performed to quantify the amount of alizarin red S extracted. Umbilical cord samples were divided into digested and pieces samples. All samples were normalized to relative cell number ( $n = 5$  for digested,  $n = 5$  for pieces,  $n = 12$  for adipose) as well as to DNA concentration ( $n = 3$  for cords, and  $n = 3$  for adipose). Error bars represent standard deviations (SD), and significance is shown when  $*p < 0.05$ .



**Figure 36. Differentiation of adipose-derived MSCs**

(A, B) Control and induced samples were stained with oil red O and toluidine blue O. In adipogenic cultures, oil red O positive lipid droplets (red) were seen compared to the lack of staining in the control culture. (C, D) Control and induced samples were stained with alizarin red S. In osteogenic cultures, mineralization was visible (red stained calcium deposition) whereas this was absent in the control culture. (E, F) Control and induced samples were stained with toluidine blue O. In chondrogenic cultures, staining of the proteoglycans (purple) was visible, whereas this was absent in control cultures.



**Figure 37. Differentiation of Wharton's jelly-derived MSCs**

(A, B) Control and induced samples were stained with oil red O and toluidine blue O. In adipogenic cultures, oil red O positive lipid droplets (red) were absent in the induced and in the control culture. (C, D) Control and induced samples were stained with alizarin red S. In osteogenic cultures, mineralization was visible (red stained calcium deposition) whereas this was absent in the control culture (E, F) Control and induced samples were stained with toluidine blue O. In chondrogenic cultures, staining of the proteoglycans (purple) was visible, whereas this was absent in control cultures.

### 5.4.3 Differentiation into the chondrogenic lineage

Chondrogenic differentiation was not quantified; only Yes/No answers for differentiation were determined. All cord samples and adipose samples showed differentiation capacity into the chondrogenic lineage. Positive identification was observed when the proteoglycan and collagenous matrix stained purple with TBO. A representative histology image of chondrogenic differentiation can be seen in Figure 36 and 37.

## 5.5 Discussion

In the series of experiments described herein, *in vitro* studies were conducted to evaluate the tri-lineage differentiation potential of MSCs isolated from two different sources, namely adipose tissue (lipoaspirate) and umbilical cord (Wharton's jelly).

### 5.5.1 Adipogenic differentiation

Various cell culture models (preadipocyte cell lines and primary cultured adipose derived stromal cells) have been used to study the molecular and cellular events that occur during adipocyte differentiation. The murine preadipocyte 3T3-L1 cell line is commonly used for investigating preadipocyte differentiation (Gregoire *et al.*, 1998, Tang and Lane, 2012). In the laboratory, adipogenic differentiation takes two to three weeks in appropriate induction media, after which intracellular lipid accumulation becomes visible with oil red O staining. This technique depends on the ability of the lipid droplets in the adipocyte to collect the red stain. The stain can then be extracted from the cells using isopropanol and quantified by measuring the absorbance values spectrophotometrically (Ramírez-Zacarías *et al.*, 1992). Stimulation of adipogenesis can be achieved by the addition of insulin and glucocorticoids. For effective stimulation into adipocytes, induction medium is supplemented with 3-isobutyl-methylxanthine (IBMX), insulin and dexamethasone. The addition of IBMX is necessary as it acts as a cAMP inducer. Ali and colleagues showed that the removal of IBMX from the induction medium almost completely blocked the normally observed increase in intracellular lipid accumulation (Ali *et al.*, 2006). IBMX, a cAMP-phosphodiesterase inhibitor, has been shown to inhibit soluble cyclic nucleotide phosphodiesterase activity causing an increase in intracellular cAMP levels by stimulating the cAMP-dependent protein kinase pathway. This increase in cAMP

levels is subsequently responsible for the synthesis of lipogenic enzymes during adipogenesis (Spiegelman and Green, 1981, Tang and Lane, 2012). Insulin acts as a potent adipogenic hormone by triggering the induction of a series of transcription factors, which facilitate differentiation of preadipocytes into mature adipocytes. It acts through the insulin-like growth factor (IGF-1) receptor (Ntambi and Young-Cheul, 2000). Dexamethasone is a synthetic glucocorticoid agonist used to stimulate the glucocorticoid receptor pathway. Studies using immortalized preadipocytes, such as the mouse 3T3-L1 cell line, revealed that combinations of IBMX with dexamethasone or insulin induced intracellular lipid accumulation. Individually, each agent was able to initiate adipogenesis. Additive effects were however produced when the factors were combined (Rubin *et al.*, 1978, Russell and Ho, 1976).

Mature adipocytes have lost the ability to undergo cell division. Preadipocytes are responsible for differentiation into mature adipocytes. Both techniques used for quantifying the efficacy of adipocyte differentiation were valuable but not comparable with one another. Colorimetric assays are useful for determining the amount of dye taken up by cells. They rely on the dye to be specific, implying that only differentiating cells will take up dye and stain positively. However, each sample was compared to its control, and background staining was found in all the controls (Figure 31). Spontaneous differentiation of the controls was not responsible for these observations as no oil droplet formation was visible in control cultures. Control cells were kept in culture (in control medium containing no inducing agents) for the duration of differentiation (21 days). After the first week, these cultures had already reached confluence and due to contact inhibition were assumed to be growth arrested. Great care had to be taken during medium changes not to disrupt the monolayers. The oil red O dye did stain the sides of the culture dishes. Extra wash steps were included to eliminate this non-specific staining as much as possible. All monolayers were assessed before staining to ensure that they were intact. The possibility of the monolayers detaching during staining exists, and staining of culture dishes may have contributed to the observed non-specific staining. Image analysis was useful for determining the percentage of the image occupied by positively stained lipid droplets. This technique also showed some sensitivity problems. It identified positive staining in controls even when no lipid droplets were observed. Staining due to differentiation was therefore separated from non-specific staining by

subtracting control values from treated values for both the techniques. The degree of differentiation varied greatly between samples from the same group (in both adipose and cord groups) and also varied between the two techniques (Figure 31). Contradicting results were obtained for the cord sample group. Although the colorimetric assay showed that these cells had the ability to differentiate, image analysis displayed no lipid droplets in these cultures. The cord samples identified as having positive lipid droplet accumulation were falsely positive due to non-specific staining (Figure 31 C, 32). Comparisons between the adipose group and the digested-, pieces-, and cord group for the colorimetric assay showed comparable results between samples normalized to relative cell number and samples normalized to DNA concentration. The adipose group showed significantly greater differentiation capacity into adipocytes compared to the cord group (Figure 33 A, B). Image analysis also showed comparable results between samples normalized to relative cell number and samples normalized to DNA concentration. Although the adipose group seemed to show greater differentiation, the differences were not statistically significant (Figure 33 C, D). Oil red O is a lysochrome (fat-soluble) diazo dye used for staining neutral triglycerides and lipids. Cultures were fixed with formaldehyde prior to quantification. Formaldehyde works by forming inter-molecular and intra-molecular cross-links between proteins. Other molecules, such as carbohydrates, lipids, and nucleic acids are thought to be trapped in this matrix of cross-linked proteins (DiDonato and Brasaemle, 2003). All differentiated samples were fixed and stored in PBS at 4°C until staining. The time period between fixation and staining varied between samples. The possibility of losing lipid droplets while the cultures were kept in storage could have occurred and may have influenced the low differentiation seen for the cultured MSCs. Large lipid droplets have previously been observed as vacant spheres in histological paraformaldehyde fixed sections. These observations were due to the inability of the lipid ester to be fixed by formalin and were extracted by organic solvents during dehydration (Fujimoto *et al.*, 2008). Although the samples did not undergo dehydration steps, the possibility of losing lipid droplets could have occurred due to extended storage times prior to staining. The serum added to the induction medium is required for triglyceride accumulation and depends on a small component in the serum, biotin. When this component is removed by exhaustive dialysis of the serum, the cells are able to undergo some changes that are part of adipose conversion, but accumulate little triglyceride.

Addition of commercial biotin can counteract this phenomenon. It has been shown that cells can begin adipogenesis in the absence of biotin, but are unable to increase their rate of acetate incorporation into triglycerides. Morphological changes from a fibroblastic to a spherical shape with no triglyceride accumulation would occur in biotin deficient cultures (Kuri-Harcuch *et al.*, 1978). Pre-adipose cell lines and primary preadipocytes rely on growth arrest as a requirement for adipocyte differentiation (Gregoire *et al.*, 1998). Cell confluence leads to growth arrest. The cultures were at varying degrees of confluence before addition of induction medium, and thus efficient growth arrest may not have occurred and may have had an influence on the differentiation capacity of the cultures. It was found that the cord-derived MSCs in the present study were unable to form adipocytes.

In the present study, differences in differentiation between single cells could not be determined. The amount and size of lipid droplets at a per cell level would be of interest for comparing preadipocyte and mature adipocyte formation, as well as the percentage of the cell population with the ability to differentiate. The techniques used were only able to quantify differentiation by giving an estimate of total differentiation per culture. Although assumptions were made regarding the distribution of cells over the culture dish, the heterogeneity of the cells would have caused uneven differentiation across the culture. One would expect colonies of differentiated cells due to their clonogenicity. The colorimetric assay took into account all the cells whereas the image analysis only quantified a few representative images, thus differentiated colonies could have been missed, giving inaccurate values. To overcome some of these limitations, alternative techniques such as using flow cytometry have been suggested. Flow cytometry allows for the study of differentiated MSCs at a single-cell level. Parameters such as intensity of staining, cell size, granularity, and cell number could be determined simultaneously along with MSC marker expression (Aldridge *et al.*, 2013).

### **5.5.2 Osteogenic differentiation**

Bone homeostasis depends on interactions between cells, substrates and molecular signals. Hormones, mitogens, and differentiation factors influence these signals (Zomorodian and Baghaban Eslaminejad, 2012). Signaling events within bone have the ability to repair damage by initiating a cascade of genetically programmed repair

processes. However, for extensive injuries these regenerative processes are unable to completely repair the damage. Therapeutic interventions are thus required. Autologous tissue transplantation is considered the best and safest strategy. However, the limited availability along with autograft morbidity, pain, and possible infection at the donor site are problematic. Allogenic grafts also have problems of graft rejection and inefficient graft integration. Mesenchymal stem cells have been proposed as appropriate cells for use in promoting bone regeneration (Zomorodian and Baghaban Eslaminejad, 2012). Friedenstein and colleagues showed that bone marrow derived stem cells displayed osteogenic differentiation capabilities (Friedenstein *et al.*, 1966). Osteoblasts show potential for the repair of fracture non- or mal-unions, for bone grafting and for joint fusions. Lee and colleagues published the first report of *in vivo* bone formation from adipose-derived stem cells (Lee *et al.*, 2003). They harvested adult stem cells from autologous fat and differentiated these adipose-derived stem cells into osteoblasts *in vitro*. They then transplanted these differentiated adipose-derived stem cells, loaded onto polyglycolic acid scaffolds, subcutaneously into Lewis rats and observed *in vivo* bone formation.

The osteogenic maturation pathway involves cell proliferation, differentiation, and matrix deposition followed by mineralization. Differentiation of MSCs into osteoblasts *in vitro* involves incubating a monolayer of MSCs with ascorbic acid,  $\beta$ -glycerolphosphate and dexamethasone for three weeks. Ascorbic acid acts as a co-factor for collagen synthesis through the hydroxylation of proline and lysine residues in collagen and induces ECM production through the synthesis of non-collagenous bone matrix proteins.  $\beta$ -glycerolphosphate is needed for calcification and mineralization of the ECM. Dexamethasone regulates osteoblastic gene expression (Fiorentini *et al.*, 2011). *In vitro* dexamethasone treatment was shown to increase alkaline phosphatase activity which is required for matrix mineralization and morphological transformation to cuboidal shaped cells (Cheng *et al.*, 1994). Alizarin red staining was used for identifying calcific deposition during matrix mineralization in osteogenic differentiation cultures. It is an early marker for differentiation. The dye taken up can be extracted and measured spectrophotometrically. The colorimetric assay used for quantification of osteogenic differentiation showed that background staining was obtained for all cord and adipose control samples. Spontaneous osteoblast differentiation for MSCs has previously been observed on plastic

substrates (Li *et al.*, 2011); however, microscopic inspection of the control cultures revealed that this phenomenon did not occur in our studies. Only a single cord- and a single adipose sample did not show osteogenic differentiation (Figure 34). To eliminate background staining, the control well values were subtracted from the induced well values. Comparisons between the culture groups showed that cords isolated via the digested method had significantly greater differentiation capacity than the cord pieces, however no significant differences were observed between cord (pooled digested cord and cord pieces) and adipose groups. The samples normalized to DNA concentration showed no significant difference in differentiation capacity between the cord- and adipose samples.

Alizarin red S staining has been used for decades to evaluate calcium-rich deposits by cells in culture. Dye extraction of alizarin red S using CPC has shown lack of sensitivity in weakly stained monolayers. A study compared CPC dye extraction to acetic acid extraction and found the acetic acid method to be three times more sensitive; however, this was a labor-intensive technique (Gregory *et al.*, 2004). Microscopic inspection along with morphology changes is not adequate for quantification of differentiation. Changes in cell morphology were difficult to identify under the calcified matrix, which formed above the cells. Additional techniques should be included. Alkaline phosphatase activity along with molecular signaling analysis would be able to better identify differences in differentiation capacity between different stem cell sources.

### 5.5.3 Chondrogenic differentiation

Chondrogenesis is the earliest phase of skeletal development and precedes bone formation in endochondral bone differentiation through the formation of hyaline cartilage. Hyaline cartilage persists in articulations following bone differentiation. Osteoarthritis is a degenerative disease of cartilage consisting of progressive destruction of articular cartilage and eventually the entire joint. The poor regenerative capacity of articular hyaline cartilage has led to attempts to support biological repair of cartilage defects (Boeuf and Richter, 2010). Autologous chondrocyte transplantation has been tested for some time, and involves surgical intervention prior to transplantation. Mesenchymal stem cells are becoming an appealing

alternative cell source for cartilage repair, involving the implantation of cartilage differentiated from autologous MSCs *in vitro*.

*In vitro* chondrogenesis is induced by artificially condensing the cells by centrifugation followed by addition of TGF- $\beta$ 3, dexamethasone, and BMP-2 to the medium. Johnstone and colleagues developed a culture system to facilitate chondrogenic differentiation for bone marrow derived MSCs (Johnstone *et al.*, 1998). This pellet culture allows for cell-cell interaction similar to those that occur during embryonic development (Fell, 1925). It has been shown that a defined medium, to which dexamethasone and TGF- $\beta$ 1 have been added, is required (Johnstone *et al.*, 1998). Dexamethasone induction resulted in a metachromatic staining pattern of aggregated cells. This suggested that cartilaginous matrix was synthesized and was confirmed with collagen type II immunohistochemistry. The use of TGF- $\beta$ 1 either alone or in combination with dexamethasone induced chondrogenesis in aggregated cells. It was later found that the addition of BMP-6 further increased the extent of chondrogenesis (Sekiya *et al.*, 2001). The growth factor TGF- $\beta$ 3 has replaced the use of TGF- $\beta$ 1 (Sekiya *et al.*, 2002b). Critical roles are played by BMP's in compaction of MSCs and for the shaping of the condensations (Zuscik *et al.*, 2008). Comparisons between BMP-2, -4, and -6 to enhance *in vitro* formation of MSCs demonstrated that BMP-2 was the most effective, resulting in increased pellet weight along with more proteoglycan and collagen type II production (Sekiya *et al.*, 2005). However, the addition of BMPs to cartilage induction medium is not necessarily required, and was omitted for the current study (Zuk *et al.*, 2001). In our study, cartilage formation was determined by proteoglycan deposition through TBO staining. All cultures from cord and adipose tissue showed differentiation capacity into the chondrogenic lineage, although no quantification of chondrogenic differentiation was done. No comparisons could be made between different samples. Cell numbers before induction were not meticulously noted, leading to variable pellet sizes. In addition, sections for each sample were not necessarily cut at the same place even though care was taken to use sections from about a third of the way into the pellets.

More detailed strategies for chondrogenic differentiation include extensive morphological examination during *in vitro* cartilage formation utilizing techniques

such as light microscopy, transmission electron microscopy, and immunohistochemistry (Ichinose *et al.*, 2005). Other studies have shown that after a week in induction medium, cell pellets consist of three layers: superficial zone, containing fibroblast-like cells, middle zone, containing apoptotic cells, and the deep zone, containing matrix producing chondrocyte-like cells. After 14 days, the middle zone disappeared and the deep zone dominated after the induction period of 21 days (Ichinose *et al.*, 2005). Biochemical analysis of chondrogenesis includes methods that quantify the total glycosaminoglycan content in pellets (Naumann *et al.*, 2002, Estes *et al.*, 2010). Boeuf and Richer reviewed the requirements for the induction of chondrogenesis in MSCs from different sources. They concluded that differences were in terms of growth factors needed and suggested that these differences may be related to differences in the growth factor repertoires expressed by the cells and/ or the pathways involved for initiation of chondrogenesis (Boeuf and Richter, 2010).

#### 5.5.4 Concluding remarks

None of the techniques used truly gave accurate and reliable quantitative results, and all were semi-quantitative. Problems with controls were encountered due to the confluent monolayers. Determining cell numbers per well came with its own challenges as the cells were fixed in monolayers. The two techniques used, namely using DNA concentration and relative cell number by DAPI staining were flawed. However results normalized by either of these were comparable and gave similar results. By determining the DNA concentration, each well as a whole could be “counted”. Sensitivity in determining low DNA concentrations was a concern. Isolation and purification of DNA from the samples was encountered with difficulty, as routinely used DNA isolation kits are applicable to unfixed cells in suspension. The differentiated cultures were fixed and adherent to the plastic culture dishes in a monolayer. The FFPE tissue kit used for DNA isolation is designed for purifying DNA from formalin-fixed, paraffin embedded tissue sections. This kit proved to be effective for formalin-fixed adherent cells and just required the cells to be scraped off the surface before use. The fluorescent dye DAPI was used for determining a relative cell number and selected for certain areas of the cultures. Due to over confluent monolayers being observed in control and bone induced wells; determining cell numbers were difficult and impossible for some cultures. The integrity of

differentiated samples was also compromised due to extended storage times post fixation and may have influenced the staining of adipocytes and bone matrix mineralization. This can in the future be avoided. Mesenchymal stem cell cultures are heterogeneous in nature, consisting of stem- and progenitor cells. Thus cells with differentiation potential would be randomly dispersed in cultures and selective imaging of stained cells could have led to colonies of differentiated cells being excluded. Sekiya and colleagues' experiments indicated that the greatest number of adipocytes was generated from cultures plated at lower densities for shorter periods prior to induction (Sekiya *et al.*, 2002a). These observations could be due to a more homogeneous population of cells being obtained and may also be applicable to osteogenic induced cultures.

Interestingly, the cord derived MSCs did not differentiate toward the adipogenic lineage. The literature with regard to adipogenic differentiation of cord derived MSCs (i.e. from Wharton's jelly) has not reported this inability of MSCs to undergo adipogenesis. However, it has been noted that MSCs derived from umbilical cord blood were unable to display an adipogenic phenotype under standard differentiation conditions (Bieback *et al.*, 2004, Kern *et al.*, 2006). The greater adipocyte differentiation capacity of adipose-derived MSCs compared to cord-derived MSCs is not surprising. The function of adipose tissue is to produce adipocytes, whereas the umbilical cord supplies the baby with nutrients. The hypothesis has arisen that differentiation sensitivity toward adipogenesis is correlated with age (Bieback *et al.*, 2008). The differentiation efficiency of MSCs may vary with age; for example, Zhu and colleagues showed that the osteogenic potential of human female adult stem cells decreased with age, while the adipogenic potential remained unchanged (Zhu *et al.*, 2009). Another study showed that UCB-MSCs showed a significantly stronger osteogenic potential but a lower ability for adipogenic differentiation than bone marrow-derived MSCs (Chang *et al.*, 2006). Further genomic and proteomic analysis should be performed to evaluate the adipogenic differentiation capacity of cord derived MSCs.

The *in vitro* differentiation potential of MSCs does not necessarily predict the *in vivo* differentiation capacity. Differentiation into adipose, bone, and cartilage lineages was only assessed morphologically and by a colorimetric assay, thus the findings of this

study need to be confirmed at the molecular level and translated to the *in vivo* setting.

## Chapter 6: Concluding discussion

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Regenerative medicine involves the use of cells (including stem cells) and other biological material (growth factors, extracellular matrix) and scaffolds (biological or synthetic) in various combinations to repair damaged tissue. Adult stem cells represent an ethical alternative to ESCs and appear to have greater differentiation potential than originally anticipated (Poulsom *et al.*, 2002, Verfaillie, 2002, Wagers and Weissman, 2004). The field of regenerative medicine requires a reliable stem cell source. Mesenchymal stem cells are ideal adult stem cells due to their ability to self-renew, multipotent differentiation capacity, as well as immunomodulatory and anti-inflammatory properties (Chamberlain *et al.*, 2007). Bone marrow has been the best characterized and most commonly used source of MSCs. However, the need has arisen to identify alternative sources. Bone marrow-MSCs have limited autologous/allogeneic use due to (i) invasive harvesting procedures; (ii) the associated donor morbidity; (iii) low cell yield after harvest; and (iv) decreased differentiation potential and frequency with age (Grontos *et al.*, 2003, Stenderup *et al.*, 2003). Adipose tissue is an abundantly available alternative source of MSCs, displaying equivalent proliferation and differentiation capacity to BM (reviewed in Mizuno, 2009). Umbilical cord blood and umbilical cord (Wharton's jelly) have attracted attention as alternative allogeneic sources due to their availability, lack of ethical problems, and are believed to harbour the youngest MSCs with high proliferative capacity and low immunogenicity (Bieback *et al.*, 2004, Bieback *et al.*, 2008, Can and Karahuseyinoglu, 2007). The question arises however as to whether MSCs derived from various tissues all present the same biological features (Si *et al.*, 2010). Differences in expansion potential under identical conditions have previously been found (Kern *et al.*, 2006).

Currently the minimal criteria to define human MSCs, as proposed by the ISCT, are used to characterize MSCs (Dominici *et al.*, 2006). Mesenchymal stem cells need to be plastic-adherent, express an MSC phenotype, and show tri-lineage differentiation potential. An important issue regarding the therapeutic use of MSCs relates to their isolation. There is a lack of standardization when isolating and characterizing MSCs. In the present study, routine isolation, culturing, and characterization methods were

established. A defined culturing medium, referred to as complete growth medium, was used for the culturing of the different sources of MSCs. Cells with a fibroblastic morphology resembling MSCs were successfully isolated from adipose tissue and Wharton's jelly using standardized protocols. However, we were unable to isolate and grow MSCs from UCB. For isolation of WJ-MSCs, two techniques were used: (1) explant culture, and (2) enzymatic digestion. A previous study showed that the nature of the isolation technique influenced cell proliferation and morphology (Salehinejad *et al.*, 2012). Enzymatic digestion modified cell size and internal complexity/granularity compared to the explant method (Margossian *et al.*, 2012). Enzymatic digestion has been described as the most efficient method to obtain the highest cell yield from Wharton's jelly (Margossian *et al.*, 2012). The explant method, although easier, and less cumbersome in processing, limits the ability to determine the initial cell yield. The present study used an additional procedure which was to sequentially replate umbilical cord pieces to obtain cells with an MSC phenotype. Thus, from a single cord, there is potential to yield a large number of stem cells. Concerns relating to the use of enzyme assays have been expressed (McCarthy *et al.*, 2011). Enzymes such as collagenase work by breaking down the connective tissue and ECM to release the resident cells. When using enzymes, it would be important to determine/predict the effect this might have on the activity of cells. In the present study the type of isolation technique used did not influence the proliferation capacity of WJ-MSCs. However, a statistically significantly higher PDT was obtained for ASCs compared to WJ-MSCs. A standardized 5-colour flow cytometry protocol was used to characterize isolated MSCs phenotypically. Most studies employ single staining, where the expression of each marker is looked at independently. The present study was able to look at the entire cell population and determine the combined phenotype. Cells were monitored over 10 passages to determine their ability to acquire and maintain an MSC phenotype. Mesenchymal stem cells from adipose tissue and Wharton's jelly displayed the classical MSC phenotype (CD73+, CD90+, CD105+, CD34-, and CD45-) in  $\geq 95\%$  of the cell population. The phenotype of frozen cells compared to fresh samples remained stable. Greatest variability in phenotype was seen in early passages. Expression of CD34 was high after isolation of ASCs and low levels remained during culturing. Various study groups have also noted positive CD34 expression of human ASCs, while others claims them to be CD34 negative. Variability in the phenotypic profile of ASCs has been reported to be

due to donor-dependent differences (Baer *et al.*, 2013). The type of isolation protocol used for WJ-MSCs did not influence the cell phenotype.

Mesenchymal stem cells are characterized by their ability to differentiate into cells found in mesenchymal tissues, namely adipocytes, chondrocytes, and osteoblasts (Pittenger *et al.*, 1999). The *in vitro* differentiation capacity was compared between ASCs and WJ-MSCs. Tri-lineage differentiation was seen in ASCs, with a significantly higher adipogenic differentiation capacity compared to WJ-MSCs. The WJ-MSCs did not differentiate into adipocytes but displayed differentiation into osteoblasts and chondrocytes. Lack of adipogenic differentiation has not been previously noted in WJ-MSCs, but has been found in UCB derived MSCs (Bieback *et al.*, 2004, Kern *et al.*, 2006). One explanation might be the nature of the differentiation medium used, and this will need to be compared directly to a panel of different conditions previously reported in the literature. When strictly adhering to the proposed standards proposed by ISCT to characterize MSCs *in vitro*, the present study failed to produce cells from Wharton's jelly that could be classified as MSCs. This was due to their lack of adipogenic differentiation. These standards have been proposed as a guideline only, and have not been updated since 2006. This study shows that MSCs isolated from different tissue source are not biologically identical. Further investigation into characterizing MSCs is needed since the phenotypic profile of MSCs is still ill defined. Identification of a single marker or panel of markers uniquely expressed on MSCs is urgently needed. The current markers are not exclusive to MSCs. Thus the purity of isolated cells may vary between samples and research groups.

Limitations of the present study include:

- Fresh and thawed cord samples were used during the study which were compared to fresh adipose samples, and may have changed the characteristics of the cells
- Volunteers' age, sex, and site where adipose tissue was harvested varied
- Detailed morphology of the tissue from which the cells were isolated was not investigated

- Different isolation protocols were not used to further investigate whether UCB contained MSCs
- An in depth molecular analysis of differentiation was not performed; employing PCR techniques and transcriptome analysis could shed light on the differentiation capacity of the cells
- Fixed differentiated samples were stored for extended periods in PBS prior to doing morphological analyses
- The sample size of the cords was limited due to difficulty experienced in receiving samples from the hospital
- Non-specific staining of dyes was observed in the colorimetric assay
- Image analysis used only five fields per well, and not the entire well
- False positive oil droplets were identified in cord samples using image analysis
- Calcium matrix produced by osteogenic differentiating cells interfered with obtaining images of the cells

Beside the differentiation capacity of MSCs, the capacity of these cells to modulate the immune system during inflammation has gained interest. A study conducted by La Rocca *et al.* (2013) studied WJ-MSCs, and found that they express immunomodulatory molecules when undifferentiated or differentiated. Umbilical cord is composed of a heterogeneous population of progenitor cells grouped as MSCs. The use of WJ-MSCs has been suggested in an allogeneic manner (La Rocca and Anzalone, 2013). The efficiency of cryopreservation conditions for WJ-MSC storage has previously been evaluated (Balci and Can, 2013). A review on the therapeutic potential of WJ-MSCs described these cells as being the best suited adult stem cell for clinical applications (Bongso and Fong, 2013). This was based on the ability of WJ-MSCs to be non-controversial and the fact that they can be harvested painlessly, and in abundance. They are proliferative, exhibit stem cell properties *in vitro*, are multipotent, hypoimmunogenic, anti-tumorigenic, possess anti-cancer properties, and have been shown to support HSC transplantation (reviewed in Bongso and Fong, 2013). Clinical translation is impeded by the inconsistency of techniques used to isolate WJ-MSCs. Besides the choice between using enzymatic digestion or cord explants, variations have been observed using different zones of umbilical cord (Conconi, 2011, Bongso and Fong, 2013). There is a need for robust comparisons

between stem cell populations obtained from the different umbilical cord compartments to identify the optimum source of MSCs from the cord. Standardization between groups needs to be achieved in order to compare results as to ensure reliable stem-like properties of isolated MSCs. Product quality, safety, and efficiency of MSCs is required to be evaluated before regulatory approval can be obtained for future clinical trials. The use of ASCs in the clinical setting is faced with similar concerns as for umbilical cord. There is a need to develop good manufacturing practices for producing clinical-grade human SVF cells or ASCs. Samples (tissues or cells) need to be screened for viral and other infectious agents, the manufacturing process needs to be standardized with the ability to define the biological characteristics of the cells, and quality control and assurance needs to be attained (Gimble *et al.*, 2010, Gimble *et al.*, 2007). There is a demand for minimally manipulated adipose-derived cell products. In an attempt to provide adipose-derived cells directly to the surgeon while in the operating room, Cytori Therapeutics (San Diego, CA, USA) have developed a closed device for harvesting the SVF.

Taken together, the present study describes the isolation of MSCs from adipose tissue and MSC-like cells from Wharton's jelly. Differences in proliferation, phenotype, and differentiation were noted. Further investigation into these differences is needed. This study did, however, reveal that biological differences do exist between stem cells from different sources. Future studies will also investigate the potential of each MSC source for therapeutic purposes.

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## APPENDIX A: Adipose tissue informed consent

### **PATIENT INFORMATION LEAFLET AND INFORMED CONSENT FORM**

**(Each patient must receive, read and understand this document before the start of the study)**

#### **STUDY TITLE**

The isolation, characterisation and differentiation of mesenchymal stem cells from umbilical cord blood, Wharton's jelly and adipose tissue.

**Dear Patient/Participant:** \_\_\_\_\_

#### **INTRODUCTION**

You are invited to participate in a research study that is being carried out by the Department of Immunology at the University of Pretoria. This information leaflet is to help you to decide if you would like to participate. Before you agree to take part in this study you should fully understand what is involved. No selection criteria will be applied. Any donor will be eligible to participate (donate tissue). If you have any questions, which are not fully explained in this leaflet, do not hesitate to ask the investigator. You should not agree to take part unless you are completely happy about all the procedures involved. Your personal health will not be compromised at all by the procedures. These procedures have already been discussed with your doctor beforehand.

#### **THE PURPOSE OF THE STUDY**

Researchers at the University of Pretoria would like to investigate the healing properties of adult stem cells for possible future application, in regenerative medicine. These adult stem cells, found in **fat (adipose tissue)**, could potentially be used to cure patients with various kinds of injuries or diseases. In order to use these cells to cure humans in the future, researchers must first study their behaviour and growth, in tissue cultures or animal models. The collection of adult stem cells does not make use of any unethical procedures.

#### **HOW IS ADIPOSE TISSUE COLLECTED**

During various normal plastic surgery operations, adipose tissue (fat) will be aspirated (sucked out), and discarded. This adipose tissue according to the surgical doctor does not serve a purpose to the patient's physique anymore. This discarded fat could serve a very important purpose to researchers in the field of regenerative medicine.

No additional fat will be collected, only the fat that the doctor would normally discard. The fat will be collected in the form of lipoaspirate (from liposuction) or from fat removed during abdominoplasty surgery.

There will be no added risks or discomfort with the collection of the adipose tissue other than normally associated with the specific procedure the patient will experience during normal operative procedures.

### **WHAT IS EXPECTED**

Consent should be given by you (the patient) to the researchers to receive your discarded fat from your doctor. The consent will also allow the researchers to grow, differentiate and study the isolated stem cells from the fat tissue.

### **CONFIDENTIALITY**

No personal information will be collected from you, the participant. Each participant will be assigned a specific code and this code will be the only information that the researchers will have. So no one will be able to identify you. Research reports and articles in scientific journals will not include any information that may identify you.

It might however be important for the doctors or researchers involved in this study to convey medical information to medical personnel or appropriate Research Ethics Committees. In such a case, you hereby authorise your investigator to release your medical records to regulatory health authorities or an appropriate Research Ethics Committee. These records will only be utilised by them in order for them to carry out their obligations toward this study, while always acting in your best interest.

### **ETHICAL APPROVAL**

The protocol involved for this study was submitted to the Research Ethics Committee. This study has received written approval from the Research Ethics Committee of the Faculty of Health Sciences at the University of Pretoria. The study is structured in accordance with the Declaration of Helsinki, which deals with the recommendations of guiding doctors in biomedical research involving humans.

### **RIGHTS OF THE PARTICIPANT**

Your participation in this study is entirely voluntary and you can refuse to participate or withdraw consent at any time without stating any reason. Your withdrawal will not affect your access to medical care or the quality of medical care that you will receive. Your participation or withdrawal from the study would not affect you in any way.

### **FINANCIAL GAIN OR LOSS**

There will be no financial gain or loss to your account, should you participate or withdraw from the study. This research could potentially lead to future profitable treatments. However, you will not have access to these profits. There will be no additional financial costs for you to participate in the study.

**The participant has no legal remedy and will not share in any financial gain that may be derived from the study**

### **INFORMATION AND CONTACT PERSON**

If at any time you would like to find out more information or have any questions regarding the study, please do not hesitate to contact the researchers.

Ms. Fiona van Vollenstee: 082 859 4239

Ms. Karlien Kallmeyer: 073 507 0103

Dr. Marnie Potgieter: 083 996 0078

Prof. MS Pepper: 012 420 3845 or 012 420 5317

## **INFORMED CONSENT**

I confirm that the person asking my consent to take part in this study has told me about the nature, process, risks, discomforts and benefits of the study. I have also received, read and understood the above written information (Information Leaflet and Informed Consent) regarding the study. I am aware that the results of the study, including personal details, will be anonymously processed into research reports. I am participating willingly. I have had time to ask questions and have no objection to participate in the study. I understand that there is no penalty should I wish to discontinue with the study and my withdrawal will not affect my access to medical care or the quality of medical care I will receive.

I have received a copy of this informed consent agreement.

Participant full names (print): \_\_\_\_\_

Participant signature: \_\_\_\_\_ Date: \_\_\_\_\_

Investigator full names (print): \_\_\_\_\_

Investigator signature: \_\_\_\_\_ Date: \_\_\_\_\_

Witness full names (print): \_\_\_\_\_

Witness signature: \_\_\_\_\_ Date: \_\_\_\_\_

Witness full names (print): \_\_\_\_\_

Witness signature: \_\_\_\_\_ Date: \_\_\_\_\_

You hereby give the researchers permission to perform routine HIV, hepatitis B and hepatitis C tests on your cord blood sample since it is important for our work that we only work with tissue that are negative for these infections. If the researchers detect HIV or hepatitis B or C in the blood sample, the codified sample details will be sent to Prof. Piet Coetzee, who will notify you. If you do not wish us to test your blood for HIV or hepatitis B or hepatitis C, or if you do not wish to know the results of these tests, we will not be able to include you in the study. In the case of an HIV positive result, you will be counselled and treated by qualified medical personnel.

Patient signature: \_\_\_\_\_

## **INFORMED CONSENT**

I confirm that the person asking my consent to take part in this study has told me about the nature, process, risks, discomforts and benefits of the study. I have also received, read and understood the above written information (Information Leaflet and Informed Consent) regarding the study. I am aware that the results of the study, including personal details, will be anonymously processed into research reports. I am participating willingly. I have had time to ask questions and have no objection to participate in the study. I understand that there is no penalty should I wish to discontinue with the study and my withdrawal will not affect my access to medical care or the quality of medical care I will receive.

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Investigator signature: \_\_\_\_\_ Date: \_\_\_\_\_

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Witness full names (print): \_\_\_\_\_

Witness signature: \_\_\_\_\_ Date: \_\_\_\_\_

You hereby give the researchers permission to perform routine HIV, hepatitis B and hepatitis C tests on your cord blood sample since it is important for our work that we only work with tissue that are negative for these infections. If the researchers detect HIV or hepatitis B or C in the blood sample, the codified sample details will be sent to Prof. Piet Coetzee, who will notify you. If you do not wish us to test your blood for HIV or hepatitis B or hepatitis C, or if you do not wish to know the results of these tests, we will not be able to include you in the study. In the case of an HIV positive result, you will be counselled and treated by qualified medical personnel.

Patient signature: \_\_\_\_\_

## APPENDIX B: Umbilical cord and cord blood informed consent form

### **PATIENT INFORMATION LEAFLET AND INFORMED CONSENT FORM**

*(Each patient must receive, read and understand this document before the start of the study)*

#### **STUDY TITLE**

The isolation, characterisation and differentiation of mesenchymal stem cells from umbilical cord blood, Wharton's jelly and adipose tissue.

**Dear Patient/Participant:** \_\_\_\_\_

#### **INTRODUCTION**

You are invited to participate in a research study that is being carried out by the Department of Immunology at the University of Pretoria. This information leaflet is to help you to decide if you would like to participate. Before you agree to take part in this study you should fully understand what is involved. Any donor will be eligible to participate (donate tissue) in this study. If you have any questions, which are not fully explained in this leaflet, do not hesitate to ask the investigator. You should not agree to take part unless you are completely happy about all the procedures involved. You or your baby's personal health will not be compromised at all by the procedures. These procedures have already been discussed with your doctor beforehand.

#### **THE PURPOSE OF THE STUDY**

Researchers at the University of Pretoria would like to investigate the healing properties of adult stem cells for possible future application in regenerative medicine. The adult stem cells, from the **umbilical cord and umbilical cord blood**, are found in the afterbirth (placenta). Umbilical cord (UC) and cord blood (UBC), could potentially be used to cure patients with various kinds of injuries or diseases. In order to use these cells to cure humans in the future, researchers must first study their behaviour and growth, in tissue cultures and/or animal models. The collection of adult stem cells does not make use of any unethical procedures.

#### **UMBILICAL CORD AND CORD BLOOD COLLECTION**

Under normal conditions, the umbilical cord (Wharton's jelly) and placenta are discarded after a baby is born, since they serve no further purpose for either the mother or the baby. Researchers could however use the normally discarded cord and the cord blood for research purposes. After the birth of the baby, the umbilical cord is cut and cord blood will be collected from the cord. A 15 cm long piece of the cord will then be cut from the cord and the rest will be discarded as normal. Collecting the cord and the cord blood does not harm the mother or the baby in any way. The collection can only take place at the time of delivery and is performed by your doctor.

There are no risks or discomfort involved with the collection of the cord or the cord blood, for you or your baby. The birth of the child would still render the normal risks and discomforts associated with your normal birth procedure.

## **CONFIDENTIALITY**

No personal information will be collected from you, the participant. Each participant will be assigned a specific code and this code will be the only information that the researchers will have. So no one will be able to identify you or your baby. Research reports and articles in scientific journals will not include any information that may identify you or your baby.

It might however be important for the doctors or researchers involved in this study to convey medical information to medical personnel or appropriate Research Ethics Committees. In such a case, you hereby authorise your investigator to release your medical records to regulatory health authorities or an appropriate Research Ethics Committee. These records will only be utilised by them in order for them to carry out their obligations toward this study, while always acting in your and your baby's best interest.

## **ETHICAL APPROVAL**

The protocol involved for this study was submitted to the Research Ethics Committee. This study has received written approval from the Research Ethics Committee of the Faculty of Health Sciences at the University of Pretoria. The study is structured in accordance with the Declaration of Helsinki, which deals with the recommendations of guiding doctors in biomedical research involving humans.

## **RIGHTS OF THE PARTICIPANT**

Your participation in this study is entirely voluntary and you can refuse to participate or withdraw consent at any time without stating any reason. Your withdrawal will not affect your or your baby's access to medical care or the quality of medical care that you or your baby will receive. Your participation or withdrawal from the study would not affect you or your baby in any way.

## **FINANCIAL GAIN OR LOSS**

There will be no financial gain or loss to you, or your baby's account, should you participate or withdraw from the study. This research could potentially lead to future profitable treatments. However, you or your baby will not have access to these profits. There will be no additional financial costs for you to participate in the study.

**The participant has no legal remedy and will not share in any financial gain that may be derived from the study**

## **INFORMATION AND CONTACT PERSON**

If at any time you would like to find out more information or have any questions regarding the study, please do not hesitate to contact the researchers.

Ms. Fiona van Vollenstee: 082 859 4239

Ms. Karlien Kallmeyer: 073 507 0103

Dr. Marnie Potgieter: 083 996 0078

Prof. M.S. Pepper: 012 420 3845 or 012 420 5317

## **INFORMED CONSENT**

### **WHAT IS EXPECTED?**

Consent should be given by you (the mother/patient) to the researchers, in order to receive your normally discarded:

- |                                 |                              |                             |
|---------------------------------|------------------------------|-----------------------------|
| i. <b>Umbilical cord</b>        | <input type="checkbox"/> Yes | <input type="checkbox"/> No |
| ii. <b>Umbilical cord blood</b> | <input type="checkbox"/> Yes | <input type="checkbox"/> No |

from your doctor after delivery of your baby. The consent will also allow the researchers to grow, differentiate and study the isolated stem cells from the umbilical cord blood.

I confirm that the person asking my consent to take part in this study has told me about the nature, process, risks, discomforts and benefits of the study. I have also received, read and understood the above written information (Information Leaflet and Informed Consent) regarding the study. I am aware that the results of the study, including personal details, will be anonymously processed into research reports. I am participating willingly. I have had time to ask questions and have no objection to participate in the study. I understand that there is no penalty should I wish to discontinue with the study and my withdrawal will not affect my or my baby's access to medical care or the quality of medical care my baby and I will receive.

**I have received a copy of this informed consent agreement.**

Participant full names (print): \_\_\_\_\_

Participant signature: \_\_\_\_\_ Date: \_\_\_\_\_

Investigator full names (print): \_\_\_\_\_ Prof. Michael S. Pepper

Investigator signature: \_\_\_\_\_ Date: \_\_\_\_\_

Witness full names (print): \_\_\_\_\_

Witness signature: \_\_\_\_\_ Date: \_\_\_\_\_

Witness full names (print): \_\_\_\_\_

Witness signature: \_\_\_\_\_ Date: \_\_\_\_\_

You hereby give the researchers permission to perform routine HIV, hepatitis B and hepatitis C tests on your cord and cord blood sample since it is important for our work that we only work with tissue that are negative for these infections. If the researchers detect HIV or hepatitis B or C in the cord and/or cord blood sample, the codified sample details will be sent to Dr. Hennie Lombaard, who will notify you. If you do not wish us to test your sample(s) for HIV or hepatitis B or hepatitis C, or if you do not wish to know the results of these tests, we will not be able to include you in the study. In the case of an HIV positive result, you will be counselled and treated by qualified medical personnel.

Patient signature: \_\_\_\_\_

## **INFORMED CONSENT**

### **WHAT IS EXPECTED?**

Consent should be given by you (the mother/patient) to the researchers, in order to receive your normally discarded:

- |                                 |                              |                             |
|---------------------------------|------------------------------|-----------------------------|
| i. <b>Umbilical cord</b>        | <input type="checkbox"/> Yes | <input type="checkbox"/> No |
| ii. <b>Umbilical cord blood</b> | <input type="checkbox"/> Yes | <input type="checkbox"/> No |

from your doctor after delivery of your baby. The consent will also allow the researchers to grow, differentiate and study the isolated stem cells from the umbilical cord blood.

I confirm that the person asking my consent to take part in this study has told me about the nature, process, risks, discomforts and benefits of the study. I have also received, read and understood the above written information (Information Leaflet and Informed Consent) regarding the study. I am aware that the results of the study, including personal details, will be anonymously processed into research reports. I am participating willingly. I have had time to ask questions and have no objection to participate in the study. I understand that there is no penalty should I wish to discontinue with the study and my withdrawal will not affect my or my baby's access to medical care or the quality of medical care my baby and I will receive.

**I have received a copy of this informed consent agreement.**

Participant full names (print): \_\_\_\_\_

Participant signature: \_\_\_\_\_ Date: \_\_\_\_\_

Investigator full names (print): \_\_\_\_\_ Prof. Michael S. Pepper

Investigator signature: \_\_\_\_\_ Date: \_\_\_\_\_

Witness full names (print): \_\_\_\_\_

Witness signature: \_\_\_\_\_ Date: \_\_\_\_\_

Witness full names (print): \_\_\_\_\_

Witness signature: \_\_\_\_\_ Date: \_\_\_\_\_

You hereby give the researchers permission to perform routine HIV, hepatitis B and hepatitis C tests on your cord and cord blood sample since it is important for our work that we only work with tissue that are negative for these infections. If the researchers detect HIV or hepatitis B or C in the cord and/or cord blood sample, the codified sample details will be sent to Dr. Hennie Lombaard, who will notify you. If you do not wish us to test your sample(s) for HIV or hepatitis B or hepatitis C, or if you do not wish to know the results of these tests, we will not be able to include you in the study. In the case of an HIV positive result, you will be counselled and treated by qualified medical personnel.

Patient signature: \_\_\_\_\_

## APPENDIX C: Ethical clearance

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- \* FWA 00002567, Approved dd 22 May 2002 and Expires 13 Jan 2012.
- \* IRB 0000 2235 IORG0001762 Approved dd 13/04/2011 and Expires 13/04/2014.



UNIVERSITEIT VAN PRETORIA  
UNIVERSITY OF PRETORIA  
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee  
Fakulteit Gesondheidswetenskappe Navorsingsetiekomitee

DATE: 30/09/2011

NUMBER	180/2011
TITLE	Isolation and characterisation of mesenchymal stem cells from human tissues
INVESTIGATOR	Karlien Kallmeyer Dept: Immunology; University of Pretoria. Cell: 0735070103 E-Mail: <a href="mailto:karlien.kallmeyer@gmail.com">karlien.kallmeyer@gmail.com</a>
SUPERVISOR	Prof Michael S Pepper E-Mail: <a href="mailto:michael.pepper@up.ac.za">michael.pepper@up.ac.za</a>
SPONSOR	None
STUDY DEGREE	MSc Medical Immunology
MEETING DATE	28/09/2011

The Protocol was approved on 28/09/2011 by a properly constituted meeting of the Ethics Committee subject to the following conditions:

1. The approval is valid for a 2 year period [till the end of December 2013], and
2. The approval is conditional on the receipt of 6 monthly written Progress Reports, and
3. The approval is conditional on the research being conducted as stipulated by the details of the documents submitted to and approved by the Committee. In the event that a need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Members of the Research Ethics Committee:

Prof M J Bester	(female) BSc (Chemistry and Biochemistry); BSc (Hons)(Biochemistry); MSc(Biochemistry); PhD (Medical Biochemistry)
Prof R Delport	(female) BA et Scien, B Curationis (Hons) (Intensive care Nursing), M Sc (Physiology), PhD (Medicine), M Ed Computer Assisted Education
Prof JA Ker	MBChB; MMed(Int); MD – Vice-Dean (ex officio)
Dr NK Likibi	MBB HM – Representing Gauteng Department of Health MPH
Dr MP Mathebula	(female) Deputy CEO: Steve Biko Academic Hospital; MBCHB, PDM, HM
Prof A Nienaber	(female) BA(Hons)(Wits); LLB; LLM; LLD(UP); PhD; Dipl.Datametrics(UNISA) – Legal advisor
Mrs MC Nzeku	(female) BSc(NUL); MSc(Biochem)(UCL, UK) – Community representative
Prof L M Ntlhe	MbChB (Natal) FCS (SA)
Snr Sr J Phatoli	(female) BCur(Eet.A); BTec(Oncology Nursing Science) – Nursing representative
Dr R. Reyniers	MBChB (Prêt), FCPaed (CMSA) MRCPCH (Lon) Cert Med. Onc (CMSA)
Dr T Rossouw	(female) MBChB (cum laude); M.Phil (Applied Ethics) (cum laude), MPH (Biostatistics and Epidemiology (cum laude), D.Phil
Dr L Schoeman	(female) B.Pharm, BA(Hons)(Psych), PhD – Chairperson: Subcommittee for students' research
Mr Y Sikweyiya	MPH; SARETI Fellowship in Research Ethics; SARETI ERCTP; BSc(Health Promotion)Postgraduate Dip (Health Promotion) – Community representative
Dr R Sommers	(female) MBChB; MMed(Int); MPharmMed – Deputy Chairperson
Prof TJP Swart	BChD, MSc (Odont), MChD (Oral Path), PGCHE – School of Dentistry representative
Prof C W van Staden	MBChB; MMed (Psych); MD; FCPsych; FTCL; UPLM - Chairperson

**DR R SOMMERS;** MBChB; MMed(Int); MPharmMed.

Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

◆ Tel:012-3541330	◆ Fax:012-3541367 / 0866515924	◆ E-Mail: <a href="mailto:mandy@med.up.ac.za">mandy@med.up.ac.za</a>
◆ Web: //www.healthethics-up.co.za	◆ H W Snyman Bld (South) Level 2-34	◆ Private Bag x 323, Arcadia, Pta, S.A., 0007

MS: dd 2011/11/08: C:\Documents and Settings\user\My Documents\Protokolle\Grade brieve\Letters 2011\180.2011.DOC

## APPENDIX D: Table comparing induction media of different investigators

Reference	Lineage	Cell density	Stain	Passage	Induction time (days)	Medium	FBS	Antibiotics	Dexamethasone	3-isobutyl-methylxanthine	Indomethacin	Ascorbate-2-phosphate	B-glycerophosphate			TGF-β1	TGF-β3	BMP-6	Proline	Pyruvate	ITS + premix	
Vieira et al. (2010)	Adipose	-	Oil red O	3	21	DMEM-Ig	10%		1 µM	500 µM	60 µM			5 µg/ml								
	Bone	-	Von Kossa		21		10%		0.1 µM			50 µM	10 mM									
	Cartilage	2.5 x 10 <sup>5</sup>	Toluidine Blue		21	DMEM-Ig	-		100 nM			50 µM			10 ng/ml						1 mM	1%
Bieback et al. (2004)	Adipose		Oil red O			Induction & maintenance	10%		1 µM	0.5 mM	0.2 mM			10 µg/ml								
	Bone	3.1 x 10 <sup>3</sup> /cm <sup>2</sup>	Von Kossa		21	CellSystems	10%		0.1 µM			0.05 mM	10 mM									
	Cartilage	2.5 x 10 <sup>5</sup>	Safranin O		4 wks	CellSystems												10 ng/ml				
Waterman et al. (2010)	Adipose		Oil Red O			DMEM-hg (induction)	10%		1 µM	0.5 mM	100 µM			10 µg/ml								
	Bone	3 x 10 <sup>4</sup> cells/well (6-well)	40 mM Alizarin Red							10 <sup>-8</sup> M												
	Cartilage	2.5 x 10 <sup>5</sup>	Safranin O			DMEM-hg + ITS			0.1 µM			50 µM	10 mM					10 ng/ml			2 mM	
Bunnell et al. (2008)	Adipose		0.5% Oil Red O						0.5 µM	0.5 mM	50 µM											
	Bone		40 mM Alizarin Red						1 nM			50 µM	2 mM									
	Cartilage		TB			DMEM-hg			10 <sup>-7</sup> M			50 µg/ml					10 ng/ml	500 ng/ml	40 µg/ml	100 µg/ml	50 mg/ml	
Bonab et al. (2006)	Adipose		Oil Red O			DMEM-Ig	10%	0.5 µM Hydrocortisone		0.5 µM	60 µM			10 µg/ml								
	Bone		Von Kossa			DMEM-Ig	10%	"	0.1 µM			50 µM	10 µM									
	Cartilage																					
Sotiropoulou et al. (2006)	Adipose		Sudan Black IV			DMEM-hg	2%	50 µg/ml Gentamicin		0.5 µM	0.5 mM	60 µM										
	Bone		Von Kossa			DMEM-Ig	10%	"	10 <sup>-7</sup> M			50 µM	10 mM									
	Cartilage																					

Reference	Lineage	Cell density	Stain	Pass age	Induction time (days)	Medium	FBS	Antibiotics	Dexam ethasone	3-isobuty l-methyl xanthine	Indome thacin	Ascorb ate-2-phosphate	B-glycero l-phosphate	Insulin	TGF-β1	TGF-β3	BMP-6	Proline	Pyruvate	ITS + premix
Rada et al. (2011)	Adipose																			
	Bone		Alizarin Red		21	α-MEM	10%	1%	10 <sup>-8</sup> M			50 µg/ml	10 mM							
	Cartilage	5 x 10 <sup>6</sup>	Safranin O		21	DMEM	10%	1%	1 mM			17 mM			10 ng/ml			35 mM	0.1 M	✓
Lund et al. (2009)	Adipose	300 cells/cm <sup>2</sup>	Oil Red O		14	DMEM/F12	10%		0.1 µM	0.45 mM	0.2 mM			170 nM					1 µM ROSIGLITAZONE	
	Bone		Alizarin Red			DMEM/F12	10%		0.1 µM			50 µM	10 mM						0.5 µM Calcitriol	
	Cartilage		Alcian Blue G8			DMEM-hg						150 µM				10 ng/ml		0.34 mM		✓
Kasten et al. (2008)	Adipose	3.5 x 10 <sup>6</sup> /24 well	Oil Red O		2	DMEM-hg	10%	1% P/S	1 µM	0.5 mM	0.2 mM			0.01 mg/ml						
	Bone		Alizarin Red			DMEM-hg	10%	P: 100 IE/ml S: 100 µg/ml	0.1 µM			0.17 mM	10 mM							
	Cartilage	1 x 10 <sup>6</sup> cells	TB			DMEM-hg			0.1 µM			0.17 mM	1.25 mg/ml BSA		10 ng/ml		0.35 mM	1 mM	1%	
Meuleman et al. (2006)	Adipose		Oil Red O		1	α-MEM			1 µM	60 µM				5 µg/ml						
	Bone		Von Kossa			α-MEM			0.1 µM			60 µM	10 mM							
	Cartilage	2 x 10 <sup>5</sup>				DMEM	1%					50 µM		0.5 µg/ml	10 ng/ml TGF-β					
Kedong et al. (2010)	Adipose	5 x 10 <sup>4</sup> /24-well	Oil Red		14	IMDM	10%		1 µM	0.1 mM	0.2 µM			10 µM						
	Bone		Von Kossa						0.1 µM			50 µM	10 mM							
	Cartilage		Connexin43						0.1 µM			50 µg/ml				10 ng/ml	500 ng/ml		100 µg/ml	50 mg/ml
Sasaki et al. (2008)	Adipose		Oil Red O		21	DMEM	10%	1%P/1% S/1%AB	0.5 µM	0.5 mM	0.1 mM									
	Bone		Von Kossa					0.1 µM				50 µM	10 mM							
	Cartilage		Toluidine Blue					0.1 µM				50 µM			10 ng/ml TGF-β		40 µg/ml	100 µg/ml		

Induction media

Reference	Lineage	Cell density	Stain	Pass age	Induction time (days)	Medium	FBS	Antibiotics	Dexam ethasone	3-isobuty l-methyl xanthine	Indomethacin	Ascorbate-2-phosphate	B-glycero-l-phosphate	Insulin	TGF-β1	TGF-β3	BMP-6	Proline	Pyruvate	ITS + premix
Zuk et al. (2001)	Adipose		Oil Red O	1	14	DMEM	10%	1%	1 μM	0.5 mM	200 μM			10 μM						
	Bone		AP or von Kossa	1	14	DMEM	10%	1%	1 μM			50 μM	10 mM							
	Cartilage	8 x 10 <sup>6</sup> cells/ml	Alcian blue	1	14	DMEM	1%	1%			50 nM		6.25 μg/ml	10 ng/ml						
Bunnell et al. (2008)	Adipose		Oil Red O or BODIPY & DAPI		12-14	α-MEM	20%	1%	0.5 μM	0.5 mM	50 μM									
	Bone		Alizarin Red (pH 4.1)		14	α-MEM	20%	1%	1 nM		50 μM	2 mM								
	Cartilage		Toluidine blue		21	DMEM-HG			10 <sup>-7</sup> M		50 μg/ml				10 ng/ml	500 ng/ml	40 μg/ml	100 μg/ml	50 mg/ml	
Wagner et al. (2005)	Adipose	1 to 2 x 10 <sup>4</sup> cells/cm <sup>2</sup>	Oil Red O		14 (stained after 21)		10%		1 μM	0.5 mM	200 μM			10 μM						
	Bone	1 to 2 x 10 <sup>4</sup> cells/cm <sup>2</sup>	AP or von Kossa		21	DMEM	10%		10 <sup>-7</sup> M		0.2 mM	10 mM								
	Cartilage	Not done																		
Reger et al. (2008)	Adipose	1 x 10 <sup>4</sup> cells/cm <sup>2</sup>	Oil Red O		21	α-MEM	16.5%	1%	0.5 μM	0.5 μM	50 μM									
	Bone	1 x 10 <sup>4</sup> cells/cm <sup>2</sup>	Alizarin Red		21	α-MEM	16.5%	1%	10 nM		50 μM	20 mM								
	Cartilage	400 cells/μl	Toluidine blue		21	DMEM-HG			10 <sup>-7</sup> M		50 μg/ml				10 ng/ml	500 ng/ml	40 μg/ml	100 μg/ml	5 ml	
Le Blanc et al. (2003)	Adipose	2.1 x 10 <sup>4</sup> cells/cm <sup>2</sup>	Oil Red O		21	DMEM-LG	10%	1%	1 μM	0.5 mM	100 μM			10 μg/ml						
	Bone	3.1 x 10 <sup>3</sup> cells/cm <sup>2</sup>	Von Kossa		21	DMEM-LG	10%	1%	0.1 μM		0.05 mM	10 mM								
	Cartilage	7.5 x 10 <sup>5</sup> cells	Alcian Green		21	DMEM-HG			0.1 μM		0.17 mM				0.01 μg/ml		0.35 mM	1 mM	✓	
Baptista et al. (2009)	Adipose		Oil Red O	3	14	DMEM	20%		1 μM	0.5 μM	200 μM			10 μM						
	Bone		Von Kossa	3	14	DMEM	10%		0.01 μM		5 x 10 <sup>-6</sup> M	10 <sup>-2</sup> M								
	Cartilage	1.5 x 10 <sup>5</sup> cells	Alcian Blue	3	21	MSC Chondrogenic differentiation BulletKit supplemented with 10 ng/ml TGF-β3 (Cambrex)														

Reference	Lineage	Cell density	Stain	Pass age	Induction time (days)	Medium	FBS	Antibiotics	Dexam ethasone	3-isobutyl methyl xanthine	Indomethacin	Ascorbate-2-phosphate	B-glycerophosphate	Insulin	TGF-β1	TGF-β3	BMP-6	Proline	Pyruvate	ITS + premix
Liu et al. (2007)	Adipose	6 cells/cm <sup>2</sup>	Oil Red O	2	3	DMEM	10%	1%	1 μM	0.5 mM	200 μM			10 μM						
	Bone		Alizarin Red S	2	14	DMEM	10%	1%	0.1 μM		50 μM	10 mM								
	Cartilage	2 x 10 <sup>5</sup> cells	COLII	2	3 & 14 21			1%	10 <sup>-7</sup> M		50 nM		6.25 μg/ml	10 ng/ml				10 ng/ml	40 μg/ml	100 μg/ml
Yoshimura et al. (2006)	Adipose	2000 cells/cm <sup>2</sup>	Oil Red O	3-5	28	DMEM	10%	1%	1 μM	0.5 mM	200 μM			10 μM						
	Bone	2000 cells/cm <sup>2</sup>	2.5% Silver nitrate	3-5	28	DMEM	10%	1%	0.1 μM		50 μM	10 mM								
	Cartilage	2000 cells/cm <sup>2</sup>	Alcian Blue 8 GX		28	DMEM	1%	1%			50 nM		6.25 μg/ml	10 ng/ml						
Lu et al. (2006)	Adipose	3000 cells/cm <sup>2</sup>	Oil Red O	2	14	DMEM	10%		1 μmol/L	0.5 mmol/L	60 μmol/L			5 μg/ml						
	Bone	3000 cells/cm <sup>2</sup>	Von Kossa	2	14	DMEM-LG	10%		100 nmol/L		0.2 mmol/L	10 mmol/L								
	Cartilage																			
Francis et al. (2010)	Adipose		Oil Red O		14	DMEM-LG	10%	1%	1 μM	0.5 mM	200 μM			10 μM						
	Bone		Alizarin Red		14	DMEM-HG	10%	1%	Used 0.01μM 1,25-dihydroxyvitamin D <sub>3</sub> instead		50 μM	10 mM								
	Cartilage	2 x 10 <sup>5</sup> cells	Safranin O	21	DMEM-LG	1%	1%			50 nM		6.25 μg/ml	10 ng/ml							✓
Li et al. (2005)	Adipose		Oil Red O		21	DMEM	10%	✓	1 μM	0.5 mM				1 μg/ml						
	Bone		Alizarin Red		21	DMEM	10%	✓	0.1 μM		50 μg/ml	10 mM								
	Cartilage	4 x 10 <sup>5</sup> cells	Alcian Blue	21	DMEM	0		✓	0.1 μM		50 μg/ml		10 ng/ml					40 μg/ml	100 μg/ml	✓
Shetty et al. (2010)	Adipose	5000-10000 cells/ 8-well plate	Oil Red O		18*	DMEM-HG	10%		1 μM	0.5 mM	60 μM			10 μg/ml						
	Bone	3000-10000 cells/ 8-well plate	Von Kossa		21															
	Cartilage	0.25-0.5 x 10 <sup>5</sup> cells	Alcian Blue		14 to 28	Osteogenic induction medium from Lonza														

\* After 3 days the adipogenic induction medium was changed to adipogenic maintenance (AM medium) medium for 3 days (contains 10 μg/ml insulin and 10% FBS in DMEM-HG), three cycles of induction/maintenance was carried out

## Induction media

Reference	Lineage	Cell density	Stain	Pass age	Inducti on time (days)	Medium	FBS	Antibiotics	Dexam ethaso ne	3-isobut yl-methyl xanthi ne	Indom ethaci n	Ascor bate-2-phosp hate	B-glycer ol-phosp hate	Insulin	TGF- $\beta$ 1	TGF- $\beta$ 3	BMP-6	Prolin e	Pyruva te	ITS + premix
Kern et al. (2006)	Adipose *	2.1x10 <sup>4</sup>	0.18% Oil Red / nucleus Mayer's hematoxylin solution	0-5	3 cycles	MSCGS or DMEM-Ig+ 10% MSCGS till 100%-post confluent/	10%		1 mM	0.5 mM	100mM			10 $\mu$ g/ml						
	Bone	3.1x10 <sup>3</sup>	Alkaline phosphatase /von Kossa/silver nitrate	0-5	2.5 week	MSCGS or DMEM-Ig+ 10% MSCGS till 70-80% confluent/ osteogenic basal medium	10%		100nM			0.2mM Ascorb ate	10mM							
	Cartilage	2.5x10 <sup>5</sup>	Safranin O	0-5	4 weeks	Complete chondrogenic differentiation medium (Cambrex)											10ng/ml			
Farrell et al. (2008)	Adipose		Oil Red O	From passa ge 4	13 days	DMEM 10%FCS		50 $\mu$ g/ml gentamycin; 1.5 $\mu$ g/ml fungizone	1 $\mu$ M	0.5 mM	0.2 mM			0.01 mg/ml						
	Bone		Von Kossa			DMEM 10%FCS			1 $\mu$ M			0.1 mM	10 mM							
	Cartilage		Thionine			DMEM serum free	No seru m					25 $\mu$ g/ml			TGF- $\beta$ 2 10ng/ml			40 $\mu$ g/ml	100mM	1%
Igura et al. (2004)	Adipose	2 x10 <sup>4</sup>	Oil Red O			DMEM high glucose	10% FBS		1 $\mu$ M	0.5 mM	0.2 mM			10 $\mu$ g/ml						
	Bone	1.5 x10 <sup>4</sup>	Von kossa		3 weeks	DMEM low glucose	FBS		0.1 $\mu$ M			50 $\mu$ M	10 mM							
	Cartilage	2 x10 <sup>5</sup>	Toluidin e blue		3 weeks	DMEM low glucose	10% FBS	X amount							X amount	BMP-2 500n g/ml				

Reference	Lineage	Cell density	Stain	Pass age	Induction time (days)	Medium	FBS	Antibiotics	Dexamethasone	3-isobutyl-methyl xanthine	Indomethacin	Ascorbate-2-phosphate	B-glycerophosphate	Insulin	TGF-β1	TGF-β3	BMP-6	Proline	Pyruvate	ITS + premix	
Lee et al. (2004b)	Adipose		Oil Red O		2 weeks	αMEM	10%		1μM	100 μg/ml	60 μM			5 μg/ml							
	Bone		Alizarin red		2 weeks	αMEM	10%		0.1mM			AA 50 μg/ml	10 μM								
	Cartilage	3 x 10 <sup>6</sup> cells/ml	Toluidine blue		3 weeks	αMEM	1%					AA 50 μg/ml		6.25μg/ml	10 ng/ml						
Mitchell et al. (2006)	Adipose	confluent	Oil Red O		9 days	DMEM/F-12 33μM biotin, 17 μM pantothenate	3%	0.25μg rosiglitazone 100U pen/100μg strep/0.25 μg fungizone	1 μM	0.5mM				Bovine Insulin 1μM							
	Bone	confluent	Alizarin Red		3 weeks	DMEM/F-12	10%		10 nM			Na 50 μg/ml	10 mM								
	Cartilage	Not Done																			
Bertolo et al. (2011)	Adipose	5x10 <sup>5</sup> cells/cm <sup>2</sup>	Oil Red O		3 weeks	DMEM/F12 100U/ml pen; /100U/ml strep; 2.5ng/ml amphotericin	5%	100U/ml pen; 1mg/ml strep; 2.5ng/ml amphotericin	1 μM	0.5 mM	0.5 mM			170 mM							
	Bone	5x10 <sup>3</sup> cells/cm <sup>2</sup>	Von Kossa		2 weeks				100 nM			0.05 mM	10 mM								
	Cartilage	5x10 <sup>5</sup> cells/cm <sup>2</sup>	Alcian Blue		4 weeks	DMEM/F12	5%		40ng/ml			50 mg/ml			5 ng/ml			50 mg/ml	1x		
D'Ippolito et al. (2004)	Adipose	10 000 cells/cm <sup>2</sup>	Sudan-IV		3 weeks	α MEM 10% horse serum; 0.5μM hydrocortisone	10%	100u/ml pen; 1mg/ml strep		0.5 mM	60 μM										
	Bone	10 000 cells/cm <sup>2</sup>	Alizarin Red-S		-	α MEM	10%	pen/strep	DMSO 0.001%			100 μM	10 mM								
	Cartilage	250 000 cells/aliquot	-		4 weeks	DMEM-HG			100 nM			50 μg/ml					10 ng/ml	40 μg/ml	Na 100 μg/ml	1x	
Kang et al. (2004)	Adipose	1:10 dilution but grown to 80% confluency	Oil Red O	80% confluence		α-MEM	10%	1% Pen/Strep	10 <sup>-9</sup> M					10 ng/ml							
	Bone		Alizarin Red S		3 weeks				10 <sup>-9</sup> M			AA 50 ng/ml	10 mM								
	Cartilage		Safranin O		3-4 weeks							AA 50 ng		6.25 μg/ml	10 ng/ml						
Curran et al. (2006)	Adipose	5x10 <sup>4</sup> cells/ml																			
	Bone				1-28 days	DMEM	10%		100 nM			100 mM	10 mM								
	Cartilage		Von Kossa			DMEM-HG	1%	FCS	100 nM			100 mM			10 ng/ml			1 mM	1%		

Reference	Lineage	Cell density	Stain	Pass age	Induction time (days)	Medium	FBS	Antibiotics	Dexamethasone	3-isobutyl-methyl xanthine	Indomethacin	Ascorbate-2-phosphate	B-glycerophosphate	Insulin	TGF-β1	TGF-β3	BMP-6	Proline	Pyruvate	ITS + premix
Le Blanc et al. (2003)	Adipose	2.1x10 <sup>4</sup> cell/cm <sup>2</sup>	Oil Red O		21 days	DMEM-LG	10%	1% antibiotic antimycotic	1 μmol/L	0.5 mmol/L	100 μmol/L			10 μg/ml						
	Bone	3.1 x10 <sup>5</sup> cell/cm <sup>2</sup>	Von Kossa		21 days	DMEM-LG	10%		0.1 μmol/L			0.05 mmol/L	10 mmol/L							
	Cartilage	7.5 x10 <sup>5</sup> cell/pell	Alcian green		21 days	DMEM-HG			0.1 μmol/L			0.17 mmol/L		6.25 μg/ml		0.01 μg/ml			1 mmol/L	Other values

**ITS + Premix:**

6.25 μg/ml insulin

6.25 μg/ml transferring

6.25 ng/ml selenous acid

1.25 mg/ml BSA

5.35 mg/ml Linoleic acid