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Exploring the Human Term Placenta as a Novel Source for Stem Cells and Their Application in the Clinic

Celena Heazlewood^{1,3*}, Matthew Cook^{1,3}, Nina Ilic² and Kerry Atkinson^{1,2,3}

¹*Mater Medical Research Institute, Level 3, Aubigny Place,
Raymond Terrace, South Brisbane,*

²*Mater Adult Hospital, South Brisbane,*

³*University of Queensland, School of Medicine, Brisbane,
Australia*

1. Introduction

The human placenta is a fetomaternal organ, consisting of both fetal (amnion and chorion) and maternal (decidua) tissues (Parolini *et al.*, 2008). This complex organ begins to develop within a few days after fertilisation and is fundamental to the development and survival of the fetus throughout gestation. The placenta also acts as the lung, kidney and digestive system for the growing fetus and protects the fetus from infection throughout development (Parolini *et al.*, 2008).

The placenta is of interest to stem cell biologists since it is rich in stem cells and their progenitors. A stem cell is defined as a cell that has the ability to self renew and that can differentiate to progeny (daughter cells) of one or more of the germ layers. Stem cells are classified as either totipotent, pluripotent or multipotent. The most primitive stem cell, with the greatest ability to differentiate, is the totipotent cell of the zygote or first blastomere (Mitalipov and Wolf, 2009). This cell occurs from the first division of the zygote and has the ability to form an entire organism. Once these totipotent cells begin to divide, they give rise to the embryo and placenta. At the 32-cell stage of the embryo, known as the morula, the cells have lost their totipotency and are pluripotent (Mitalipov and Wolf, 2009, Witkowska-Zimny and Wrobel, 2011). These pluripotent cells contribute to all three germ layers in the developing embryo, the endoderm, mesoderm and ectoderm. Stem cells with limited differentiation ability are known as multipotent stem cells and can differentiate into a number of cell types within the same germ layer. Multipotent stem cells are committed to a particular organ or tissue and are the most mature stem cell type (Witkowska-Zimny and Wrobel, 2011).

1.1 Types of stem cells

1.1.1 Pluripotent stem cells

Embryonic stem (ES) cells derived from the inner cell mass of the very early embryo, are capable of giving rise to all three germ layers, and are pluripotent stem cells. Induced

pluripotent stem cells (iPS) have many characteristics in common with ES, but are derived by re-programming adult stromal cells (Mitalipov and Wolf, 2009). As their name implies, they too are pluripotent and both types can divide indefinitely, and have the potential to develop benign teratomas consisting of tissues of all three germ layers. Regardless of this potential complication, neural cells differentiated from human ES cells are already in clinical trial (www.geron.com). The differentiated progeny of both ES and iPS cells (for example, neurons, cardiomyocytes, hepatocytes) are likely to be rejected by the immune system of an allogeneic recipient and the use of such progeny is likely to require administration of immune suppressive agents after their administration to prevent their immune-mediated rejection. Additionally, there are a number of ethical concerns particularly with the use of ES cells because of the inevitable destruction of the human embryo in the generation of ES cells. This concern does not apply to human iPS cells which can be generated from any postnatal human tissue, including those that are routinely disposed of, such as term placenta after safe delivery of the baby.

1.2 Multipotent stem cells

1.2.1 Haematopoietic stem cells

The best known example of a multipotent stem cell is the haematopoietic stem cell derived from the bone marrow, umbilical cord blood or mobilised peripheral blood. Less well-known is the fact that the placenta is an important source of HSC, at least during mid-gestation in the mouse (Gekas *et al.*, 2008, Gekas *et al.*, 2010). Haematopoietic stem cells (HSC) are the source of all blood cell types and continuously replenish the haematopoietic and immune systems throughout life. They are the best characterised adult stem cell and are the only stem or progenitor cells in routine clinical use today (Appelbaum, 2007). The transplantation of HSC is a potentially curative therapy for immunodeficiencies such as severe combined immune deficiencies, haematological malignancies such as leukaemia, myeloma, and myelodysplasia, and bone marrow failure syndromes such as severe aplastic anaemia. HSC transplantation (HSCT) was pioneered in the 1950s using bone marrow (BM) as the source of HSC, with the first successful allogeneic transplant performed in 1968. Mobilised peripheral blood (mPB) HSC, collected by apheresis, are now the most commonly used source of stem cells for HSC transplantation. However, both of these sources are restricted by the availability of a suitable human leukocyte antigen- (HLA-) matched related or unrelated living donor. The use of BM- or mPB- HSC from a matched unrelated donor or a partially mismatched family member has a higher incidence of potentially fatal, graft-versus-host disease (GVHD), a condition that occurs due to immune attack by donor leukocytes on recipient tissues. Umbilical cord blood (UCB) is now recognised as a promising alternative tissue source of HSC and it has some advantages over conventional sources since it is readily available and easy to collect. Most importantly, UCB can be used in transplants with less than optimal donor-recipient HLA-matching, thus providing broader access compared to BM- and mPB- HSC. The isolation and clinical applications of HSC derived from UCB will be discussed later in this chapter.

1.2.2 Mesenchymal stem cells

Another multipotent stem cell was discovered within the bone marrow by Friedenstein more than forty years ago (Friedenstein *et al.*, 1976). These cells are now commonly known

as mesenchymal stem/stromal cells (MSC) and are cells that give rise to tissues of the mesodermal lineage, including bone, cartilage, muscle, tendons and adipose tissue. Their many advantages include their relative ease of isolation, expansion potential, stable phenotype and compatibility with different delivery methods and formulations (reviewed in "Therapeutic applications of mesenchymal stromal cells" (Brooke *et al.*, 2007)).

It is known that traditionally derived bone marrow (bm) MSC are a rare cell population (~0.001% of BM mononuclear cells) *in vivo*, resulting in a low MSC yield when isolated. Hence, *ex vivo* expansion is required to gain sufficient numbers for clinical applications. In general, MSC are isolated using a density gradient or cell lysis, after which the mononuclear cells are cultured in basal medium such as Dulbecco's modified Eagle's medium and 10 % - 20 % fetal calf serum (FCS) (Pittenger *et al.*, 1999, McBride *et al.*, 2003, Lodie *et al.*, 2002). Cells are subsequently maintained in culture for several days during which contaminating, non-adherent haematopoietic cells such as macrophages are depleted. Human MSC have a characteristic (but not unique) cell surface phenotype of CD90⁺, CD105⁺, CD73⁺, CD44⁺, HLA I⁺, CD45⁻, CD34⁻ CD11b⁻, HLA II⁻ (Pittenger *et al.*, 1999, Javazon *et al.*, 2003, Peister *et al.*, 2004). MSC are unique amongst nucleated mammalian cells in that they stimulate little allogeneic reactivity when administered to MHC-unmatched adult immune competent recipients, perhaps due to their lack, at least in the human, of expression of co-stimulatory cell surface molecules such as CD80 and CD86 (Weiss *et al.*, 2008, Wang *et al.*, 2009). Furthermore, they are actively suppressive of T cell, dendritic cell and B cell function (Weiss *et al.*, 2008, Wang *et al.*, 2009, Jiang *et al.*, 2005) and this is presumably linked to their ability to down-modulate exuberant inflammation, which can subsequently result in pathological remodeling and excessive fibrosis.

It has been proposed that current tissue culture methods used to expand MSC reduce multipotency and result in lower migratory/engraftment capacity of the expanded MSC. It has also been shown that humans and animals show a decreased rate of production of bone marrow mesenchymal stem and progenitor cells with increasing age (Caplan, 1994). Several studies have provided evidence of a strong correlation between age and the proliferative potential exhibited by MSC *in vitro* (Stenderup *et al.*, 2003, Bergman *et al.*, 1996, D'ippolito *et al.*, 1999). Thus, the progenitor pool may be depleted following extensive proliferation. Consequently, this results in a reduced ability to ensure regeneration after injury or disease depending on the age of the MSC (Ringe *et al.*, 2002). Such a decline in the quality of the cells is suboptimal for therapeutic application.

For these reasons, novel sources of MSC are now being investigated for clinical use in diseases in which the regenerative and immunomodulatory functions of MSC may be useful (Barlow *et al.*, 2008b, Chang *et al.*, 2006a, Jones *et al.*, 2007, Brooke *et al.*, 2009). A readily available and younger source that can be obtained by a non-invasive procedure, and which yields large numbers of MSC for *ex vivo* expansion would be an ideal alternative to adult bone marrow.

MSC derived from tissues normally disposed of, such as the term placenta and other gestational tissues that are fetal derived (In 'T Anker *et al.*, 2004, Yen *et al.*, 2005, Bailo *et al.*, 2004, Wulf *et al.*, 2004, De Coppi *et al.*, 2007) have been investigated to see if they fulfill these criteria. This chapter will describe various gestational tissue sources for human MSC as alternatives to bone marrow, the isolation of MSC from these sources and their application in the clinic.

2. The human term placenta

The human term placenta represents an attractive source of MSC due to its ready availability, its easy access without invasive procedures, and lack of the ethical issues that surround the use of embryonic stem cells. The placenta as a whole, represents both fetal (amnion and chorion) and maternal (decidua) components (Figure 1) and is vital for the development and survival of the fetus throughout gestation.

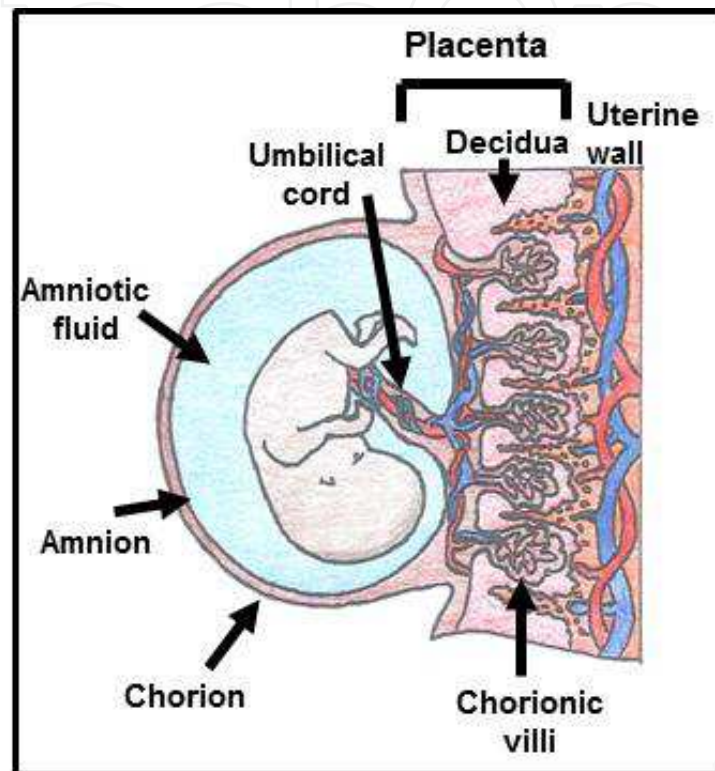


Fig. 1. A schematic diagram showing the developed human placenta. The diagram was adapted from the Merck Manual website. <http://www.merckmanuals.com>

Our group has isolated MSC from the whole placenta (amnion, chorion and decidua). Whole term placentas were collected from consenting healthy volunteer donors scheduled for elective Caesarean sections to minimise microbial contamination. Briefly, the whole placenta was cut into approximately 2 cm² pieces followed by enzymatic digestion using Collagenase Type 1 and DNase I. Ficoll density gradient or cell lysis was performed and cells placed in culture for *ex vivo* expansion. We found that human placental (hp) MSC differed to human bone marrow (hbm) MSC in proliferative capacity, with hbmMSC proliferating more slowly than hpMSC. Human pMSC were shown to be safe when administered into healthy mice at the same or higher dose than those currently used in clinical trials with hbmMSC (Barlow *et al.*, 2008a). Importantly, we also showed that the immune suppressive capacity of hpMSC to decrease T cell alloreactivity in mixed lymphocyte reaction (MLR) was equivalent to that of hbmMSC (Jones *et al.*, 2007). This thus suggested that human placenta is a potentially viable alternative source for human MSC and with this knowledge we are currently investigating placental-derived MSC in several human clinical trials (Brooke *et al.*, 2009).

3. Other gestational tissue sources of MSC

MSC derived from whole term placenta are of maternal origin (Barlow *et al.*, 2008a). Over the past decade much has been discovered about MSC in the developing fetal environment. Fetally-derived MSC are theoretically attractive because they generally have not been exposed to viruses and toxins, may contain less genetic abnormalities than adult tissue-derived MSC, and may have greater proliferative capacity and a greater retention of “stemness” memory. It has been suggested that they have properties intermediate between embryonic and adult stem cells (Guillot *et al.*, 2006). Thus, they may be a superior MSC source for clinical trials than the traditional source which is adult bone marrow.

This section will focus on the isolation of MSC from amniotic fluid (Mareschi *et al.*, 2009, Nadri and Soleimani, 2007, Roubelakis *et al.*, 2007), the amniotic membrane (Soncini *et al.*, 2007, Portmann-Lanz *et al.*, 2006, Diaz-Prado *et al.*, 2010), the chorion (chorion laeve and chorionic villi) (Soncini *et al.*, 2007, Portmann-Lanz *et al.*, 2006, Igura *et al.*, 2004, Poloni *et al.*, 2008), the decidua (Macias *et al.*, 2010, Aghajanova *et al.*, 2010), umbilical cord/Wharton’s jelly (Mitchell *et al.*, 2003, Gonzalez *et al.*, 2010, Reinisch *et al.*, 2007, Petsa *et al.*, 2009) and umbilical cord blood (Mareschi *et al.*, 2001, Bieback *et al.*, 2004, Romanov *et al.*, 2003). This section will also discuss the isolation of haematopoietic stem cells from the umbilical cord blood and the application of these cells.

3.1 Amniotic fluid

Amniotic fluid helps protect the fetus throughout gestation. This unique environment allows the fetus to move freely within the uterus and protects the fetus from mechanical injury.

Amniocentesis is a diagnostic procedure that samples amniotic fluid from 14 weeks gestation until birth. This can be used to isolate amniotic fluid MSC (AF-MSC) for genetic screening purposes. It has been found that amniotic fluid contains a heterogeneous population of many cells including a large portion of epithelioid E-type cells, amniotic fluid-specific AF-type cells and fibroblast F-type cells (Witkowska-Zimny and Wrobel, 2011, Prusa and Hengstschlager, 2002). Although many cell types exist within the amniotic fluid, MSC have been found. It has been estimated that approximately 1% of cells in culture obtained from human amniocentesis are MSC. Only a few studies have successfully isolated single cell-derived MSC clones from amniotic fluid (Tsai *et al.*, 2004, De Coppi *et al.*, 2007, Antonucci *et al.*, 2009, Phermthai *et al.*, 2010, Witkowska-Zimny and Wrobel, 2011).

Amniotic fluid derived-MSC show typical MSC characteristics as well as expression of OCT4, a primitive stem cell marker (Roubelakis *et al.*, 2007). This suggests these cells may be more primitive than adult BM-MSC. Moreover, it has also been reported these cells have a high proliferative potential with over 250 population doublings without doubling time changes and can be differentiated into endodermal and ectodermal lineages *in vitro*. Although these properties seem theoretically more advantageous, it is debatable whether amniotic fluid is a practical and reliable source for generating MSC on a regular basis for clinical trials (Tsai *et al.*, 2004, De Coppi *et al.*, 2007, Antonucci *et al.*, 2009, Phermthai *et al.*, 2010, Witkowska-Zimny and Wrobel, 2011).

3.2 The amniotic membrane

The amnion is derived from the fetus and is the innermost membrane of the placenta. The amniotic membrane encases the amniotic fluid and fetus, and is highly flexible as it expands throughout gestation. It is a thin, avascular membrane and contains an epithelial cell layer and mesenchymal stromal cell layer. The amniotic epithelial cell layer is a single layer of flat, cuboidal and columnar cells in close contact with the amniotic fluid. The amnion epithelial cells are attached to a distinct basal lamina that is connected to the amniotic mesoderm (Blackburn, 2003, Parolini *et al.*, 2008). The amniotic mesoderm layer consists of macrophages and fibroblast-like mesenchymal cells (Parolini *et al.*, 2008). It is from this layer of the amniotic membrane that amniotic MSC (AMSC) can be isolated.

To isolate fetal-derived MSC-like cells from the amniotic membrane, enzymatic digestion is used. The amniotic membrane can be peeled from the chorionic membrane (Figure 1), washed until blood has been removed (until it appears almost translucent) and cut into smaller pieces. It is digested first using trypsin to remove the epithelial layer of cells and second, enzymatically digested to release the stromal cells. A ficoll density gradient or cell lysis can be performed and the mononuclear cells placed in culture. Similar to AF-MSC, these cells are fetally derived and exhibit similar characteristics to BM-MSC (Soncini *et al.*, 2007).

3.3 The chorion

The human chorion consists of the chorionic laeve (membrane), chorionic plate and chorionic villi. The chorionic laeve is the outer fetal membrane composed of layers of polygonal cells consisting of both mesoderm and trophoblast regions (Blackburn, 2003). The chorionic laeve is closely associated but not attached to the amniotic membrane and gives rise to the chorionic plate and villi. During development, the chorionic villi grow outwards into the endometrium to anchor at the decidua basalis (see below). This phenomenon occurs through dividing cytotrophoblasts and syncytiotrophoblasts and ultimately gives rise to a network of villi called the chorionic villi. Chorionic villi develop from the chorionic plate and stretch outwards (like finger-like projections) and attach to the decidua basalis to anchor the placenta in the uterus. The mesenchymal cells form a connective-tissue like support for the blood vessels growing into the villi and can also be found within this region.

To isolate fetal-derived MSC-like cells from the chorionic laeve, enzymatic digestion is suggested. The chorionic laeve is peeled from the amniotic membrane and cut at the chorionic plate. Similar to the amniotic membrane, the chorionic laeve is washed until blood clots have been removed and cut into smaller pieces. It is then digested using collagenase and DNase to release the stromal cells. A ficoll density gradient or cell lysis can be performed and the mononuclear cells placed in culture. Despite the chorionic laeve arising from the fetus, we have found from FISH and genotyping analyses from term chorionic membranes, that maternal cells are present in the cultured MSC population. It is predicted that this is a result of the decidua capularis (maternal derived) fusing to the chorionic laeve early in pregnancy. Hence, we suggest that the chorionic laeve is not an optimal source for fetally derived MSC.

3.4 Chorionic villi

The chorionic villi are fetally derived and intercalates with the maternal decidua. It is responsible for the exchange of nutrients from the mother to the fetus. The chorionic villi are

highly vascularised and consist of syncytiotrophoblasts, cytotrophoblasts and mesenchymal stromal cells. To isolate MSC, a diagnostic test, chorionic villus sampling, can be performed. This procedure is invasive to both mother and baby as it is performed *in utero*. Therefore, a less invasive method is to collect chorionic villi samples after birth (from term placenta). Due to the close interaction with maternal decidua, it is suggested that a dissecting microscope is used to mechanically separate fetal villi from maternal decidua. Despite these efforts, it can be a relatively ineffective method for isolating fetally derived cells with maternal cell contamination rapidly occurring in the cultured cell population. Hence, we do not recommend using this tissue source for isolating fetally derived MSC.

3.5 The decidua

After implantation of the blastocyst (~6-10 days post fertilisation), regional specialisation occurs within the endometrium to accommodate the developing embryo and placenta. The maternal decidua arises from this and forms 3 layers, decidua capularis, decidua basalis and decidua parietalis. The decidua capularis is adjacent to the embryo and fuses to the chorionic laeve as the embryo develops. At term this region of the decidua can not be distinguished from the chorion laeve. The decidua basalis is located between the myometrium and chorionic villi and can be difficult to distinguish. The decidua parietalis forms part of the uterine lining and is a better decidual source for isolating maternal MSC because it contains few invasive fetal cells (trophoblasts).

To obtain decidua parietalis, a suction or vacuum curettage of the uterine wall is used once the baby has been delivered. This is the most sterile technique but may cause complications to the mother such as post-natal uterine bleeding. The tissue is then digested and cells placed in culture.

3.6 Umbilical cord / Wharton's jelly

The life line that connects the fetus to the mother is the umbilical cord. The human umbilical cord allows vital nutrients and oxygen to be exchanged from the mother to the fetus. Within the umbilical cord are two arteries and one vein; these are surrounded by a gelatinous mucoid connective tissue known as Wharton's jelly. These tissues are derived from the extra-embryonic mesoderm, derived from the fetus (Witkowska-Zimny and Wrobel, 2011). The role of Wharton's jelly is to protect and insulate the umbilical cord vessels, and is composed of myofibroblast-like stromal cells, collagen fibers and proteoglycans. In 2003, primitive stem cell types were found to reside within the Wharton's jelly of the umbilical cord (Mitchell *et al.*, 2003). These cells are referred to as umbilical cord mesenchymal stromal/stem cells (UC-MSC).

There are several methods for the isolation of MSC from the umbilical cord. As with the isolation of whole marrow, the isolation of MSC from the umbilical cord includes enzymatic digestion of the tissue, followed by either a density gradient centrifugation or cell lysis. These cells can be isolated in large numbers, approximately 1.5×10^6 cells/cm of the umbilical cord (Weiss *et al.*, 2005). It has been observed that UC-MSC proliferate faster than bmMSC and may be cultured for more than 80 population doublings with no indication of senescence or changes in morphology (Mitchell *et al.*, 2003).

3.7 Umbilical cord blood

The umbilical cord blood is known as a rich source for both MSC and HSC. MSC within the cord blood have been referred to as unrestricted somatic stem cells (USSC). However, there has been controversy over the efficiency and yield of USSC within the cord blood. It is proposed that possible explanations for the controversy involve the variability between donors, the isolation technique, and the use of different culture conditions by independent studies (Da Silva Meirelles *et al.*, 2008). Though the isolation of USSC from umbilical cord blood can be difficult, there have been some recent breakthroughs in attempts to optimise the isolation process. Flynn and colleagues (Bieback *et al.*, 2004, Chang *et al.*, 2006b, Flynn *et al.*, 2007, Kern *et al.*, 2006, Sparrow *et al.*, 2002) found the isolation process to be most efficient when the umbilical vein was cannulated and blood collected into a sterile bag containing either citrate phosphate dextrose or citrate-based anti-coagulant [0.6% acid citrate dextrose formula-A acid anti-coagulant and BSA (0.5% fraction V)], called ACD-A buffer (Flynn *et al.*, 2007, McGuckin *et al.*, 2003). Once the umbilical cord blood has been collected it should be processed within 15 hours as USSC yield can decrease dramatically over time (Bieback *et al.*, 2004). Cord blood is processed using a ficoll density gradient and the mononuclear cells collected and placed in culture.

3.8 Isolation of HSC from the cord blood

The first successful UCB-derived HSCT was performed in 1989 by Gluckman and colleagues to treat a six year-old child with Fanconi anaemia (Gluckman *et al.*, 1989). Since then, UCB has become recognised as a promising alternative HSC source for HSCT. Some advantages that UCB has over bone marrow or mobilised peripheral blood from living donors is that it is readily available and available from cord blood banks throughout the world (Bradley and Cairo, 2005). Most importantly, UCB can be used in transplants with less than optimal donor-recipient HLA-matching, providing a broader application compared to BM and mPB. The reason behind this versatility is the immunologically naive nature of UCB which stems from its ontogenetic primitiveness compared to BM and mPB (Haylock and Nilsson, 2007).

UCB-derived HSC, and indeed all human HSC, are classically identified by cell surface expression of CD34, a cell surface glycoprotein (Andrews *et al.*, 1992, Okuno *et al.*, 2002, Osawa *et al.*, 1996). However, CD34⁺ populations still contain a large population of committed progenitor cells with less than 1% of this population representing truly primitive HSC, as identified in transplantation assays (Wognum *et al.*, 2003). Thus, the CD34 marker is often used in combination with other markers such as Lin⁻, CD38⁻ and CD90⁺ (Park *et al.*, 2008). In addition to these cell surface markers, other techniques have been developed to identify populations enriched for HSC based on some of the functional characteristics of these cells. These include dye exclusion due to efflux pumps of the fluorescent dyes Hoechst-33342 (Ho) and Rhodamine-123 (Rho) (Bertoncello *et al.*, 1988, Bertoncello and Williams, 2004, Goodell *et al.*, 1996, Li and Johnson, 1995, Schroeder, 2010, Wognum *et al.*, 2003). In the case of Ho, HSC actively pump the dye out of the cell and therefore can be fluorescently selected as Ho^{-/low} (also known as side-population [SP] cells). Likewise, Rho is used to detect the low metabolic activity of HSC and they are identified as Rho^{low}. These techniques are used to both enrich and characterise HSC populations. However, the best and most accurate test of HSC quality is based on self-renewal potential and the ability to

give rise to all cells of the haematopoietic lineage in an *in vivo* setting. This characteristic is assayed by measuring the ability of HSC to repopulate and contribute to haematopoiesis in lethally irradiated animals for long periods (usually greater than 3 months in mice).

While UCB has several advantages over BM and mPB as a HSC source, the application of UCB is limited by HSC yield. Consequently, the HSC dose (cells/kilogram) from a single cord is low for clinical transplantation. This is particularly evident in the adult setting, where multiple cords are often needed to permit a successful transplant. Therefore, there has been a strong focus in HSC expansion processes that may increase cell yield prior to transfusion.

4. Preclinical and clinical studies using cord blood HSC

Various culture methods have been proposed for both HSC expansion and for the production of mature cell end-products with clinical utility such as erythrocytes (particularly for trauma applications) and neutrophils (for neutropenia in the post-HSCT period). Historically, two main approaches have been taken towards achieving these aims.

1. Using *in vitro* culture systems supplemented with various combinations of haematopoietic growth factors.
2. Using a specific feeder cell monolayer to provide a supportive microenvironment.

This section will focus on HSC expansion techniques that use MSC as a supportive feeder layer and critique these in both pre-clinical and clinical settings.

The notion of using MSC to support HSC in culture arises from the HSC niche. Our understanding of the HSC niche has dramatically improved in the last three decades since Schofield first postulated the idea of a specialised micro-environment where stem cells exist (Schofield, 1978). This environment provides HSC with the necessary cues to maintain stem cell homeostasis by ensuring quiescence in a healthy state, or proliferation in the case of cytopenia or infection. These cues regulate HSC via cytokines, growth factors, extracellular matrix proteins, adhesion molecules and cell-cell interactions. Many of these signals are provided by the cells that make up the HSC niche, most notably those of the mesenchymal lineage. Thus, this innate supportive role characteristic of MSC has provided the basis for their use in *ex vivo* HSC expansion systems.

The most common source of MSC in MSC-HSC expansion systems is the BM. However, MSC have also been shown to be effective when sourced from other tissues including human placenta (Zhang *et al.*, 2004), umbilical cord (Bakhshi *et al.*, 2008, Huang *et al.*, 2007, Wang *et al.*, 2004) and adipose tissue (Nakao *et al.*, 2010). Recent papers have demonstrated that many of the specific cell-cell interactions between HSC and stromal cells are critical and may be essential for HSC regulation both *in vivo* (Steiner *et al.*, 2009) and *in vitro* (Jing *et al.*, 2010, Song *et al.*, 2010, Wagner *et al.*, 2008, Wagner *et al.*, 2007, Wein *et al.*, 2010). Indeed, a majority of studies have shown that cell-cell contact between HSC and MSC is essential for their *ex vivo* expansion. There is also evidence that the most primitive HSC directly interact with stromal cells (Song *et al.*, 2010, Zhang *et al.*, 2006). Although, MSC may provide growth factors themselves, one disadvantage of this technique, at least in the human system, is that the co-cultures still require additional supplementation with growth factor cocktails (Andrade *et al.*, 2010, Da Silva *et al.*, 2005, Mcniece *et al.*, 2004).

Clinical trials using co-culture expanded HSC are few in number. The proprietary Replicell technology developed by Aastrom Biosciences Inc. was shown to be feasible but not definitively effective in enhancing myeloid, erythroid or platelet engraftment in the clinical setting (Jaroscak *et al.*, 2003, Pecora *et al.*, 2001). The system uses stromal co-cultures while also providing a continuous supply of culture medium containing fetal calf serum, horse serum, PIXY321 (a GM-CSF/IL-3 fusion protein), flt3 (FL) and erythropoietin (EPO) (Stiff *et al.*, 2000). One of the most comprehensive co-culture clinical trials has recently been initiated by the company Mesoblast PTL using a BM-derived multipotent progenitor cell (an MSC-like cell) product to expand UCB cells. As with clinical trials using cytokine-expanded HSC, the study transfused one unmanipulated UCB unit together with one 14-day expanded unit. Expansion using this method enhanced neutrophil recovery by 14 days and the grafts were shown to elicit less GVHD compared to that of published reports using unmanipulated UCB transplants (Mesoblast ASX announcement, 06 Nov 2009, www.mesoblast.com) (Kelly *et al.*, 2009). While long-term follow up results are yet to be reported, it will be interesting to determine whether the therapeutic value of the expanded unit is purely short-term myeloid support, as is the case in the Delaney's cytokine-mediated expansion trial (Delaney *et al.*, 2010), or whether the expanded unit also provides durable long-term engraftment.

5. Manufacturing process of human placental-derived MSC under GMP conditions, quality assurance and regulatory considerations

In this section we discuss the manufacturing processes that are required for using current good manufacturing practice (cGMP), quality assurance and regulatory considerations when conducting clinical trials with placenta-derived mesenchymal stem cells in Australia.

We have chosen to utilise term human placental-derived mesenchymal stem/stromal cells (hpMSC) for a series of clinical trials. Placentas obtained during elective term Caesarean sections were used as our source for hpMSC manufacture in order to minimise the risk of microbial contamination, a major concern with *ex vivo* expansion of cells for therapeutic use.

With no need to MHC-match the donor to the intended recipient, hpMSC from a single manufacturing campaign can be utilised in numerous clinical trials and for number of patients (Brooke *et al.*, 2009). However, hpMSC manufacture currently represents a complex, specialised, time-consuming and labour-intensive exercise (Ilic *et al.*, 2011). At present MSC manufacture requires an "open" system due to the multiple steps required for isolation and expansion of MSC from placenta. Although class II safety cabinets or clean rooms can be utilised, the extended period of expansion (up to 6 weeks in our process) introduces the risk of microbial contamination. We have utilised extensive in-process and end-product testing prior for release of product for clinical use to minimise these risks (Ilic *et al.*, 2011).

The Code of Good Manufacturing Practice (cGMP) standard (or its close equivalent) is applied across the entirety of the hpMSC manufacturing process, including acquisition of the starting cell population (placental tissue), isolation of cells, processing, storage and transport. A Quality Management System (QMS) is required to provide support for the manufacturing process based on the International Organization for Standardization (ISO) standard requirements.

The new Biologicals Framework was established recently by the Therapeutic Goods Administration (TGA) in Australia (<http://www.tga.gov.au/industry/btb.htm>). Its purpose is to improve the regulation of human tissue and cellular therapies and provide improved clarity by applying different levels of pre-market regulation to biological products based on the risks associated with the use of each product. According to the Biologicals Framework, hpMSC are categorised as Class 3 products. As a result, any trial utilising hpMSC (Class 3 product) can be conducted under the TGA's Clinical Trial Notification (CTN) Scheme. The CTN scheme is designed to combine rapid approval of clinical trial protocols with ongoing monitoring and supervision by a Human Research Ethics Committee (HREC) acting in accordance with nationally agreed guidelines developed by the Australian National Health and Medical Research Council (NHMRC). It is important to note that the TGA does not review any data relating to the trial under this scheme. The Human Research Ethics Committee (HREC) reviewing a new clinical trial protocol utilising hpMSC must have sufficient experience among committee members in order to effectively review the protocol ensuring that the proposed trial has scientific validity, and that participants' rights and well-being are protected according to the Australian National Health and Medical Research Council's National Statement on Ethical Conduct in Human Research 2007, the Declaration of Helsinki and International Conference on Harmonisation Good Clinical Practice ICH GCP(CPMCP/ICH/135/95).

Along with the study protocol, a Participant Information Sheet and Consent Form (PISCF) and other supporting documents including an Investigators Brochure is submitted for review by the HREC and the TGA, and is used by study personnel to facilitate their understanding of the key features of the protocol, in particular, the dosing and methods of administration of the hpMSC.

5.1 Pre-manufacturing and quality management

All hpMSC donors are subject to screening requirements according to the AusCord (Australian National Network of Umbilical Cord Blood Banks and Cord Blood Collection Centres) Guide to Selection of Mothers and Cord Blood Donors. Prior to the collection of the placenta, the donating mother undergoes screening serology for infectious disease markers and completes an in-depth medical questionnaire (as per AusCord Guidelines). The same process is repeated at 180 days after placental donation and information is sought about the health of the baby to identify if any medical conditions have been identified that may exclude the donated placenta.

An operational unit provides support to advanced clinical research based upon high level cell manipulation, such as *ex vivo* expansion of hpMSC, through its Quality Management System. Our Quality Management System comprises the organisational structure, procedures, processes and resources that control quality activities within the operational unit. It is defined in a series of policy statements in the Quality Manual and it is implemented through Standard Operating Procedures. Standard Operating Procedures (SOPs) are used to ensure that work having an effect on service and product quality, either directly or indirectly, is carried out in a consistent and satisfactory manner. Clear and effective procedures are developed and maintained by relevant staff. Procedures are regularly reviewed for accuracy, relevance and consistency with the policies, requirements of the relevant standards, guidelines and best practices.

5.2 Manufacturing

The placenta is obtained from healthy mothers undergoing elective Caesarean sections with their full informed consent prior to delivery. The placenta is subsequently double bagged, placed in a cool box and transferred to our manufacturing facility for processing. Our protocol for the isolation of MSC from placenta utilises a GMP grade collagenase-based digestion of tissue which has been dissected and washed to remove blood before isolation of cells. After digestion, large particulate matter is removed by low speed centrifugation and cell suspensions are collected and filtered into fresh tubes using 70 μm filters. The cells are then pelleted by centrifugation, resuspended and erythrocytes are subjected to rapid lysis with water. The cells are washed with Hank's Balanced Salt Solution (HBSS) and the final cell pellet is resuspended in Dulbecco's Modified Eagle Medium (DMEM-LG), 25% fetal calf serum (FCS), and 50 $\mu\text{g}/\text{ml}$ gentamycin. Cells are initially seeded into eight T175 cm^2 tissue culture flasks and cultured in a humidified incubator at 37°C, 5% CO_2 . A scheme of the production schedule is shown in Figure 2.

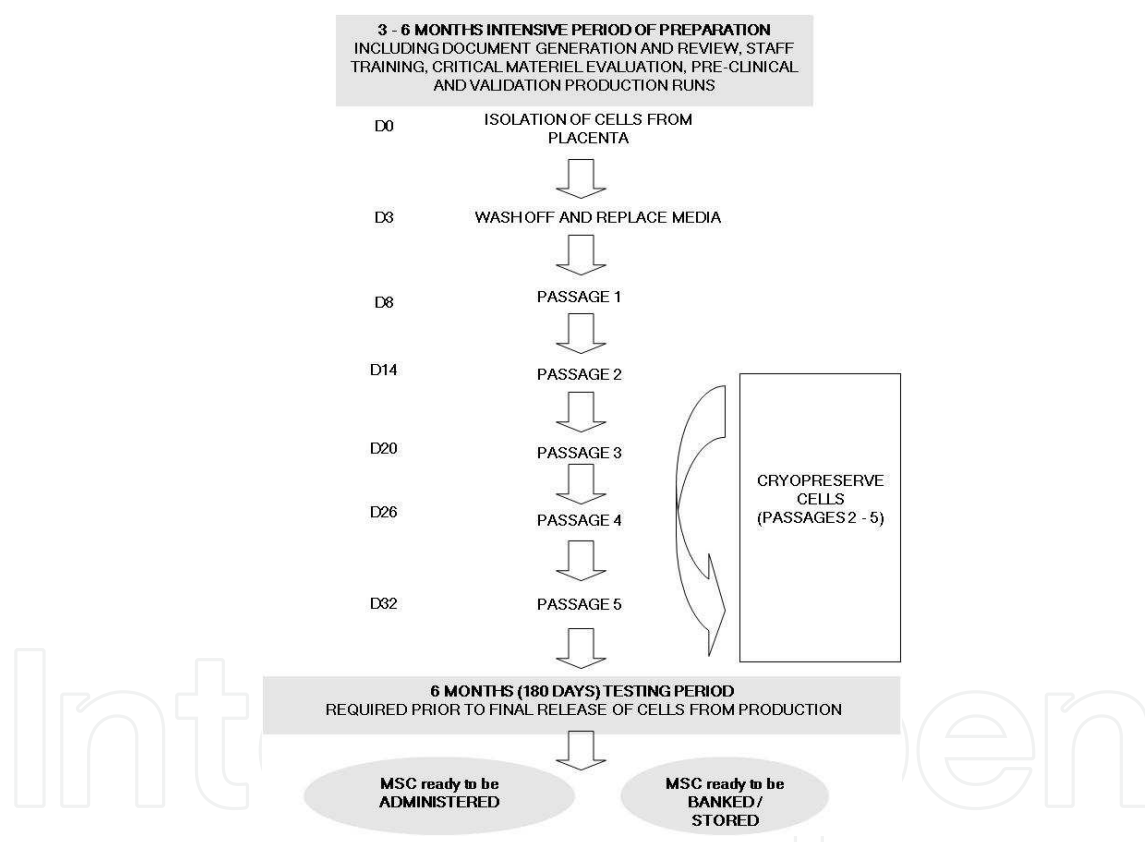


Fig. 2. Human placenta-derived MSC manufacturing flow diagram. D, day of manufacturing process

5.3 Release criteria

Prior to placenta donation, the mother must fulfil the criteria in the health questionnaire and undergo screening serology testing for infectious disease markers, the results of which must be negative. At day 180, a health questionnaire for both mother and baby are performed and both must be satisfactory. The mother's infectious disease markers are repeated and, again, must be negative (Table 1). In addition, for the cryopreserved cells to be available for

subsequent use in a clinical trial, the following release criteria must be met: MSC passages (P) zero to five (P0-P5) must be sterile after 14 days microbiology culture; the MSC must be greater than 70% viable (using Trypan Blue exclusion); MSC purity is determined by flow cytometry, and must be >85% CD73+, >85% CD105+, and <1% CD45+. A normal karyotype analysis must be demonstrated on MSC from P2 - P5; Gram stain (P2-P5) must be negative; mycoplasma testing (P2-P5) must be negative, and endotoxin testing (P2- P5) must show a level of < 2 EU/ml (Table 1).

Test	Pre-donation	P0	P1	P2	P3	P4	P5	Day
Completed	Screening							180**
Gram stain	N/A	N/A	N/A	Required	Required	Required	Required	N/A
Sterility	N/A	N/A	N/A	Required	Required	Required	Required	N/A
Mycoplasma	N/A	N/A	N/A	Required	Required	Required	Required	N/A
Endotoxin	N/A	N/A	N/A	Required	Required	Required	Required	N/A
FACS phenotype	N/A	N/A	N/A	Required	Required	Required	Required	N/A
Karyotype	N/A	N/A	N/A	Required	Required	Required	Required	N/A
Donor serology*	Required	N/A	N/A	N/A	N/A	N/A	N/A	Required
Donor health questionnaire	Required	N/A	N/A	N/A	N/A	N/A	N/A	Required

* For infectious disease markers. ** Donor/mother and the baby follow-up. N/A = Not applicable. P = Passage.

Table 1. Human placenta-derived MSC Quality Control Testing used by the Stem Cell Biology, Regenerative Medicine and Stem Cell-based anti-Cancer Therapeutics Group, MHS/MMRI, Brisbane, Australia.

6. Placental-derived MSC in the clinic

6.1 Application in clinical trials

Clinical trials of MSC therapy in humans have shown promising results in several clinical settings. Many patients have now received MSC by intravenous infusion for multiple clinical indications world-wide. A search on ClinicalTrials.gov listed a total of 192 MSC clinical studies in July 2011, including 171 studies with their status indicated. Clinical diseases treated to date have included acute graft-versus-host disease (GVHD) following allogeneic HSC transplantation, Crohn's disease, insulin-dependent diabetes mellitus and renal transplantation (Kebriaei and Robinson, 2011). The tissue repair capability of MSC is also being investigated in clinical trials for cardiac muscle repair after acute myocardial infarction, congenital bone disorders such as osteogenesis imperfecta, severe bone fractures, meniscal tears and liver repair in patients with cirrhosis. Studies have also been carried out using MSC to treat various metabolic disorders, ischemic stroke and neurological disorders.

Currently, four clinical trials using bone marrow-derived MSC are completed or in progress in Australia. Our group was the first to conduct clinical trials with human placenta-derived MSC. We are currently conducting three clinical trials using hpMSC and have four additional clinical trials in preparation.

6.2 Phase I clinical trial of hpMSC co-transplanted with umbilical cord blood transplants

In 2007, we submitted a HREC application for our first Phase I clinical trial, which was a multicentre, open label dose-escalation study of volunteer unrelated, MHC-unmatched placenta-derived MSC in recipients of unrelated umbilical cord blood HSC transplants. In this setting it was hypothesised that the transplanted MSC would support engraftment of HSC and reduce the frequency and severity of graft-versus-host disease (GVHD). The total time from submission of the application until final approval was 1 year. During this time, a request was made by our institutional HREC for an external audit to be conducted on the manufacturing processes outlined in the study protocol. This was performed by the Australian Red Cross Blood Service (ARCBS) and a two-way clinical trial agreement was established between the two participating hospitals. This agreement included an appropriate indemnification for each of the participating sites and ensured reporting of any adverse events related to the administration of the hpMSC.

6.2.1 Course of clinical trial

One day after the completion of pre-transplant myeloablative conditioning with cyclophosphamide and total body irradiation, a 20 year old Caucasian male with acute myeloid leukemia in second remission was given 1.20×10^8 human placental MSC ($1 \times 10^6/\text{kg}$) intravenously. These were suspended in 30 ml and infused over 7 min using a 200 μm in-line filter. No adverse events were noted. Five hours later the patient received two cord blood units. Post-thaw, the total nucleated cell dose from the two cord blood units was $3.6 \times 10^7/\text{kg}$ and the total CD34⁺ cell dose was $1.2 \times 10^5/\text{kg}$. The MSC were MHC-unmatched with both the recipient and the two cord units. The MSC donor serology tests were negative for cytomegalovirus (CMV), as were the two cord blood donors (Brooke *et al.*, 2009).

6.2.2 Post-transplant clinical course

Cyclosporine and mycophenylate mofetil were used as prophylaxis for graft-versus-host diseases (GVHD). The patient developed *Streptococcus viridans* septicemia on day 7 post-transplant and was treated accordingly. Subsequently, the patient developed a skin rash on day 14 and the skin biopsy was consistent with acute GVHD and resolved with treatment. Neutrophil engraftment with an absolute neutrophil count $> 500/\mu\text{l}$ occurred at day 38. CMV reactivation occurred at day 45 and the patient was treated with ganciclovir. However, at day 52 staphylococcal bacteremia occurred with the subsequent development of fever, fluid overload, respiratory distress and hypoxia. On day 68 the patient died of respiratory failure, thought due to interstitial pneumonitis. The patient did not become platelet-independent by the time of his death and the treating physicians did not consider any of the post-transplant complications to be related to the MSC infusion (Brooke *et al.*, 2009).

6.2.3 Lessons learned from this trial

One patient was enrolled in this study and later died from pneumonitis related to CMV reactivation. It is important to note that the donor of the hpMSC was CMV-negative prior to the collection of the placenta and again at the follow-up screening period. This particular study did not yield significant clinical results. Unfortunately, this particular study has now been closed as umbilical cord blood transplants are no longer being performed in adults at the specific hospitals involved.

However, it did highlight some of the impracticalities in incorporating the manufacturing protocol within a given clinical trial protocol. As a result, a new manufacturing protocol was established in 2009. This manufacturing protocol allowed us to continue manufacturing hpMSC independent of a clinical trial and therefore established a master cell bank of hpMSC. This protocol was approved by our institutional HREC under the provision that any clinical trial utilising the hpMSC as the investigational product was to be reviewed by the Human Research Ethics Committee.

6.3 Phase I clinical trial of hpMSC in patients with idiopathic pulmonary fibrosis

In 2010 we initiated a phase I study to evaluate the potential role of placenta-derived MSC in the treatment of idiopathic pulmonary fibrosis (IPF). MSC represent an attractive and novel therapeutic agent for lung diseases, as the lungs are the first site in which MSC lodge after intravenous injection (Figure 3). IPF is a relatively common chronic, fibrosing lung disease of unknown aetiology that results in severe, refractory and progressive breathlessness. MSC have theoretical benefits to patients with IPF because of their immunomodulatory capacity to decrease fibrosis. It is possible that any therapeutic role for MSC in this disease will be mediated by their ability to remodel extracellular matrix, or their ability to suppress the immune response through contact-dependent and soluble mediators, or both.

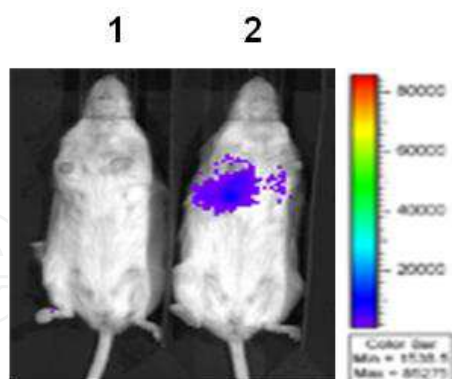


Fig. 3. *In vivo* luciferase activity of MSC after intravenous injection. Images were taken 38 min after injection with an exposure period of 10 min. Mouse 1: No cells administered (negative control), Mouse 2: 1×10^6 MSC transgenic for luciferase.

6.3.1 Course of clinical trial and monitoring

Our hpMSC were transported in a dry shipper to the participating hospital where they were thawed and infused intravenously. This is another Phase I study (since we regard hpMSC as a “first-in-man” reagent that need to be investigated in a Phase I trial for each different

patient population) to assess the safety of hpMSC in IPF patients, as well as to perform an MSC dose-escalation study, with the approval of the Data Safety Monitoring Committee to initiate the second dose-cohort. The first 4 patients receive 1×10^6 MSC/kg and the second 4 patients receive 2×10^6 MSC/kg. To date 3 patients have been infused in Cohort 1 without any adverse events due to the hpMSC. Specifically, no serious adverse events relating to infusional toxicity or ectopic tissue formation have been reported. Instructions for monitoring for infusional toxicity are represented in Table 2. Infusional toxicity is defined as any one of the criteria observed in Table 2 at any time within the 4-hr post-infusion period.

Vital Sign	Measurement
Systolic Blood Pressure (mmHg)	< 90 > 180 > 25% decrease > 25% increase
Diastolic Blood Pressure (mmHg)	< 50 > 100 > 25% decrease > 25% increase
Heart Rate (bpm)	< 60 > 110 > 25% decrease > 25% increase
Body Temperature (°F)	> 101.5 > 2° decrease > 2° increase

Table 2. Monitoring for infusional toxicity

6.3.2 Lessons learned from this trial

Thus far, human placenta-derived MSC appear safe with no adverse events noted after intravenous infusion. If safety is confirmed in this Phase I trial we will proceed to a Phase II trial powered for efficacy.

6.4 Phase I clinical trial of hpMSC for Achilles tendinopathy

In 2011 we initiated our first study in the treatment of chronic refractory tendinopathy. Tendinopathy is a common condition associated with pain and tendon dysfunction. Tendinopathy often occurs in young, active adults. As life expectancy increases, so does the incidence of tendinopathy. This in turn will place large costs on the health system budget. The initial management of all tendinopathies is usually conservative and includes activity modification, medication, injections and exercises. If prolonged disability occurs, surgical treatment is considered which is expensive and involves periods of immobilisation. Current treatment is relatively ineffective, as tendons have a poor capacity to repair themselves. Therefore, stem cell therapies have been extensively researched in preclinical models as a possible treatment.

Several animal studies have shown that MSC can repair the tendon defect, and regenerate the tendinopathic tissue (Nourissat *et al.*, 2010, Chen *et al.*, 2009, Lim *et al.*, 2004). It may be that the main mechanism of MSC repair in this case is to enable differentiation into tenocytes. The biomechanics of the resulting tendon can be improved further by applying mechano-stimulation (e.g. exercise). This regenerative technique has not shown any complications in the published preclinical animal studies and seems a promising treatment in man.

6.4.1 Course of clinical trial

This is a phase I, open-label, single centre, non-randomized dose-escalation evaluation of the safety and feasibility of MSC treatment for patients diagnosed with refractory Achilles tendinopathy. Up to nine patients will be enrolled in the study. All patients will receive ultrasound-guided precision intratendinous injection of hpMSC into their damaged Achilles tendon. Injections will occur at least 4 weeks after the previous patient so that any early adverse effects from the previous hpMSC application can be closely monitored and assessed, both clinically and via diagnostic power doppler ultrasound examination.

There will be 3 cohorts, each with 3 patients, to assess the safety of the MSC dose. The first cohort of 3 patients will receive a single dose of 1.0×10^6 placenta-derived MSC (1.0 ml of solution containing 1.0×10^6 MSC per ml) each. The next cohort of 3 patients will receive 4.0×10^6 MSC (1.0 ml of solution containing 4.0×10^6 MSC per ml) each. The final cohort of 3 patients will receive 1.6×10^7 MSC (1.0 ml of solution containing 1.6×10^7 MSC per ml) each. An interim safety analysis will be carried out by the Data Safety Management Committee (DSMC) after each group of 3 has received their individual injection. This will occur following the 4 week post injection assessment, of the third member of each group. Should no serious adverse events be documented due to, or likely due to, the MSC injection, the subsequent group will receive a local guided precision injection at the next ascending dose.

The injection phase of the study will take at least 9 months to complete. Therefore, from this clinical trial, there will be data to show if intra-tendinous injection of MSC is a safe treatment of otherwise treatment-refractory Achilles tendinopathy.

6.4.2 Post-injection clinical course

The primary purpose of this trial is to provide evidence of the safe delivery of intra-tendinous injection of MSC. This will be assessed at 2 days post injection (via telephone), 2 weeks, 4 weeks, 10 weeks and 26 weeks and will include ultrasound assessment of the tendon (after 4, 10 and 26 weeks).

The second purpose is to measure the possible effectiveness of MSC in reducing the chronic morbidity associated with Achilles tendinopathy after 4, 10 and 26 weeks after intratendinous injection. Evidence for improvement in ultrasound and power doppler ultrasound assessment of tendon structure will include the following parameters: (i) tendon thickness (sagittal plane), (ii) echogenicity, (iii) discontinuity, (iv) neovascularisation and (v) other abnormalities.

6.4.3 Expected outcomes

It is proposed MSC may offer a safe and highly cost effective treatment for chronic refractory tendinopathy, which could increase population activity levels, improve quality of life, and minimise dependence on costly long term medication and allied health treatments. MSC-initiated tendon regeneration could reduce the requirement for surgical treatments, thereby reducing the risks and costs of surgery and post-operative immobility. MSC injections also have the potential to augment and accelerate orthopaedic surgical tendon repair, either intraoperatively or by percutaneous injection, and offer an alternative treatment for individuals in whom tendon surgery has failed or is not possible.

To the best of our knowledge, this is the first trial of the use of allogeneic placenta-derived mesenchymal stem cells in the treatment of chronic refractory tendinopathy. Although we have chosen to use MSC derived from whole placenta for our current clinical trials, we are currently conducting research that explores the possibility of more advantageous MSC residing within the placenta. It is hypothesised that fetal MSC derived from the term placenta may have different biological properties from maternal MSC, given the differences in age at the time of their development. Such differences may have implications in the potential use of MSC as therapeutic agents. Therefore, this research may prove to be useful for choosing the optimal gestational product MSC for our future clinical trial program.

7. Conclusion/summary

Human gestational tissues show great promise as alternative stem cell sources. They are readily available and provide a basically unlimited supply of donor tissue for generating both MSC, and in the case of cord blood, HSC therapeutic products.

8. Acknowledgements

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This book contains the total of 19 chapters, each of which is written by one or several experts in the corresponding field. The objective of this book is to provide a comprehensive and most updated overview of the human placenta, including current advances and future directions in the early detection, recognition, and management of placental abnormalities as well as the most common placental structure and functions, abnormalities, toxicology, infections, and pathologies. It also includes a highly controversial topic, therapeutic applications of the human placenta. A collection of articles presented by active investigators provides a clear update in the area of placental research for medical students, nurse practitioners, practicing clinicians, and biomedical researchers in the fields of obstetrics, pediatrics, family practice, genetics, and others who may be interested in human placentas.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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