

The potential of fetal cell therapy for osteogenesis imperfecta using placenta derived stem cells

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Abstract

Human mesenchymal stromal/stem cells (MSC) isolated from aborted first trimester fetal bone marrow (BM) hold promise for use in tissue engineering applications and cell-based therapies due to their advantageous characteristics compared to their adult BM-MSC counterparts; faster growth kinetics, active telomerase, smaller size and higher differentiation potency. However, their isolation is restricted ethically and technically and therefore there is a need to identify a cell source with high therapeutic potential that is easily accessible in the clinic without ethical restrictions. The placenta is a potential source of readily-obtainable chorionic stem cells (CSC) throughout pregnancy. The aim of my thesis was to study the evolution of the CSC phenotype (i.e. change of stem cell characteristics) during gestation and to assess their capacity for bone repair in a mouse model of osteogenesis imperfecta (*oim*). I hypothesised that early fetal placental chorionic stem cells (e-CSC) were physiologically superior to their term counterparts, late chorionic stem cells (l-CSC), with advantages for use in fetal stem cell therapy of osteogenesis imperfecta (OI).

In the first chapter I showed that e-CSC and l-CSC shared a common phenotype, which was intermediate between adult BM-MSC and human embryonic stem cells, with characteristics of both. I also showed the phenotype of CSC evolves during gestation with e-CSC displaying characteristics of an earlier state of stemness compared to l-CSC, such as smaller size, faster kinetics, unique expression of *OCT4A variant 1* and higher levels of *Nanog*, *Sox2*, *c-Myc* and *Klf4* expression, as well as the capacity to differentiate into lineages of the three germ layers through embryoid body formation.

In the second chapter I showed the more primitive *in vitro* characteristics of e-CSC translated to higher tissue repair *in vivo* compared to l-CSC; accelerating healing when applied to a skin wound and increasing bone quality and plasticity following neonatal transplantation into the *oim* model. I subsequently used the *oim* model to assess the therapeutic potential of e-CSC for use in fetal cell therapy for OI. I showed compared to non-transplanted mice, *oim* transplanted with e-CSC had a two third reduction in fracture incidence, more ductile bones and increased trabecular bone volume. Prevention of fractures was attributed to the differentiation of exogenous cells to osteoblasts that expressed mature osteoblast genes and

synthesised human type 1 collagen (COL1A2). However, this beneficial effect may also have resulted from an indirect effect of the transplanted cells on the endogenous cells of the host mouse, since transplanted mice had upregulation of endogenous genes involved in endochondral ossification and osteoblast differentiation.

Altogether, my thesis characterises early and late human fetal chorionic stem cells, providing insight into the ontogenesis of stemness phenotype during fetal development and shows the first trimester placenta is a practical source of stem cells that can be used to treat OI during bone development.

Declaration of Originality

All experiments included in this thesis were performed by the author unless otherwise stated below or in the text.

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Publications from thesis

First authored publications

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Jones GN, Moschidou D, Lay K, Abdulrazzak H, Vanleene M, Shefelbine SJ, Polak J, de Coppi P, Fisk NM and Guillot PV. (2012) Upregulating CXCR4 in human fetal mesenchymal stem cells enhances engraftment and bone mechanics in a mouse model of osteogenesis imperfecta. Stem Cells Translational Medicine 1: 70-78.

Jones GN, Abdulrazzak H, Moschidou D, Kalirai BS, Vanleene M, Osatis S, Shefelbine SJ, Horwood N, Marenzana M, De Coppi P, Bassett D, Williams GR, Fisk NM and Guillot PV. (2013) Potential of human fetal chorionic stem cells for the treatment of osteogenesis imperfecta (*in revision with Stem Cells and Development*).

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Co-authored publications

Moschidou D, Mukherjee S, Blundell MP, Drews K, Jones GN, Abdulrazzak H, Nowakowska B, Phoolchund A, Lay K, Ramasamy TS, Cananzi M, Nettersheim D, Sullivan M, Frost J, Moore G, Vermeesch JR, Fisk NM, Thrasher AJ, Atala A, Adjaye J, Schorle H, De Coppi P and Guillot PV. (2012) Valproic acid confers functional pluripotency to human amniotic fluid stem cells in a transgene-free approach. Molecular Therapy 20: 1953-1967.

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Abbreviations

-/-	homozygous for mutation
+/-	heterozygous for mutation
AD	autosomal dominant inheritance
AE	amniotic epithelial cells
AFSC	amniotic fluid stem cells
AR	autosomal recessive inheritance
<i>bp</i>	<i>base pair</i>
<i>Brtl</i>	Brittle mouse
BSA	bovine serum albumin
BV/TV	bone volume/total tissue volume
cDNA	complementary DNA
CSC	chorionic stem cells
Ct	threshold cycle
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E	embryonic day
EB	embryoid body
EC	embryonic carcinoma
EDTA	ethylenediaminetetraacetic acid
EG	embryonic germ
ESC	embryonic stem cells
e-CSC	early chorionic stem cells
GFP	green fluorescent protein
GMP	good manufacturing practice
HDAC	histone deacetylase
hESC	human embryonic stem cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hfMSC	human fetal mesenchymal stromal/ stem cells

HLA	human leukocyte antigen
HRP	horseradish peroxidase
HSC	hematopoietic stem cells
HUVEC	human umbilical vein endothelial cells
ICM	inner cell mass
IFN- γ	interferon gamma
i.p.	intraperitoneal
iPSC	induced pluripotent stem cells
IUT	<i>in utero</i> transplantation
i.v.	intravenous
kDa	Kilo Dalton
l-CSC	late chorionic stem cells
MASC	multipotent adult stem cells
MHC	major histocompatibility complex
MIAMI	marrow-isolated adult multilineage-inducible
M-MuLV	Moloney-murine leukemia virus
mRNA	messenger RNA
MSC	mesenchymal stromal/ stem cells
OI	osteogenesis Imperfecta
<i>oim</i>	osteogenesis imperfecta murine
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PGC	primordial germ cells
PMSF	phenylmethanesulfonylfluoride
qRT-PCR	quantitative real time PCR
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcription PCR
s.d.	standard deviation
s.e.m.	standard error of the mean
siRNA	small-interfering RNA

UC-MSC	umbilical cord tissue mesenchymal stem cells
USSC	unrestricted somatic stem cells
VPA	valproic acid
VSEL	very small embryonic like cells

CHAPTER 1 - Introduction

1.1. Stem cells have properties useful for cell therapy

1.1.1. Stem cells

Stem cells from embryonic, fetal, neonatal and adult sources have huge potential for therapeutic applications. Embryonic stem cells (ESC) have been derived from the inner cell mass of embryos (Thomson, Itskovitz-Eldor et al. 1998; Adewumi, Aflatoonian et al. 2007), embryonic germ (EG) cells arise from cultured primordial germ cells (PGC) (Matsui, Zsebo et al. 1992; Resnick, Bixler et al. 1992; Shambloott, Axelman et al. 1998), and embryonic carcinoma (EC) cells are malignant counterparts of ESC, derived from teratocarcinomas (Martin 1980; Andrews 1984). Stem cells derived from embryonic sources can self-renew indefinitely *in vitro* and are pluripotent, capable of giving rise to cells from all three germ layers (ectoderm, mesoderm and endoderm). However, there are significant ethical concerns surrounding their use as well as risk of tumorigenicity if cells are not pre-differentiated prior to application. In contrast, somatic stem cells are found in various adult (bone marrow, fat, liver), neonatal (cord, amnion, chorion) and fetal tissues (blood, liver, bone marrow, placenta, amniotic fluid) (Friedenstein, Chailakhjan et al. 1970; Fortier 2005; Abdulrazzak, Moschidou et al. 2010). These cells can proliferate *in vitro*, but not indefinitely, are non-tumorigenic and have multipotent differentiation capacity, forming a restricted number of cell lineages. These cells may also provide beneficial trophic effects through cytokine and growth factor production and modulate the immune response (Vats, Bielby et al. 2005; Guillot, Cui et al. 2007).

1.1.2. Mesenchymal stem cells

Adherent fibroblast-like stromal cells with osteogenic and clonal potential were first identified in adult bone marrow by Friedenstein *et al.* (Friedenstein, Petrakova et al. 1968; Friedenstein, Chailakhjan et al. 1970; Owen and Friedenstein 1988), and were subsequently termed mesenchymal stem cells (MSC) (Caplan 1991). MSC have since been isolated from a range of adult and fetal tissues and display distinct variations in cell size, growth rate, senescence, differentiation potency, immunosuppressive ability and gene expression profiles (Wagner, Wein et al. 2005; da Silva Meirelles, Chagastelles et al. 2006; Kern, Eichler et al.

2006; Guillot, Gotherstrom et al. 2007; Peng, Jia et al. 2008; Abdulrazzak, Moschidou et al. 2010). The criteria to define an MSC is proposed as non-haematopoietic, plastic adherent, with multipotential differentiation to the mesenchymal lineages (bone, cartilage and adipose tissues) and expression of a specific panel of surface antigens; CD73 (SH3 and SH4), CD90 (thy-1), CD105 (SH2) as well as lack of expression of the pan-leukocyte marker CD45, hematopoietic progenitor marker CD34, monocyte and macrophage hematopoietic cell marker CD14 (Dominici, Le Blanc et al. 2006). However, the exact definition of an MSC remains controversial, since cell surface epitopes can vary considerably depending on the extracellular environment (i.e. stem cell niche) (Kuhn and Tuan 2010) and although MSC are non-pluripotent, the transdifferentiation of MSC to non-mesodermal lineages has been widely reported (Poulsom, Alison et al. 2002). In addition, MSC possess immuno-modulatory properties through secretion of soluble factors such as indoleamine 2,3-dioxygenase, prostaglandin, nitric oxide, IL-6 and human leukocyte antigen (HLA)-G, as well as via cell-cell interactions (Ghannam, Bouffi et al. 2010).

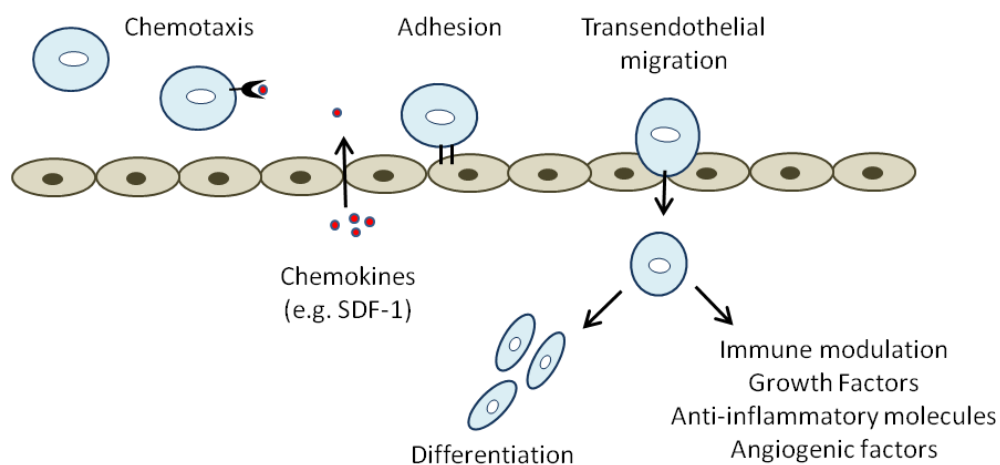


Figure 1 – MSC migrate, differentiate and have trophic effects at sites of tissue injury

MSC traffick through blood vessels to sites of tissue injury in response to migratory cues from chemokines (e.g. SDF-1), where they adhere and migrate across the endothelium and differentiate or release trophic factors to repair the tissue. Adapted from (Chavakis, Urbich et al. 2008).

MSC have generated substantial interest for clinical applications, such as in cell therapy, due to their ability to home to sites of injury (Chavakis, Urbich et al. 2008; Karp and Leng Teo 2009), migrate across the transendothelial barrier (Schmidt, Ladage et al. 2006), undergo clonal expansion (Morrison and Kimble 2006), differentiate to specialised cells that replace damaged or defective cells (Pittenger, Mackay et al. 1999; Fuchs and Segre 2000) and also modulate the local tissue environment via paracrine anti-inflammatory, proangiogenic, anti-apoptotic and immune suppressive effects that aid tissue repair (Caplan and Dennis 2006; Ghannam, Bouffi et al. 2010; Kuhn and Tuan 2010) (**Fig.1**). In addition, MSC from adult and fetal tissues have potential for allogeneic use due to their low immunogenicity in response to lack of expression of human leukocyte antigen (HLA) class II, modulation of a variety of immune cells, including T cells, B cells, natural killer cells, dendritic cells and neutrophils, and induction of regulatory T-cells that induce tolerance (Le Blanc, Tammik et al. 2003; Ghannam, Bouffi et al. 2010).

1.1.3. Stem cell homing

Schofield *et al.* first described the stem cell niche as a dynamic microenvironment that maintains stem cell quiescence and promotes proliferation and differentiation in response to local and systemic signals (Schofield 1978). Activation as well as trafficking and homing of MSC to sites of injury is controlled by release of inflammatory chemokines and cytokines as well as growth factors from injured tissues (Fox, Chamberlain et al. 2007; Chavakis, Urbich et al. 2008), which provide migratory cues and facilitate adhesion and transendothelial migration (Brooke, Tong et al. 2008). Stem cell therapy aims to use these natural homing mechanisms to direct transplanted cells to the target tissue and therefore identification of a cell source with high intrinsic migratory capacity would be advantageous. For example, fetal lung derived MSC had greater migratory capacity than adult stem cells to various stimuli, although the same was not found for hfMSC from bone marrow and therefore migratory potential may be source dependent (Maijenburg, Noort et al. 2010).

Injured and inflamed tissues produce a variety of factors including hepatocyte growth factor (HGF) (Son, Marquez-Curtis et al. 2006), basic fibroblast growth factor (bFGF) (Schmidt, Ladage et al. 2006), platelet derived growth factor (PDGF) (Fiedler, Roderer et al. 2002; Zhu,

Mitsubishi et al. 2006), bone morphogenic proteins BMP-2 and BMP-4 (Fiedler, Roderer et al. 2002), insulin-like growth factor I (IGF-1) (Li, Yu et al. 2007), and matrix metalloproteinases (MMPs) (Son, Marquez-Curtis et al. 2006). Importantly, stromal-derived factor (SDF-1 or CXCL12) is a dominant chemokine in the bone marrow and in combination with its receptor, CXCR4, has a key role in homing of MSC to various injured tissues (Ji, He et al. 2004; Wynn, Hart et al. 2004; Togel, Isaac et al. 2005; Son, Marquez-Curtis et al. 2006; Itoh, Satou et al. 2009).

The interaction of SDF-1 and CXCR4 causes cytoskeleton rearrangements and integrin activation within the MSC, which results in their migration towards SDF-1, followed by transmigration across the endothelial barrier (Dar, Kollet et al. 2006). This SDF-1-CXCR4 axis is thought to be essential for the migration of MSC to sites of fracture. For example, Granero Molto *et al.* used mice with a stabilised tibia fracture to show migration of MSC to the site of injury was dependent on the BM-MSC expression of CXCR4, since CXCR4-selected cells did not home to the fracture site (Granero-Molto, Weis et al. 2009). SDF-1/CXCR4 signalling has also been shown to aid bone healing through recruitment of MSC. For example, AMD3100, a CXCR4 antagonist, reduced fracture repair (Toupadakis, Wong et al. 2012), whilst inhibition of SDF-1 using either a neutralizing antibody or a mouse partially lacking SDF-1 resulted in reduced bone formation and repair (Kitaori, Ito et al. 2009). Increasing the interaction of CXCR4 and SDF-1 could improve bone repair in cell based therapies.

1.1.4. Stem cell self renewal

Stem cell proliferation/clonal expansion can be either symmetric (giving rise to two unspecialised daughter stem cells) or asymmetric replication (giving rise to one stem cell and one committed/differentiated cell) (Morrison and Kimble 2006). Self renewal describes the proliferation of a stem cell to create more identical stem cells. The unlimited self renewal of ESC has been clearly demonstrated and attributed to *c-Myc* signalling (Rossant 2008; Smith, Singh et al. 2010; Wiese and Eilers 2011). However, controversy remains over the true ability of MSC to self renew, since we lack evidence that MSC proliferate indefinitely and instead

show senescence and loss of initial multipotentiality (Sacchetti, Funari et al. 2007; Kuhn and Tuan 2010).

The limited expansion potential of adult BM-MSC and low yield isolated from bone marrow aspirates, has meant obtaining sufficient numbers for use in regenerative medicine is difficult. There is research being carried out to maximise yield by adjusting the microenvironment of the cells to enhance proliferation, for example culturing in serum from donors of a young age (Lohmann, Walenda et al. 2012). Nevertheless, identifying a source of stem cells with greater expansion potential than adult MSC would allow greater numbers of cells to be generated for clinical use. BM-MSC have been found in the early fetus and compared to their adult counterparts have greater expansion potential due to faster kinetics and later senescence (Campagnoli, Roberts et al. 2001; Guillot, Gotherstrom et al. 2007). Bone marrow human fetal MSC (hfMSC) also have greater proliferation capacity when compared to neonatal umbilical cord derived MSC (Zhang, Teoh et al. 2009). However, fetal bone marrow can only be collected after termination of pregnancy, which raises ethical issues and prevents their autologous use in cell-replacement therapies or tissue engineering settings. There is subsequently a need to identify a source of fetal stem cells with high proliferative capacity that can be collected without pregnancy termination.

1.1.5. Stem cell differentiation

The ability of MSC to differentiate to more specialised cells has resulted in their use in cell-based tissue engineering and regenerative medicine (Fortier 2005). Adult BM-MSC were first shown to differentiate along osteogenic, chondrogenic and adipogenic lineages (Pittenger, Mackay et al. 1999) and have subsequently shown differentiation beyond mesodermal lineages (Poulsom, Alison et al. 2002). Bone marrow hfMSC have a broad differentiation potential beyond the mesodermal lineages, as well as basal expression of hESC markers (Campagnoli, Roberts et al. 2001; Guillot, Gotherstrom et al. 2007). Subsequent studies have shown other fetal stem cells as well as extra-embryonic stem cells are broadly multipotent, differentiating to some ectoderm and endoderm lineages as well as mesodermal and expressing an array of pluripotency genes (**Table 1**) (Abdulrazzak, Moschidou et al. 2010). However, unlike hESC these cells are not pluripotent and do not form tumours, which

Table 1 – Phenotype comparison of stem cells. Characteristics of fetal (blood, liver, bone marrow) and extra-embryonic (cord blood, Wharton’s jelly, amniotic fluid, amnion, chorion) derived stem cells compared to embryonic stem cells and adult bone marrow mesenchymal stem cells (BM-MSC). Listed are the cell phenotypes. E, embryonic; MSC, mesenchymal; H, haematopoietic; EP, epithelial; VSEL, very small embryonic like; P, pluripotent; M, multipotent; U, unipotent; n.d., not determined. (* these characteristics are not listed). Population doublings (PD) and doubling time (DT) shown for expansion over a, 50 days; b, 8 passages; c, 14 passages; d, 5 passages. (Table taken from (Abdulrazzak, Moschidou et al. 2010)).

	embryonic stem cells	foetal tissues			extraembryonic						
		blood	liver	bone marrow	cord blood	Wharton’s jelly	amniotic fluid	amnion-derived epithelial cells	amnion-derived MSC	chorion-derived MSC	adult BM MSC
cell phenotype (s)	E	MSC/H*	MSC/H*	MSC/H*	MSC/H*	MSC	MSC/AFS/VSEL*	EP	MSC	MSC	MSC
doubling time (DT)/ population doublings (PD)	48–24 h (DT)/ indefinite (PD)	21.9 ± 1.1 h (first-trimester MSC) (DT)/ 28.4 ^a (PD)	46 h (second- trimester MSC) (DT)	32 h (second- trimester MSC) (DT)	39–43 h (DT)/ 20 ^b (PD)	85–11 h (DT)	25–38 h (second- trimester MSC) 36 h (AFS) (DT)/ 66 (MSC)/over 250 AFS (PD)	38.4 h (DT)	30 ^c (PD)	21.25 ± 3.2 h (DT)/30 ^d	54–111 h (DT)/ 7.1 ^a (PD)
feeder/matrigel requirement	+	–	–	–	–	–	–	–	–	–	–
potency	P	P/M	P/M	P/M	P/M	P/M	P/M	P/M	P/M	P/M	M
Oct-4	+++	++	++	+	+	+	+	+	+	+	±
Sox2	+++	n.d.	n.d.	n.d.	+	–	–	+	n.d.	n.d.	±
Nanog	+++	++	++	++	+	+	±	+	n.d.	+	±
c-Myc	+++	n.d.	n.d.	n.d.	n.d.	n.d.	+	+	n.d.	n.d.	n.d.
Klf-4	+++	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TRF length/telomerase activity	+++	++	++	++	++	+	n.d.	–	n.d.	+	–
alkaline phosphatase	+++	n.d.	n.d.	++	+	+	+	–		n.d.	±
c-Kit (CD117)	+++	+	+	+	±	+	+	±	±	–	–
Rex-1	+++	++	+	++	+	n.d.	+	+	–	+	±
SSEA-4	+++	++	++	++	+	+	+	+	–	+	–
SSEA-3	+++	++	++	++	+	–	–	+(low)	–	+	–
Tra-1-81	+++	++	++	++	n.d.	+	–	+	–	+	–
Tra-1-61	+++	++	++	++	n.d.	+	±	+	–	+	–
MHC class I	+(low)	+(low)	+(low)	+(low)	+	+	+	+(low)	+(low)	+	+
MHC class II (HLA-DR)	–	–	–	–	±	–	±	–	–	–	–
teratoma formation in immune compromised mice	yes	n.d.	n.d.	n.d.	n.d.	n.d.	no	no	n.d.	n.d.	no

is critical for their safe therapeutic application. Full analysis of the pluripotency genes has yet to be determined for all sources and there remains debate in the literature as to whether the pluripotent isoform *Oct4A* is truly expressed by these cells or if instead *Oct4B* found in non-pluripotent cells has been detected (Atlasi, Mowla et al. 2008; Ryan, Pettit et al. 2011).

Bone marrow hfMSC have shown superior osteogenic differentiation capacity to adult BM-MSC counterparts (Guillot, De Bari et al. 2008), which was attributed to the intermediate phenotype of the hfMSC between hESC and adult stem cells, with stemness characteristics of both (Guillot, O'Donoghue et al. 2006; Guillot, Gotherstrom et al. 2007). The hfMSC also showed greater osteogenic differentiation capacity, as shown by mineralisation of cellular scaffolds in immune-deficient mice, compared to perinatal MSC derived from umbilical cord (Zhang, Teoh et al. 2009). This may suggest primitive stem cell characteristics (i.e. stemness) are lost with increasing age of the tissue from which they are isolated. However, in this study cells were not derived from the same source, and therefore differences in stem cell phenotype may have been due to cell source rather than gestational age (in 't Anker, Noort et al. 2003).

1.1.6. Trophic effects of stem cells

MSC have the ability to produce a broad spectrum of soluble factors such as chemokines, cytokines and growth factors, which can aid tissue repair and also modulate the immune response. The immunosuppressive activity of MSC has been attributed to direct inhibition of T-lymphocyte (Bartholomew, Sturgeon et al. 2002; Di Nicola, Carlo-Stella et al. 2002) and natural killer cell proliferation (Sotiropoulou, Perez et al. 2006), but also indirect activation of monocytes (Beyth, Borovsky et al. 2005; Groh, Maitra et al. 2005) and regulatory T-lymphocytes (Prevosto, Zancolli et al. 2007). These properties make MSC ideal for treatment of autoimmune diseases such as graft versus host disease (Trento and Dazzi 2010; Francois and Galipeau 2012), but also for use in cell therapy in combination with allogeneic cells.

The paracrine and autocrine secreted factors from MSC influence the microenvironment to promote tissue repair via a combination of indirect trophic effects (Caplan and Dennis 2006). For example, the release of cytoprotective molecules such as anti-apoptotic factors can promote cell proliferation over cell death and reduce tissue fibrosis and scarring (Uemura, Xu

et al. 2006), whilst proangiogenic molecules can enhance angiogenesis to deliver more oxygen, micronutrients and inflammatory cells to the site of injury to aid tissue repair (Kinnaird, Stabile et al. 2004; Mirotsou, Jayawardena et al. 2011). Localised anti-inflammatory factors also aid tissue repair by returning the tissue to its normal homeostatic function and preventing excessive detrimental inflammation (Ohnishi, Yanagawa et al. 2007). In cell therapy the secretion of soluble factors by MSC has been shown useful since MSC conditioned media alone improved cardiac function of acutely infarcted hearts (Takahashi, Li et al. 2006) and increased chondrocyte proliferation and bone growth in the *oim* model (Otsuru, Gordon et al. 2012). Transplanted cells may also produce trophic factors that stimulate endogenous stem cell activity; increasing host cell commitment and enhancing tissue repair (Phinney 2007; Dominici, Rasini et al. 2009). The indirect paracrine action of MSC in cell-based therapies could be equally or possibly more useful to tissue repair than their direct differentiation.

1.2. Developmental origin of stem cells

Pluripotent epiblast stem cells distribute during gastrulation of the embryo into the three primary germ layers of the embryo, the primordial germ cells and the extra-embryonic mesoderm, which forms the umbilical cord and the mesenchymal part of the chorioallantoic placenta (Smith, Gesteland et al. 1994; Downs and Harmann 1997). In addition to the somatic cells found in the bone marrow, it was recently hypothesised that a sub-population of epiblast/germ line derived cells exist in murine bone marrow descended from precursors of germ cells, primordial germ cells (PGC), due to their small morphology, high nuclear/cytoplasmic ratio, CD133+/Lin-/CD45- and expression of pluripotency markers *OCT4*, *NANOG* and *SSEA4* (Kucia, Dawn et al. 2004; Wojakowski, Tendera et al. 2004; Kucia, Machalinski et al. 2006; Ratajczak, Mierzejewska et al. 2013). These cells were termed very small embryonic-like (VSEL) stem cells and similar cells have subsequently been found in human umbilical cord blood (Ratajczak, Zuba-Surma et al. 2008), human liver, heart and bone marrow (D'Ippolito, Diabira et al. 2004; Beltrami, Cesselli et al. 2007) and from *in vitro* cultures of various adult tissues (Vacanti, Roy et al. 2001; Kogler, Sensken et al. 2004; Wernet, Trapp et al. 2010; Santourlidis, Wernet et al. 2011).

Kucia *et al.* hypothesised that epiblast/germ line derived cells were deposited in tissues prior to or during their migration through the developing blastocyst, which were then retained in a stem cell niche that maintained their stemness (**Fig.2**) (Kucia, Machalinski et al. 2006). The expression of pluripotency markers has been demonstrated for stem cells derived from human fetal tissues (Guillot, Gotherstrom et al. 2007; Zhang, Teoh et al. 2009; Ryan, Pettit et al. 2011), although the presence of a subpopulation of fetal stem cells with a germ/epiblast developmental origin has yet to be elucidated.

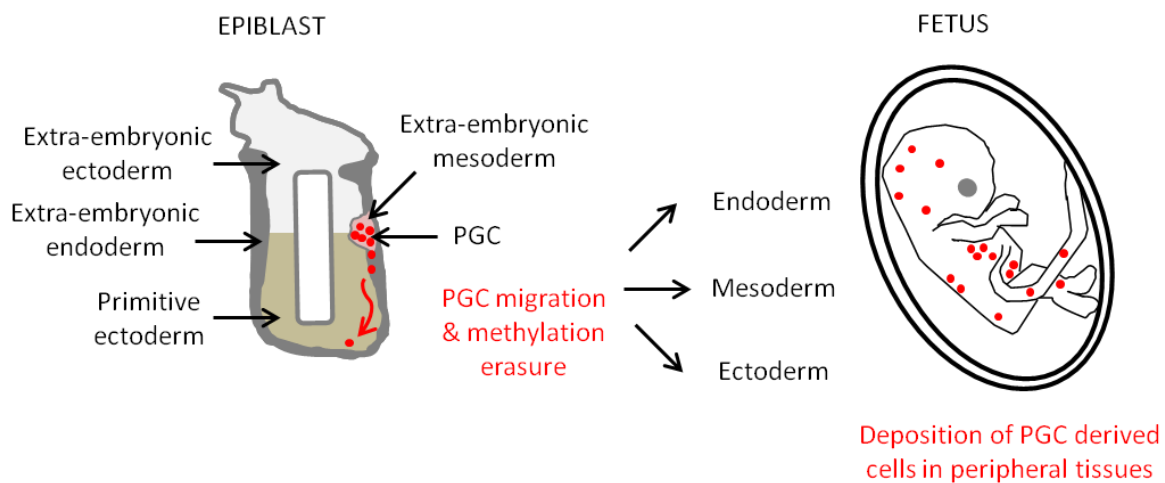


Figure 2 – The developmental deposition hypothesis of PGC during migration.

During early embryogenesis early primordial germ cells (PGC; red) arise in the extra-embryonic mesoderm. It is hypothesised that whilst PGC migrate to the genital ridge and undergo methylation erasure some PGC are lost in the germ layers (endoderm, mesoderm and ectoderm), causing PGC derived stem cells to be retained in peripheral tissues of the fetus.

1.3. Stem cells in regenerative medicine

1.3.1. Tissue engineering

Regenerative medicine describes a cross-disciplinary field of scientific research that aims to repair or replace damaged tissues or organs and restore their function. It combines a number of approaches including cell therapy, gene therapy and tissue engineering (Daar and Greenwood 2007). Tissue engineering combines living cells with non-living biocompatible materials to produce tissue like-structures (Langer and Vacanti 1993). Tissue engineering has made considerable progress in recent years, with translation to the clinic in some parts (e.g. cartilage repair), development of prototypes for other tissues (e.g. blood vessels) and even incorporation of new technologies (e.g. 3D printing) (Berthiaume, Maguire et al. 2011). However, hurdles for tissue engineering include scaling up production and obtaining sufficient cell numbers, identifying the appropriate cell source and matrices to use which are both safe and reliable, using adequate tissue differentiation protocols to avoid tumorigenic transformation of cells *in vivo*, preventing immune rejection of allogeneic cells and subsequent graft-versus host disease (Motaln, Schichor et al. 2010). First trimester bone marrow MSC have been used to seed tissue-engineered bone scaffolds, which were grafted in rats with critical-sized femoral defects and successfully resulted in closure of femoral defects by promoting woven and compact bone formation (Zhang, Teoh et al. 2010).

1.3.2. Gene therapy

Gene therapy aims to silence abnormal genes and/or increase normal gene expression where a genetic mutation is responsible for the disease pathology. Ex vivo gene delivery uses genetically modified autologous bone marrow MSC or induced pluripotent stem cells (iPSC) generated from skin fibroblasts of the patient. These cells are transduced with retroviral or adenoviral vectors of the normal gene along with tissue specific promoters, which are then transplanted back into the patient and deliver the normal gene (Deyle, Khan et al. 2012). This is an autologous approach therefore immune rejection is unlikely, however, the extraction of bone marrow is painful and MSC numbers are low. Whilst iPSC from skin are reprogrammed using transgenes, which has low efficiency, lacks stability of the pluripotent phenotype and is associated with viral induced tumorigenicity (Takahashi and Yamanaka 2006; Mukherjee and

Thrasher 2011). Therefore there is a need to isolate MSC from a more accessible source that can be banked for later autologous application, for example from extra-embryonic membranes normally discarded at birth or following prenatal diagnostics.

Antisense gene approaches are required if the mutant RNA or protein results in the disease pathology. These approaches include oligodeoxynucleotides, ribozymes, hammerhead ribozymes, RNAi or siRNA to promote degradation of mutant mRNA transcripts. However, problems arise if there is wide genetic heterogeneity in the disease as the design of vectors specific to individual mutations is both complicated and time consuming (Niyibizi, Wang et al. 2004; Fuccio, Iorio et al. 2011; Ljunggren, Lindahl et al. 2011).

1.3.3. Cell therapy

In contrast, cell therapy has the potential to treat diseases without the need to tailor treatments for individual mutations. The rationale of cell therapy (**Fig.1**) is to introduce stem cells, which home to sites of injury (e.g. fractures), differentiate and replace defective cells with healthy cells (e.g. osteoblasts), that synthesise the correct protein (e.g. collagen type I) and also restore normal tissue function via trophic factors (e.g. anti-inflammatory, immunosuppressive and pro-angiogenic effects) (Guillot et al. 2008a). Cell therapy relies on the ability of stem cells to migrate to sites of injury, adhere and cross the transendothelial barrier, self-renew and hence expand to sufficient numbers for tissue repair, differentiate to specific cell lineages and secrete soluble factors to modulate the microenvironment and favour tissue repair (Chavakis, Urbich et al. 2008). In principle cell therapy could offer a long term treatment as stem cells have the ability to be stored long term in tissue niches (e.g. bone marrow) and be later activated when needed for tissue repair (Schofield 1978). However, the ability of transplanted stem cells to engraft and remain functional long term in host tissues is largely unknown. Several factors influence the efficiency of cell therapy, such as time of transplantation, dosing regimen, cell source, pre-conditioning of donor cells and potential of the cells to be banked according to good manufacturing practice (GMP) standards.

1.3.4. Fetal cell therapy

Capitalizing on the small size of the fetus and the immature fetal immune system prenatal stem cell transplantation may have advantages over the postnatal approaches. The fetal environment is also permissive to endogenous stem cell migration during development, which may aid migration of donor cells to sites of tissue injury. For disorders that begin *in utero* and can be diagnosed during pregnancy, prenatal stem cell transplantation has advantages over postnatal approaches by preventing manifestation of the disorder earlier in life before damage or even perinatal death occurs (Le Blanc, Gotherstrom et al. 2005; Guillot, Abass et al. 2008; Panaroni, Gioia et al. 2009). In addition, a fetal-to-fetal approach to cell therapy may be optimal as fetal MSC are more primitive than adult MSC, showing greater expansion potential, smaller size, better osteogenic potential and greater immunogenicity (Guillot, Gotherstrom et al. 2007).

1.3.5. Transplantation regime

The route of injection has been found to influence cell therapy success with intra-venous (i.v.) transplantation of MSC showing higher engraftment and greater improvement in liver fibrosis than intrahepatic or intra-peritoneal (i.p.) injection (Zhao, Li et al. 2012), which may be due to phagocytosis of intra-peritoneal injected MSC by macrophages in the spleen (Wang, Shao et al. 2012). However, the venous route causes accumulation of cells in the lungs (Wang, Shao et al. 2012) and is considerably more technically challenging and invasive than the peritoneal route, in particular for prenatal cell therapy. Furthermore, in larger animals such as sheep i.v. transplantation is not superior to i.p. (Tanaka, Masuda et al. 2010), although this study used HSC. Therefore transplantation via the i.p. route is likely to be favoured in studies using prenatal or neonatal mice.

1.3.6. Cell banking

The potential to bank cells for both autologous and allogeneic application is likely to play an important role in bringing the translation of stem cell therapy to the clinic as a standardised treatment for a wide variety of disease pathologies. Stem cell banks for umbilical cord blood

have already been established worldwide and there are now numerous private stem cell banks that will bank various cells from dental pulp to menstrual blood. However, the safety and efficacy of using these alternative sources in the clinical setting have yet to be tested (Stacey 2012). Recently, research has begun to investigate banking the extra-embryonic tissues normally discarded after birth. A facility with GMP production of human placenta derived MSC for human clinical trials has already been established (Brooke, Rossetti et al. 2009) and there is now a need to optimise, standardise and up-scale the production of these stem cells using large-scale bioreactors (Parolini, Alviano et al. 2010). Determining the optimal cell source for tissue banking and therapeutic application is also essential. The large size of the placenta tissues and accessibility at all clinics means they may become a fundamental MSC source for stem cell banking.

1.4. Extra-embryonic stem cells potential use in cell therapy

1.4.1. The extra-embryonic tissues

Multipotent stem cells have been isolated from various fetal tissues; including bone marrow, blood and liver, as well as from extra-embryonic tissues such as umbilical cord, placenta and amniotic fluid (**Table 1**). Collection of fetal tissues in the first trimester is technically challenging and requires pregnancy termination, an obstacle to autologous applications. Fetal tissues are difficult to procure, the small size of the fetus limits total number of cells that can be collected and their clinical use is restricted by ethical legislations (In 't Anker, Scherjon et al. 2004; Michejda 2004; Miao, Jin et al. 2006). Whilst hfMSC from fetal blood can be collected by cordocentesis during pregnancy, this procedure is highly invasive and carries a risk of miscarriage (Chan, Kumar et al. 2008). Fetal stem cell therapy could offer a promising alternative to current cell therapy using adult MSC (Abdulrazzak, Moschidou et al. 2010), but for the translation of fetal cell therapy to the clinic, it is essential to identify a cell source that is available in large numbers, without ethical or legal constraints surrounding their collection and use. For example, prenatal and perinatal stem cells that can be harvested from extra-embryonic tissues.

The placenta and extra-embryonic membranes are readily obtainable within the clinic, being discarded at birth and also collectable during ongoing pregnancy from chorionic villous sampling (CVS), a routine prenatal diagnostic procedure (Ilancheran, Moodley et al. 2009). The extra-embryonic membranes surround not only the fetus, but also the amniotic fluid, which can also be collected during pregnancy via amniocentesis and at term. Amniotic fluid contains a heterogeneous population of cell types from the fetal amnion membrane, fetal skin and fetal alimentary, urogenital and respiratory tracts (Murphy and Atala 2013). Finally the umbilical cord, which transports fetal blood to the placenta via umbilical vessels, can be collected safely and non-invasively at birth for extraction of fetal blood containing haematopoietic stem cells, and also for collection of MSC from Wharton's jelly; the connective tissue that surrounds the umbilical cord vessels (Weiss, Medicetty et al. 2006; Secco, Zucconi et al. 2008).

The placenta is a large fetal-maternal organ that contains a maternal decidua layer, a fetal amnion layer composed of amniotic epithelial cells from the epiblast and amniotic mesenchymal stromal cells from the hypoblast, and a chorion layer containing both fetal and maternal cells (**Fig.3**). The chorionic tissue of the placenta is derived from both the outer trophoblast layer of the embryo, which differentiates to cytotrophoblastic and syncytiotrophoblastic cells and invades the maternal decidua, and also from the embryonic epiblast cells that are allocated to the extra-embryonic mesoderm during gastrulation and form the fetal mesenchymal part of the mature chorioallantoic placenta (Parolini, Alviano et al. 2008; Ilancheran, Moodley et al. 2009; Insausti, Blanquer et al. 2010; Murphy and Atala 2013). The smooth chorion forms due to partial regression of the chorionic villi that surround the amniotic sac in early gestation and loosely fuses with the amnion at 17-20 weeks gestation to form the amnio-chorionic membrane (Ilancheran, Moodley et al. 2009).

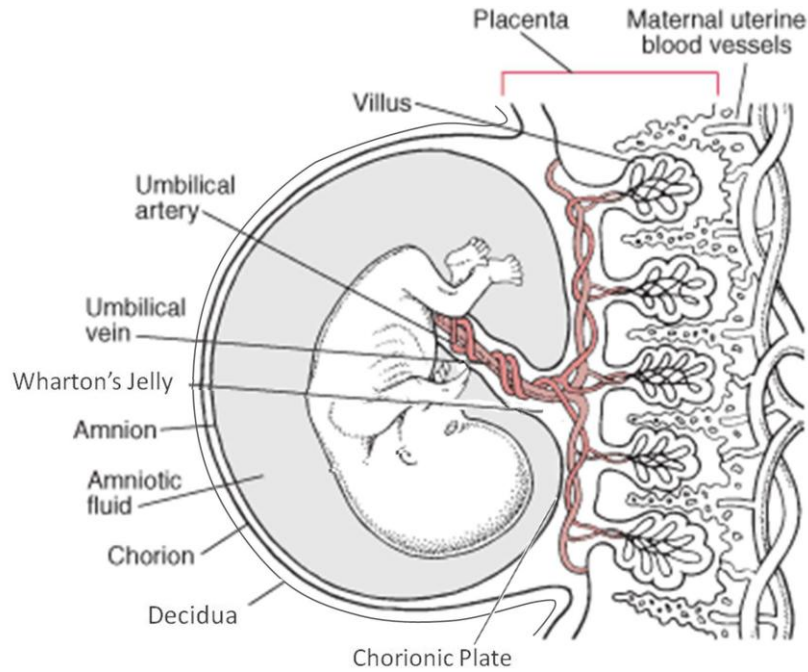


Figure 3 – The placenta and extra-embryonic membranes

The placenta is fully formed by 18-20 weeks gestation and contains chorionic villi carrying fetal blood to the maternal uterine vessels. The placenta is attached to the fetus at the chorionic plate via the umbilical cord, which contains umbilical veins and arteries as well as surrounding Wharton's jelly. The extra-embryonic membranes comprise of amnion and smooth chorion, which are surrounded by the maternal decidua and enclose the fetus and amniotic fluid. Adapted from (Brown 2007).

1.4.2. Amniotic fluid

Amniotic fluid can be collected at term (Murphy and Atala 2013), but in general is collected in the second trimester following routine amniocentesis. Just 2ml of amniotic fluid can contain up to 20,000 cells, 1% of which are stem cell factor *c-Kit* (CD117) positive and show a phenotype similar to hfMSC with characteristics intermediate of hESC and adult BM-MSC (De Coppi, Bartsch et al. 2007). Amniotic fluid stem cells (AFSC) are non-tumorigenic and have potential for application in the clinic due to their broad differentiation potential to a number of lineages from each of the three germ layers (e.g. hepatocytes, osteoblasts, and neurons). AFSC have been described as broadly multipotent, as opposed to pluripotent,

because they eventually senesce, so do not indefinitely self-renew, and they cannot differentiate to all the lineages of the 3 germ layers (Kaviani, Perry et al. 2001; Kaviani, Guleserian et al. 2003; Tsai, Lee et al. 2004; Tsai, Hwang et al. 2006; De Coppi, Bartsch et al. 2007; Cananzi, Atala et al. 2009; Ditadi, de Coppi et al. 2009).

The application of AFSC has been demonstrated *in vivo* by their neuroprotective effect in a mouse model of ischemic stroke (Rehni, Singh et al. 2007) as well as their cardioprotective effects in rodent models of myocardial infarction (Bollini, Cheung et al. 2011; Lee, Wei et al. 2011). The trophic mechanisms of tissue repair by AFSC have also been demonstrated by the increased proliferation and decreased apoptosis of tubular epithelial cells in the kidneys of mice with acute tubular necrosis (Perin, Sedrakyan et al. 2010). AFSC hold much promise for use in cell based therapies and tissue engineering, hence current research is now characterising and investigating the tissue repair capacity of stem cells derived from associated extra-embryonic tissues.

1.4.3. Wharton's jelly

MSC isolated from umbilical cord tissue (i.e. Wharton's jelly) have a similar phenotype to other fetal stem cells with high proliferative potential compared to adult BM-MSC (Schugar, Chirieleison et al. 2009) and expression of MSC genes as well as hESC genes, albeit at a considerably lower level (Fong, Richards et al. 2007; Fong, Chak et al. 2011). The umbilical cord MSC (UC-MSC) can differentiate into lineages of all three germ layers including osteocytes (Wang, Wang et al. 2004), hepatocytes (Anzalone, Lo Iacono et al. 2010) and neurons (Mitchell, Weiss et al. 2003; Anzalone, Lo Iacono et al. 2010). These cells have a high harvest yield and can be used for allogeneic applications (Schugar, Chirieleison et al. 2009). UC-MSC have also been used to suggest that primitive stem cell characteristics decline with age, since UC-MSC had lower osteogenic differentiation potency than hfMSC from first trimester bone marrow (Zhang, Teoh et al. 2009). Although differences may be due to the different tissue sources of origin rather than age.

1.4.4. Amnion

Amniotic epithelial stem cells (AE) have been isolated from the amnion membrane which encloses the amniotic fluid. Similar to AFSC, AE show characteristics intermediate between MSC and hESC, expressing both mesenchymal and pluripotency markers (Yen, Huang et al. 2005; Battula, Treml et al. 2008) with differentiation to some lineages from all three germ layers (Miki, Lehmann et al. 2005; Miki and Strom 2006; Ilancheran, Michalska et al. 2007). AE have also shown similarities to hESC through their formation with mouse ESC of xenogeneic chimeras (Tamagawa, Ishiwata et al. 2004), although importantly for clinical application AE were non-tumorigenic in SCID/beige mice (Miki, Lehmann et al. 2005). Similarly amnion derived MSC have shown capacity to differentiate beyond mesodermal lineages (In 't Anker, Scherjon et al. 2004; Soncini, Vertua et al. 2007) to neurogenic lineages (Sakuragawa, Kakinuma et al. 2004; Chang, Hwang et al. 2010; Manochantr, Tantrawatpan et al. 2010) and endodermal lineages including hepatocytes (Tamagawa, Oi et al. 2007), cardiomyocytes (Zhao, Ise et al. 2005) and endothelial cells (Alviano, Fossati et al. 2007).

AE have also been isolated from the first trimester, but in contrast to CVS or amniocentesis, their collection requires pregnancy termination which restricts their clinical application. Interestingly, first trimester AE show a potentially more primitive phenotype than AE isolated at term. This was shown by a greater proportion of first trimester AE being positive for TRA-1-60 and TRA-1-81 with higher expression of *Nanog* and *Sox2*, but not *Oct4* than their term counterparts (Izumi, Pazin et al. 2009). This suggests that primitive stem cell characteristics could be lost with increasing gestational age.

1.4.5. Chorion

The literature on term chorion-derived stem cells is insufficient with limited characterisation of immuno-phenotype and a focus primarily on isolation techniques and identification of their vascular niche within the placenta (Soncini, Vertua et al. 2007; Poloni, Rosini et al. 2008; Castrechini, Murthi et al. 2010; Bacenkova, Rosocha et al. 2011; Rus Ciuca, Soritau et al. 2011). In addition many studies of term chorionic cells report cells of both fetal and maternal origin (In 't Anker, Scherjon et al. 2004; Soncini, Vertua et al. 2007), with some groups

isolating only maternal origin cells (Barlow, Brooke et al. 2008; Brooke, Rossetti et al. 2009; Semenov, Koestenbauer et al. 2010; Rus Ciuca, Soritau et al. 2011; Jaramillo-Ferrada, Wolvetang et al. 2012), and others failing to exclude maternal cell origin (Yen, Huang et al. 2005; Miao, Jin et al. 2006; Castrechini, Murthi et al. 2010; Bacenkova, Rosocha et al. 2011; Fariha, Chua et al. 2011; Kim, Shin et al. 2011). Whilst studies that used chorionic villous from the chorionic plate of term placenta were able to isolate fetal origin stem cells, since this tissue is not in direct contact with the maternal decidual cells (Abumaree, Al Jumah et al. 2012). Likewise work on first trimester chorionic stem cells has shown fetal origin by karyotyping (Poloni, Rosini et al. 2008), although not carried out in all studies (Portmann-Lanz, Schoeberlein et al. 2006; Spitalieri, Cortese et al. 2009; Portmann-Lanz, Schoeberlein et al. 2010).

Notwithstanding this problem of contaminating decidual maternal stem cells, comparisons of term placenta /chorionic stem cells with adult bone marrow MSC (Soncini, Vertua et al. 2007; Brooke, Rossetti et al. 2009) have indicated that these cells may be more primitive than adult MSC with evidence of greater self renewal *in vitro* when growth kinetics were analysed (Barlow, Brooke et al. 2008; Jaramillo-Ferrada, Wolvetang et al. 2012), as well as expression of some pluripotency markers (Yen, Huang et al. 2005; Bacenkova, Rosocha et al. 2011; Fariha, Chua et al. 2011) and potential to differentiate beyond mesodermal lineages to hepatogenic (Kim, Shin et al. 2011) and neurogenic lineages (Portmann-Lanz, Schoeberlein et al. 2006; Portmann-Lanz, Schoeberlein et al. 2010). This suggests term placenta derived stem cells may be similar to fetal stem cells and have an intermediate phenotype.

Late gestation placenta derived stem cells have already shown potential for use in cell based therapies. For example, they inhibited growth of multiple myeloma in bone and stimulated bone formation in an immune-deficient model of medullary myeloma bone disease (Li, Ling et al. 2011). Stem cells isolated from term placenta also had an anti-fibrotic effect in rats with carbon tetrachloride injured livers (Lee, Jung et al. 2010) and in a mouse model of lung fibrosis (Cargnoni, Gibelli et al. 2009). The same repair effects were seen *ex vivo* when the cells were applied to a perfused human lung (Nazarov, Lee et al. 2012). Term placenta stem cells were also capable of reversing hyperglycemia in streptozotocin-induced diabetic mice and differentiating to islet-like cell clusters that secreted insulin (Kadam, Muthyala et al.

2010). Term placenta stem cells have also demonstrated immuno-suppressive properties both *in vitro* in a mixed lymphocyte reaction (Karlsson, Erkers et al. 2012; Lee, Jung et al. 2012) and *in vivo* where neutrophil infiltration was decreased in a mouse model of bleomycin-induced lung fibrosis (Cargnoni, Gibelli et al. 2009).

Fetal stem cells isolated from CVS can be collected in the first trimester as early as the 10th week of gestation, which allows sufficient time for cell expansion, differentiation, transduction, etc. for use in autologous prenatal cell therapy. This may be an advantage over the mid-trimester AFSC for preventing progression of congenital fetal diseases and subsequent organ damage as early as possible (Shaw, David et al. 2011). Similar to amniocentesis, CVS is an invasive procedure with a 1% miscarriage risk, which although low, may prevent banking of these cells in healthy pregnancies (Tabor and Alfirevic 2010). In terms of supply for allogeneic applications or cell banking, first trimester suction termination of pregnancy is the commonest operation in the world and although ethically contentious for clinical application, placenta would be an ideal source of stem cells to collect from pregnancy termination, being larger and easier to separate than other fetal tissues.

Cultured first trimester chorionic derived stem cells show a phenotype similar to AFSC with a fibroblast-like morphology, greater cell expansion than adult BM-MSC, differentiation to osteocytes, adipocytes, chondrocytes and neuronal cells and expression of MSC markers as well as some markers found in hESC (*Oct4* and *Rex1*) (Portmann-Lanz, Schoeberlein et al. 2006; Poloni, Rosini et al. 2008; Spitalieri, Cortese et al. 2009). Immuno-regulatory capacity has also been shown for these cells, which inhibited proliferation of stimulated T-lymphocytes (Poloni, Maurizi et al. 2012). First trimester chorionic stem cells have also demonstrated beneficial paracrine effects, showing release cytokines that reduced neurotoxin-induced apoptosis in a Parkinson's disease cell model (Nazarov, Lee et al. 2012). Although few studies have tested the survival and functions of the first trimester placenta stem cells in rodent models, their capacity to be expanded large-scale in human serum and maintain a stable karyotype means they are a promising source for regenerative medicine (Poloni, Rosini et al. 2008; Poloni, Maurizi et al. 2012). Whether the earlier gestational age of the chorionic stem cells translates to better tissue repair compared to stem cells isolated at birth needs to be answered in order to select the optimal source for cell therapy application.

1.5. Osteogenesis imperfecta

1.5.1. The 11 types of OI

Osteogenesis Imperfecta (OI), or brittle bone disease, is a genetic disorder of connective tissue caused by mutations in collagen type I or genes associated with its biosynthesis (**Fig.4**). OI occurs in 1 in 15,000–20,000 births (Marini 1998) and is defined as a heterogeneous group of disorders that vary in severity and result in bones being more prone to fracture. There is no cure for OI and current treatments merely manage the symptoms or offer temporary improvements in bone strength rather than treat the underlying cause (Van Dijk, Pals et al. 2010).

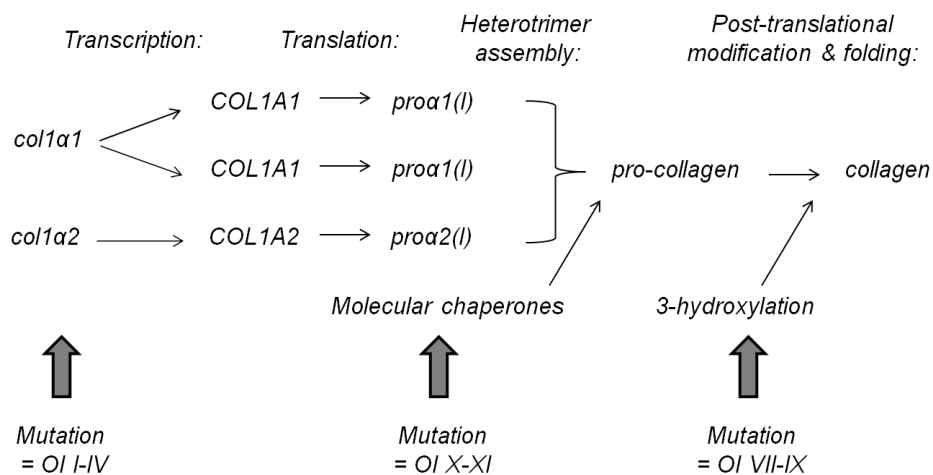


Figure 4 - The biosynthesis of collagen type I

*The biosynthesis of collagen type I; *col1a1* and *col1a2* genes are transcribed and translated to pro α 1(I) and 1 pro α 2(I), assembled by molecular chaperones, then modified with 3-hydroxylation and folded to form collagen type I. Mutations in *col1a1* and *col1a2* genes cause type I-IV OI, mutations in molecular chaperones cause type X-XI OI and mutations in the 3-hydroxylation process result in type VII-IX OI. Arrows represent mutations. OI: Osteogenesis imperfecta.*

There are 11 types of OI (**Table 2**) of which the clinical manifestations range in severity from the perinatal lethal type II to the mild non-deforming type I. OI patients are more susceptible to fractures due to reduced bone mass, which increases the brittleness and fragility of their bones (Stoss and Freisinger 1993; Cohen-Solal, Zylberberg et al. 1994; Dagleish 1997; Van Dijk, Pals et al. 2010). Other symptoms of OI can include short stature, bone deformities

including scoliosis and vertebral compressions, joint laxity, blue or grey sclera, dentinogenesis imperfecta, hearing problems, triangular shaped face, cardiopulmonary problems and reduced life expectancy (Basel and Steiner 2009).

Table 2 - Types of OI, inheritance and clinical symptoms. OI types I-XI inheritance pattern causative genetic defect, disease severity, age of diagnosis and clinical symptoms presented; including incidence of fractures, stature, eye sclera colour and whether dental problems are present. OI: Osteogenesis Imperfecta.

OI	Severity	Diagnosis	Clinical Symptoms				Genetic Defect	Ref
			Fractures	Stature	Sclera Colour	Dental Issues		
Autosomal Dominant Inheritance:								
I	Mild	Child-adulthood	A few	Normal/mildly short	Blue	No	COL1A1	(Sillence, Rimoin et al. 1979)
II	Lethal	<i>In utero</i>	Multiple	Short	Dark	NA	COL1A1/ COL1A2	(Sillence, Rimoin et al. 1979)
III	Severe	<i>In utero/at birth</i>	Multiple	Short	Grey	Yes	COL1A1/ COL1A2	(Sillence, Rimoin et al. 1979)
IV	Mild-moderate	Birth-adulthood	Variable	Moderately short	Grey/white	Yes/No	COL1A1/ COL1A2	(Sillence, Rimoin et al. 1979)
V	Moderate-severe	Birth-adulthood	Multiple with hypertrophic callus	Short/mildly short	Normal white	No	Unknown	(Glorieux, Rauch et al. 2000)
Autosomal Recessive Inheritance:								
VI	Moderate-severe	Birth-adulthood	Multiple	Moderately short	Normal white	No	SERPINF1	(Glorieux, Ward et al. 2002)
VII	Moderate-severe	Birth	Multiple	Mildly short	Normal white	No	CRTAP	(Barnes, Chang et al. 2006; Morello, Bertin et al. 2006)
VIII	Severe-lethal	<i>In utero/at birth</i>	Multiple	Short	Normal white	Yes	LEPRE1	(Cabral, Chang et al. 2007)
IX	Severe-lethal	Birth-childhood	Multiple	Moderately short	Normal white	Yes	PPIB	(van Dijk, Nesbitt et al. 2009)
X	Severe	Birth-childhood	Multiple	Moderately short	Blue	Yes	SERPINH1	(Christiansen, Schwarze et al. 2010)
XI	Severe	Birth	Multiple	Short	Normal white	No	FKBP10	(Alanay, Avaygan et al. 2010)

1.5.2. Clinical management of OI

Current clinical management of OI involves a multidisciplinary approach. Non-surgical methods aim to treat fractures through bracing, splinting and physiotherapy as well as improving hearing, dental and psychological issues, although this approach can increase mobilisation, joint movement and muscle function it cannot prevent fractures (Sillence, Morley et al. 1995; Niyibizi, Wang et al. 2004). Whilst surgery aims to prevent and correct deformities, such as using intramedullary telescopic rods to stabilise long bones (Cho, Kim et al. 2011), spine stabilisation to correct scoliosis and basilar impression surgery to correct migration of the spine (Bishop 2010; Monti, Mottes et al. 2010). However, surgery only stabilises the progression of deformity and is associated with risks during surgery as well as complications afterwards such as rod migration, increased risk of osteoporosis and immobilization (Esposito and Plotkin 2008).

Pharmacological therapies, namely bisphosphonates, have become the standard treatment for children with OI. Bisphosphonates are internalised by osteoclasts and inhibit their bone resorption, thereby increasing bone mass, density and cortical width through accumulation of the defective bone matrix (Munns, Rauch et al. 2005; Russell, Lacey et al. 2011). However, controlled trials show the effectiveness of bisphosphonates reduces long term, with no significant improvements in growth, muscle strength, bone pain or lower extremity long bone fractures (Letocha, Cintas et al. 2005). Furthermore neither surgery nor bisphosphonates address the genetic mutations that affect collagen biosynthesis and subsequently lead to bone fragility.

Some studies have shown growth hormone in combination with bisphosphonates may prove more effective than bisphosphonates alone, with greater bone mineral density and growth velocity measured in OI children, although fracture incidence was not improved (Antoniuzzi, Monti et al. 2010). Alternatively antibodies, such as denosumab, anti-sclerostin and DKK1-IgG, have also been shown to increase bone formation over bone resorption, thereby increasing matrix deposition and bone mass (Forlino, Cabral et al. 2011). However, the safety and long term efficacy of these methods is not yet known and the bone matrix deposited remains defective.

1.5.3. Rodent models of OI

Rodent models of OI (**Table 3**) have provided a tool to not only better understand the disorder, but also to investigate the effectiveness of novel therapies, before they are brought to the clinic. The most popular rodent model, with over 57 publications to date, is the model of type III OI; osteogenesis imperfecta murine (*oim*). These mice have reduced growth, multiple fractures, skeletal deformities, enlarged growth plates and decreased bone strength, length and volume. *Oim*^{-/-} (i.e. *oim*) are homozygous for a naturally occurring Glycine deletion at nucleotide 3983 of the *Colla2* gene. As a result these mice cannot produce pro α 2(I) collagen, causing the replacement of normal heterotrimeric COL1(α 1)₂(α 2) with homotrimeric COL1(α 1)₃, which accumulates in the extracellular matrix and causes bone fragility (Chipman, Sweet et al. 1993).

Table 3 – OI murine models

Murine models of OI, the type of OI they most resemble, the causative genetic mutation and how the mutation is inherited. OI: Osteogenesis imperfecta. M-MuLV: Moloney murine leukaemia virus. AD: autosomal dominant. AR: autosomal recessive. ^{+/+}: Heterozygous for mutation. ^{-/-}: Homozygous for mutation.

Mouse Model	OI Type	Genetic Mutation	Inheritance	Reference
<i>Mov13</i> ^{+/+}	I	M-MuLV insertion	AD	(Bonadio, Saunders et al. 1990)
<i>Mov13</i> ^{-/-}	II	M-MuLV insertion	AR	(Bonadio, Saunders et al. 1990)
<i>Sp7</i> ^{-/-}	II	Knock-out <i>Sp7</i>	AR	(Baek, Lee et al. 2009)
Transgenic	II	<i>Col1a1</i>	AD	(Stacey, Bateman et al. 1988)
<i>Oim</i> ^{-/-}	III	Glycine deletion <i>colla2</i>	AR	(Chipman, Sweet et al. 1993)
<i>Aga2</i> ^{+/+}	III	<i>Col1a1</i> substitution	AD	(Lisse, Thiele et al. 2008)
<i>Brt1</i> ^{+/+}	IV	Knock-in <i>colla1</i>	AD	(Forlino, Porter et al. 1999)
<i>Brt1</i> ^{-/-}	IV	Knock-in <i>colla1</i>	AR	(Forlino, Porter et al. 1999)
<i>OI-transgenic</i>	II-IV	Human COL1A1 minigene	AD	(Khillan, Olsen et al. 1991)
<i>Crtap</i> ^{-/-}	VII	KO <i>Crtap</i>	AR	(Morello, Bertin et al. 2006)
<i>Lepre1</i> ^{-/-}	VIII	KO <i>Lepre1</i>	AR	(Vranka, Pokidysheva et al. 2010)
<i>Ppib</i> ^{-/-}	IX	KO <i>Ppib</i>	AR	(Choi, Sutor et al. 2009)

Other key rodent models investigated include the *Mov13* mice, which were created by inhibition of *Colla1* allele transcription using the Moloney murine leukemia virus. Homozygote *Mov13*^{-/-} have embryonic lethality, whereas heterozygote *Mov13*^{+/-} mice display mild type I OI phenotype; reduced bone strength and increased tissue porosity, due to insufficient collagen fibril synthesis (Bonadio, Saunders et al. 1990). Knock-in type IV OI murine model Brittle (*Brtl*) mice have a Glycine³⁴⁹ to Cystine mutation in one *colla1* allele created using the *Cre/lox* recombination system. Heterozygote *Brtl*^{+/-} mice have 30% chance of perinatal lethality, reduced body size, reduced bone mineral density, and long bone deformities and fractures. Whereas, homozygote *Brtl*^{-/-} mice have no perinatal lethality, normal bone mineral density and only a moderately reduced body size due to homozygosity for the mutant allele resulting in a more homozygous extracellular matrix, which is subsequently better organised and therefore less brittle. Different mixtures of normal and mutant pro α 1(I) collagen chains may contribute to the range of severities seen in *Brtl*^{+/-} mice as well as patients with type IV OI (Forlino, Porter et al. 1999).

1.6. Prospective novel therapies for OI

1.6.1. Gene therapy

In contrast gene and cell therapy, improve bone fragility by acting during collagen formation to correct helical conformation and fibril assembly. Gene therapy aims to silence abnormal genes and/or increase normal gene expression. In autosomal recessive cases of OI (**Table 2**), where there is a deficiency in proteins required for post-translational modifications and folding (e.g. absence of 3-hydroxylation or deficiency in FKBP10 or Serpin H1 collagen chaperones), gene supplementation to over-express the normal collagen gene may be useful. Whereas in autosomal dominant OI types the defect is in the quantity or structure of type I procollagen; the presence of both mutant and normal procollagen chains results in matrix heterogeneity and the different severities of the OI phenotype. Therefore additional antisense gene approaches are required to promote degradation of mutant mRNA transcripts. However, the *Colla1* and *Colla2* genes span 51 and 52 exons respectively and over 800 different mutations have been reported within these genes to cause OI. Therefore the genetic heterogeneity of OI means vector design to specific mutations is both complicated and time

consuming (Niyibizi, Wang et al. 2004; Forlino, Cabral et al. 2011). Subsequently the reality of bringing gene therapy to the clinic as a standardised treatment for OI is unlikely in the near future and cell therapy holds the most promise for treating this disease.

1.6.2. Cell therapy

The potential of cell therapy for OI has been tested in various rodent models with different cells, methods of transplantation and analysis techniques (**Table 4**). The *OI-transgenic* murine model with a moderate (type IV OI) to severe (type II OI) phenotype was the first used to assess the potential of cell therapy to treat OI (Pereira, O'Hara et al. 1998). The mice had reduced growth, multiple fractures and reduced bone mineral and collagen content due to insertion of a mini-gene of human *pro α 1(I)* with a 41 exon deleted region that synthesised shortened pro α 1(I) chains causing degradation of normal pro α 1(I) chains (Khillan, Olsen et al. 1991). Pereira *et al.* infused wild type MSC isolated from bone marrow or whole bone marrow intra-peritoneal into 3 week old *OI-transgenic* after irradiation and showed donor cells were engrafted 1 and 2 months after transplantation in the bone, bone marrow, cartilage and other non-hematopoietic tissues such as the lung, brain, skin and spleen (Pereira, O'Hara et al. 1998). A small, but significant increase in collagen content and mineral content was also recorded in the transplanted mice, but how the donor cells contributed to these changes was not investigated.

Subsequent experiments by Niyibizi's group using the *oim* mouse model showed both engraftment of donor cells and differentiation down the osteogenic lineage. GFP tagged osteoprogenitors from wild type mouse bones were transplanted into neonatal *oim* via the superficial temporal vein and were detected on the *oim* endosteal bone surfaces, cortical bone, spongiosa, bone marrow and areas of active bone formation four weeks after transplantation. Differentiation of donor cells to osteoblasts and osteoprogenitors following transplantation was confirmed by gene expression analysis; transplanted bone had high expression of osteoblast-specific genes (e.g. *Osteocalcin* and *Osterix*), whereas

Table 4 - The history of cell therapy with murine models of OI

In chronological order the cell therapy experiments carried out on murine models of OI; with details of the type of cells used, the mouse model, the age of transplantation and analysis, whether mice were irradiated before transplantation and the route of cell delivery. Results shown include whether transplanted cells engrafted and differentiated in the recipient bones and any change to bone properties or mouse phenotype recorded. OI: Osteogenesis imperfecta. WT: wild type. MSC: mesenchymal stromal/stem cells. E: embryonic day. i.p: intraperitoneal. i.v: intravenous. ^{+/+}: Heterozygous for mutation. ^{-/-}: Homozygous for mutation.

Mouse Model	Cells	Age	Irradiate	Route	Age Culled	Results				Ref
						Cells Engraft	Donor Cell Differentiate	Bone Pathology	Phenotype	
<i>OI-transgenic</i>	WT bone marrow (whole/MSC)	3 weeks	Yes	i.p.	8-12 weeks	Yes	Not measured	Not measured	Not measured	(Pereira, O'Hara et al. 1998)
<i>Oim^{-/-}</i>	WT osteoprogenitors	2 days	Yes	i.v.	2&4 weeks	Yes	Yes	Not measured	Not measured	(Wang, Li et al. 2006)
<i>Oim^{-/-}</i>	WT clonal bone marrow MSC	2 days	Yes	i.v.	2&4 weeks	Yes	Yes	Not measured	Not measured	(Li, Wang et al. 2007)
<i>Oim^{-/-}</i>	Human fetal blood MSC	E13.5 - 15	No	i.p.	2,4,8,12 weeks	Yes	Yes	Increase bone thickness, plasticity & length.	Decreased long bone fractures	(Guillot, Abass et al. 2008)
<i>Brtl^{+/+}</i>	WT whole adult bone marrow	E13.5 - 14	No	i.p.	8 weeks	Yes	Yes	Increase bone strength	No perinatal lethality	(Panaroni, Gioia et al. 2009)
<i>Oim^{-/-}</i>	Human fetal blood MSC	E13.5 - 15	No	i.p.	8 weeks	Yes	Yes	Improved plasticity, matrix stiffness & organisation	Decreased long bone fractures	(Vanleene, Saldanha et al. 2011)
<i>Oim^{-/-}</i>	Human fetal blood MSC	3-5 days	No	i.p.	8 weeks	Yes	Yes	Decreased bone brittleness	Decreased long bone fractures	(Jones, Moschidou et al. 2012)

osteoprogenitor-specific genes were expressed more highly in the bone marrow (e.g. Osteopontin, Sox9 and *Runx2*) (Wang, Li et al. 2006). Although protein analysis did not find the presence of the missing COL1A2 protein, a subsequent study by the same group did find COL1($\alpha 1$)₂($\alpha 2$) heterotrimers 4 weeks after injection of single cell-expanded marrow progenitor cells in neonatal *oim* (Li, Wang et al. 2007). This data shows transplantation of normal cells into a mouse model of OI results in their migration to bone and subsequent differentiation to functional osteoblasts.

Since these studies clinical trials have used cell therapy to treat OI patients; four published, two completed and one presently recruiting with most studies carried out in the U.S.A and Sweden. One study showed transplantation of bone marrow MSC in OI children resulted in gains in body length and bone mineralization, although these effects were lost over time and donor cell engraftment was low at less than 2% (Horwitz, Gordon et al. 2002). This group is currently recruiting participants for further clinical trials to evaluate the safety and effectiveness of repeated infusions of MSC in children with OI.

1.6.3. Fetal cell therapy

The use of fetal stem cells to treat OI prenatally before irreversible damage is caused was shown in a clinical trial whereby allogeneic fetal liver derived MSC were transplanted *in utero* in a human fetus with OI. The transplantation led to better than expected results with no visible immune rejection, engraftment of cells in bone nine months later and regularly arranged and configured trabecular bone. However, again engraftment was low at only up to 7% and results were confounded by concomitant use of bisphosphonates (Le Blanc, Gotherstrom et al. 2005).

The potential of prenatal therapy to treat OI using human fetal MSC was assessed in the *oim* mouse model. *Oim* were transplanted prenatally at E13.5-15 with fetal MSC isolated from first trimester human fetal blood. At 8 weeks transplanted *oim* had an overall two-thirds reduction in long bone fractures when compared to age-matched *oim* controls with significant reduction in fracture incidence for all long bones at all time points (4, 8 and 12 weeks) analysed. Furthermore there was normalisation of growth plate height and increase in bone

length, strength and cortical thickness (Guillot, Abass et al. 2008). An independent study found that the dramatic reduction in fracture rate of these mice may be attributed to a reduction in bone brittleness, rather than increased bone strength (Vanleene, Saldanha et al. 2011).

Analysis of the mechanisms underlying these therapeutic effects revealed that the donor cells homed preferentially to bones, compared to other organs, where they were found in higher numbers at sites of fracture callus and active bone formation. Cells engrafted in bones, expressed late markers of osteoblasts (e.g. *Osteocalcin*) and produced the Collagen type 1 alpha 2 (COL1A2) chain protein, which is absent in non-transplanted *oim* mice. This led to modification of the bone matrix composition, resulting in increased bone matrix stiffness and improved organisation of lamellar bone (Guillot, Abass et al. 2008; Vanleene, Saldanha et al. 2011; Vanleene, Porter et al. 2012).

However, engraftment levels of donor cells were low at only 3-5% and the long term effects of transplantation could not be measured due to strict severity limits on *oim* under British law (Guillot, Abass et al. 2008). In addition fetal MSC isolated from first trimester blood are collected either by cardiocentesis during termination of pregnancy, which is ethically and technically challenging and prevents autologous application, or from the umbilical cord during ongoing pregnancy, which is highly invasive and carries a risk of miscarriage (Chan, Kumar et al. 2008).

A similar study was carried out in the *Brtl* mouse, a model of autosomal dominant OI, and similar effects were shown. Donor cells were found engrafted in bone 8 weeks after prenatal transplantation of wild type adult bone marrow into *Brtl* mice. These cells contributed to up to 20% of all collagen type I synthesised, which was associated with increased cortical thickness and improvements in bone stiffness, ultimate load and yield load. In addition transplantation *in utero* eliminated perinatal lethality seen in heterozygote *Brtl*^{+/-} mice (Panaroni, Gioia et al. 2009).

This study showed the potential of cell therapy to treat autosomal dominant types of OI in addition to recessive types. It also highlighted the possibility to treat perinatal lethal type II

OI with a prenatal approach. The improvements observed occurred despite just 1-2% engraftment of donor cells (Panaroni, Gioia et al. 2009). This engraftment rate was lower than that seen following fetal blood MSC transplantation in *oim* (3-5%) (Guillot, Abass et al. 2008) or after fetal liver MSC transplantation in an OI fetus (7%) (Le Blanc, Gotherstrom et al. 2005). This suggests a fetal-to-fetal approach may have advantages over adult-to-fetal approach in terms of engraftment levels.

In addition, human fetal MSC are more primitive than adult stem cells with higher kinetics and later senescence, longer telomeres, greater telomerase activity and expression of pluripotency markers (e.g. *OCT4*, *NANOG*, *SSEA-4*, *SSEA-3*, *TRA-1-60*, *TRA-1-81* and *REXI*). Subsequently, they have greater expansion potential. Second, fetal MSC are smaller in size, which is likely to aid their ability to pass through the endothelial wall of the blood vessels, allowing improved migration to sites of injury. Fetal MSC also have a unique immune-privileged status. For example, liver derived fetal MSC did not elicit alloreactive lymphocyte proliferation either when undifferentiated, differentiated or IFN- γ stimulated to upregulate HLA I and HLA II (Gotherstrom, Ringden et al. 2003). Finally, fetal MSC more readily differentiate to osteoblasts, which is critical to produce normal type I collagen fibrils, improve bone matrix organisation and subsequently improve the skeletal fragility caused by OI (Vanleene, Porter et al. 2012).

1.7. The hurdles of cell therapy for OI

1.7.1. Low engraftment of transplanted cells

Although cell therapy shows great potential, several pitfalls constrain their clinical application. First the clinical effectiveness of cell therapy is challenged by low level of engraftment in target organs. For example, engraftment levels in a given bone chip ranged from 0.3-28% following neonatal transplantation of adult murine MSC in *oim* mice, despite irradiation of the recipient mice (Li, Wang et al. 2007). Whilst *in utero* transplantation of human fetal blood MSC in *oim* mice that were not irradiated was associated with 3-5% engraftment levels in bone (Guillot, Abass et al. 2008; Vanleene, Saldanha et al. 2011). Clinical experience is similar, with less than 2% engraftment of bone marrow MSC in

transplanted children with OI (Horwitz, Prockop et al. 1999; Horwitz, Prockop et al. 2001; Horwitz, Gordon et al. 2002), and up to 7% engraftment recorded following prenatal allogeneic fetal liver MSC transplantation in a human fetus with OI (Le Blanc, Gotherstrom et al. 2005).

Donor cell engraftment levels are important to treat autosomal recessive OI types as they have a protein deficiency, but are more critical to treat autosomal dominant types of OI. This is because levels of differentiated donor cells must be high enough to significantly alter the ratio of normal to mutant proteins synthesised and counteract the structural instabilities caused by these abnormal pro-collagen proteins (Forlino, Cabral et al. 2011). The proof of this principle has been demonstrated in patients that are mosaic carriers of autosomal dominant OI and subsequently have both affected and normal somatic and germline cells. Despite 40-75% of their osteoblasts presenting a collagen I mutation the patients have normal skeletal growth, density and histology and often are only identified as a carrier when they have more than one child with OI (Cabral and Marini 2004).

Direct differentiation of donor cells in the *oim* model has been implicated in the therapeutic benefits seen following transplantation (Wang, Li et al. 2006; Guillot, Abass et al. 2008; Li, Ling et al. 2011; Vanleene, Saldanha et al. 2011), therefore strategies to increase donor cell engraftment are critical. However, simply increasing the number of cells transplanted has been shown to have little effect on engraftment rate, with one study showing maximal engraftment levels in lethally irradiated mice at just 15% (Marino, Martinez et al. 2008). The importance of the CXCR4-SDF1 pathway in homing of MSC to fractures was shown *in vitro* whereby SDF-1 increased chemotaxis of MSC to injured bone dose dependently (Kitaori, Ito et al. 2009). Also work in a mouse model with a stabilised tibia fracture, showed only CXCR4⁺ MSC and not CXCR4⁻ MSC migrated to the site of injury (Granero-Molto, Weis et al. 2009).

A number of studies have reported strategies to manipulate stem cell homing by upregulating CXCR4 expression and subsequent migration towards SDF-1 gradients. For example, CXCR4 expression has been increased by IGF-1 (Li, Yu et al. 2007), hypoxic pre-conditioning or pre-treatment with desferrioxamine (DFX) (Hung, Pochampally et al. 2007;

Hung, Pochampally et al. 2007), pre-treatment with PDGF (Fiedler, Etzel et al. 2004), IGF-I or IGF-II (Fiedler, Brill et al. 2006) or cytokine treatment: e.g. tumor-necrosis factor-alpha (TNF α) (Ohira, Itatsu et al. 2006) and interleukin (IL-6), which like IL-1, PDGF and TGF α are released during the early stages of fracture (Dimitriou, Tsiridis et al. 2005). Fetal MSC express high levels of intracellular CXCR4, but only a small number of cells present CXCR4 on the cell surface. Priming human fetal blood MSC with SDF1 externalised CXCR4, and was associated with increased chemotaxis *in vitro* and a three-fold upregulation of engraftment in *oim* and wild type bone and bone marrow (Jones, Moschidou et al. 2012). Testing if the increased engraftment of donor cells translates to therapeutic benefits in the *oim* mouse model is the next step to optimising cell therapy for OI.

1.7.2. Immune rejection of transplanted cells

A second hurdle for cell therapy is the rejection of allogeneic cells by the host immune system. Fetal and adult MSC do not express HLA class II and express low levels HLA class I, therefore they are unlikely to elicit an immune reaction (Le Blanc 2003; Le Blanc, Tammik et al. 2003). In addition these cells have immune-suppressive properties, which prevents their rejection from the host immune system by inhibiting T, B, NK and dendritic cell proliferation and activating regulatory T cells (Trento and Dazzi 2010; Francois and Galipeau 2012). Allogeneic bone marrow MSC has been successfully injected in collagen-induced arthritic mice and prevented bone and cartilage damage (Augello, Tasso et al. 2007). Whilst HLA-mismatched fetal liver MSC were transplanted *in utero* in a fetus with OI and engrafted and differentiated *in vivo* with no immunologic reaction against the donor cells (Le Blanc, Gotherstrom et al. 2005). However, before translation of stem cells to the clinic their immuno-modulatory properties should first be tested after expansion in clinical grade GMP conditions, since differences in immunosuppression and adhesion molecules were found in adult BM and adipose MSC after fetal bovine serum was replaced in the media with human platelet lysate (Menard, Pacelli et al. 2013).

1.7.3. Identification of a clinically translatable cell source

Finally a major hurdle to the translation of cell therapy in the clinic is to find and test an appropriate cell source. Collection procedures to aspirate bone marrow and isolate adult MSC are often painful and highly invasive and cell numbers are low with only one estimated MSC per 250,000 bone marrow cells. The limited expansion potential of adult MSC is also a practical disadvantage in terms of clinical application. Stem cells from fetal organs and blood isolated after termination have higher expansion potential and more potent differentiation, but often require termination of pregnancy, hence their clinical use is restricted by ethical legislations (Guillot, Gotherstrom et al. 2007). Therefore, to bring cell therapy for OI closer to the clinic a more suitable cell source is required, such as cells isolated from extra-embryonic fetal tissues like placenta and amniotic fluid, which are available in all clinics, are easy to physically manipulate and their use is unlikely to cause ethical controversy (Abdulrazzak, Moschidou et al. 2010).

1.8.Aims and hypothesis

Placental chorionic tissue is readily available in clinics and uniquely represents a cell source that can be safely collected without pregnancy termination in the first trimester and at term. It is therefore an ideal source to test the hypothesis that stem cells isolated earlier in gestation are more primitive than late gestation counterparts with advantages for use in regenerative medicine. The aim of this study was to first investigate whether early fetal placental chorionic stem cells (e-CSC) were physiologically superior to their late gestation counterparts (l-CSC) and secondly, if this was the case, to assess the capacity of e-CSC for bone repair in the *oim* mouse model to determine the potential of these cells for use in cell therapy of OI.

CHAPTER 2 - Materials and Methods

2.1. Cell culture techniques

2.1.1. Placenta cell isolation and culture

Human early chorionic stem cells (e-CSC) ($n=5$, 8^{+3} to 12^{+0} weeks gestational age) were previously isolated in the lab from placental chorionic villi (**Fig.5A**) after chorionic villous sampling prior to termination of pregnancy [Dr Dafni Moschidou]. Human late chorionic stem cells (l-CSC) were isolated from the placental chorionic plate (**Fig.5B**) of the extra-

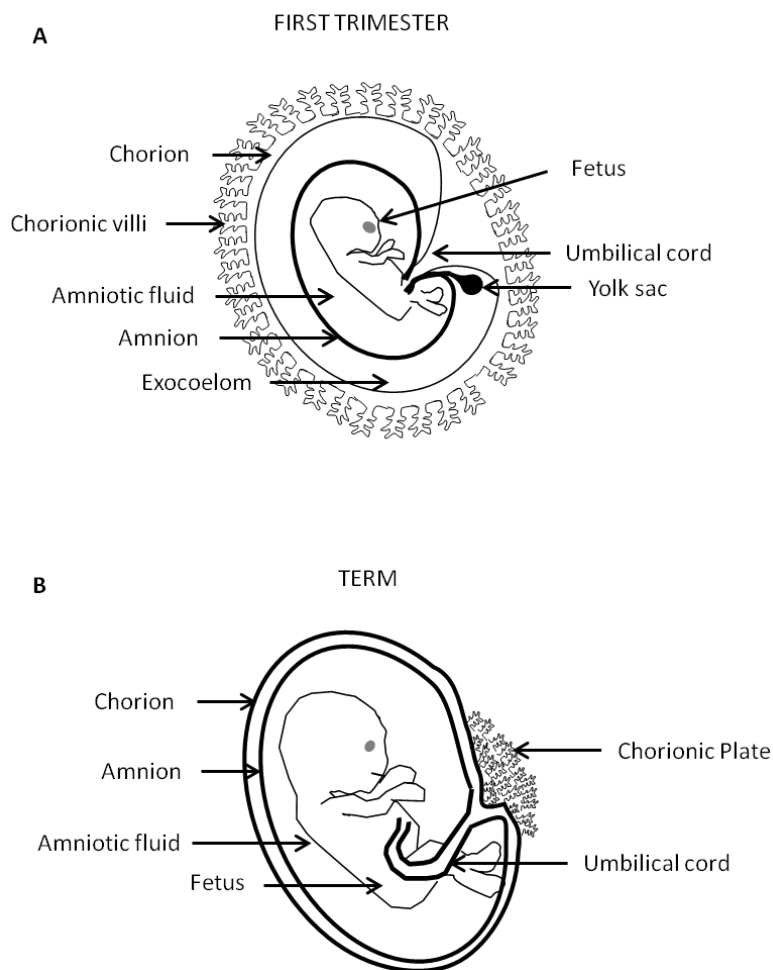


Figure 5 – Chorionic villous tissue is confined to the chorionic plate in late gestation.

(A) Chorionic villi surround the gestational sac in the first trimester. (B) By term the chorionic villi have been confined to the chorionic plate as the amniotic cavity has expanded and fused with the chorion to form the amnio-chorionic membrane.

embryonic membranes obtained from caesarean section deliveries ($n = 5$). The chorionic villous tissues (5 cm^2 for l-CSC and the totality of the sample for e-CSC) were separated from the amnion, minced mechanically, and trypsinized using 0.05% Trypsin EDTA (Invitrogen) for 20 min. Cells were subsequently centrifuged (5 min at 1500 rpm), counted and plated (10^4 cells per cm^2) in expansion media; high glucose Dulbecco's modified Eagle's medium (DMEM-LG) (Invitrogen) with 10% fetal bovine serum (BioSera) and 2 mM L-glutamine, 50 IU/ml penicillin, 50 mg/ml streptomycin (Gibco BRL Life Technologies). Cells were

incubated at 37°C in 5% CO_2

and serially passaged at 70-80% confluence. Cell passaging involved PBS washes, then trypsinization for 2 min, followed by centrifugation for 5 min at 1500 rpm and replating in expansion media at a 1:3 split. Cells were frozen using the same protocol, except instead of replating cells were frozen in freezing media; which contained 10% Dimethyl sulfoxide (DMSO; Sigma), 40% FBS and 60% expansion medium. Isolated cells were plastic adherent and c-KIT⁺ and all cells were used at passage 2-9. Ethical approval was given by the Research Ethics Committees of Hammersmith & Queen Charlotte's Hospitals and the joint University College London Hospitals. Committees on the Ethics of Human Research (08/H0714/87).

2.1.2. Osteogenic Differentiation

Cells were plated at 10^4 cells per cm^2 and grown in expansion media (see above) for 1 day. Expansion media was then replaced with high glucose DMEM supplemented with 10 mM β -glycerophosphate, 0.2 mM ascorbic acid and 10^{-8} M dexamethasone, which was replaced every 3 days for 14 days. Then cells were fixed with 10% formalin and stained with von Kossa or alizarin red, or pelleted and extracted for RNA or protein (see below) for qRT-PCR or western blot analysis respectively. All analysis was compared to undifferentiated cells. RNA and cDNA was extracted as previously described and the qRT-PCR reactions were carried out as above for osteogenic specific primers (**Table 5**), normalised to *GAPDH* and compared to basal expression levels in non-differentiated cells.

2.1.3. Adipogenic differentiation

Cells were seeded at a concentration of 10^4 cells per cm^2 on tissue culture plastic plates and cultured expansion media (see above) for 1 day. The media were then changed to high glucose DMEM supplemented with 0.5 μM hydrocortisone, 0.5 μM isobutyl methylxanthine and 60 μM indomethacin (all Sigma-Aldrich), which was replaced every 3 days for 2 weeks. Then cells were pelleted for RNA or washed in PBS, fixed with 10% formalin, rinsed with 60% isopropanol (BDH Laboratory Supplies), then stained for 10 minutes with oil-red-O (Sigma-Aldrich). Oil-red-O was prepared at a 1% concentration in isopropanol (BDH

Laboratory Supplies, Dorset, UK) then diluted in a 3:2 ratio of stock solution to distilled water. The cells were then washed 4 times with distilled water and photographed using an Olympus CK40-SLP microscope and an Olympus C3040-ADL camera (Olympus). RNA and cDNA was extracted as previously described and the qRT-PCR reactions were carried out as above with PPAR γ specific primers (**Table 5**), normalised to *GAPDH* and compared to basal expression levels in non-differentiated cells.

2.1.4. Neurogenic differentiation

Cells were seeded at a concentration of 5000 cells/cm² on tissue culture plastic plates and coverslips and cultured in expansion media (see above) for 1 day. The media were then changed to high glucose DMEM supplemented with 0.5% FBS, 1% penicillin/streptomycin, 2mM L-glutamine and 0.1% baicalin (Sigma), freshly made. C17.2 mouse neural progenitor cells (from Henrik Hagberg and Pierre Gressens) were added in a co-culture setting to promote differentiation, using co-culture membrane inserts (Thermo Scientific Nunc, Loughborough, UK). The cells were allowed to differentiate for 5 days and assessed for expression of the neuronal markers β -TUBULIN and MAP2 and for the NMDA receptor NR1 (**Table 6**) using confocal immunostaining, as previously described. Positive controls were differentiated neural progenitors [from Henrik Hagberg and Pierre Gressens] and negative controls were undifferentiated e-CSC and l-CSC cells.

2.1.5. Hepatogenic differentiation

Cells were seeded at a concentration of 5000 cells/cm² on tissue culture plastic plates and coverslips coated with Matrigel (BD Biosciences) and cultured in high glucose DMEM supplemented with 1% penicillin/streptomycin (Invitrogen), 2mM L-Glutamine (Invitrogen) and 10% FBS for 3 days. The media were then changed to high glucose DMEM supplemented with 15% FBS, 1% penicillin/streptomycin, 2mM L-glutamine, 300 μ M monothioglycerol (Sigma), 20ng/ml hepatocyte growth factor (Peprotech), 10ng/ml oncostatin M (Peprotech), 10⁻⁷ dexamethasone (Sigma), 100ng/ml FGF4 (Peprotech) and 1X ITS (insulin, transferrin, selenium, Sigma). The cells were allowed to differentiate for 21

days and then fixed and stored in PBS for immuno-fluorescence. The differentiation media were collected and analysed for the presence of urea secreted by the differentiated cells. Urea was subsequently measured using the urea/ammonia determination kit (R-Biopharm AG, Darmstadt, Germany) according to the manufacturer's instructions, with positive control being urea (as per manufacturer's instructions) and negative control undifferentiated e-CSC and l-CSC. Cells were subsequently assessed for expression of the hepatocyte markers ALBUMIN and ALPHA FETO-PROTEIN (AFP) using confocal microscopy. Positive controls were HepG2 cells [from Wei Cui] and negative controls were undifferentiated e-CSC and l-CSC cells.

2.1.6. Cell size

Size of passage 4-5 cells in suspension was determined using Image J software and calibrated with a haemocytometer.

2.1.7. Growth Kinetics

Cells were plated in triplicate at 10^4 cells per cm^2 and counted in trypan blue to exclude dead cells in a haemocytometer every 2 days for 12 days without passaging. Doubling time (DT) was calculated via the formula: $DT = t / (\log_2[y/m])$, with t =time in culture, y =number of cells at end of culture, m =number of cells at beginning of culture.

2.1.8. Embryoid body formation and analysis

As part of a collaborative work within the lab group [with Dr Dafni Moschidou and Aya Harada] undifferentiated e-CSC and l-CSC were induced to differentiate *in vitro* into EBs by incubating confluent cells with differentiation medium containing knockout Dulbecco's modified eagle's medium (Gibco BRL Life Technologies) supplemented with 1 mM L-mercaptoethanol, 1% non-essential amino acids stock (all Gibco BRL Life Technologies) and 20% FBS (BioSera). At confluence, cells were incubated with 1 mg/ml type IV collagenase (Invitrogen) before being dissociated into small clumps on low attachment Petri dish in

differentiating medium and allowed to develop into EBs for 12 days. EB suspensions were transferred to gelatin-coated coverslips before fixation in 4% PFA and analysis under confocal microscopy for expression of lineage markers from all three germ layers using specific primary (**Table 6**) then secondary antibodies (**Table 7**). Positive controls were EBs derived from induced pluripotent stem (iPS) cells, which originated from human amniotic fluid stem cells.

2.1.9. Isolation and culture of fetal stem cells for transplantation

Collection, isolation and culture of human early and late chorionic stem cells (e-CSC and l-CSC) were carried out as described in Chapter 1. The e-CSC transplanted (n=1) was 9+5 gestation age and l-CSC (n=1) was taken at term delivery. Human fetal blood (n=1) was collected previously by the group at 10+4 gestation age by cardiocentesis under ultrasound guidance during termination of pregnancy (Campagnoli, Roberts et al. 2001; Guillot, Abass et al. 2008; Guillot, Cook et al. 2008). Research was approved by the Research Ethics Committee of Hammersmith & Queen Charlotte's Hospital. Cells were selected by plastic adherence and cultured in Dulbecco's modified Eagle's medium high glucose (DMEM-LG) (Sigma) supplemented with 10% fetal bovine serum (BioSera), 2mmol/l L-glutamine, 50IU/ml penicillin, 50mg/ml streptomycin (Gibco-BRL). Cells were expanded at 70-80% confluence on plastic dishes and used at passage 4-8.

2.2. RNA and DNA manipulation methods

2.2.1. RNA extraction and cDNA synthesis

Total RNA was extracted from cells using RNeasy mini RNA isolation kit (Qiagen) followed by cDNA synthesis using Pd(N)₆ random hexamers and M-MLV reverse transcriptase in the presence of dNTPs (Promega) (10 min, 25°C; 60 min, 42°C and 10 min, 75°C).

2.2.2. RT-PCR

Reverse-transcriptase PCR (RT-PCR) was performed with cDNA using specific primers (**Table 5**), dNTPs, x10 buffer and Taq polymerase for 40 cycles according to the manufacturer's protocol (Qiagen).

2.2.3. qRT-PCR

Quantitative real time polymerase chain reaction (qRT-PCR) was performed using SYBR green dye (Qiagen or Applied Biosystem) or Taqman[®] Gene Expression Master Mix (Applied Biosystem) and specific primers (**Table 5**), then analysed either with MJ-opticon or ABI Prism 7700 Sequence Detection system (Applied Biosystem). Samples were normalized against the internal control (*GAPDH* or *β-ACTIN*) to generate the $2^{-\Delta Ct}$ for each sample. For pluripotency analysis the $2^{-\Delta\Delta Ct}$ was plotted ($\log_{10}$ scale) relative to the expression level in human embryonic stem cells (hESC) line H9 [from Wei Cui]. For differentiation analysis the $2^{-\Delta\Delta Ct}$ of differentiated cells was plotted as a fold change from the basal level of gene expression.

2.2.4. DNA extraction and SRY PCR

Male gender of cells was determined by DNA extraction using TRIzol (Invitrogen), followed by ethanol precipitation, washing and DNA dissolution in 8mM NaOH. Then PCR was performed in the presence of dNTPs (Promega) and Taq polymerase (Bioline) with *SRY* specific primers (**Table 5**), followed by gel electrophoresis on 1.5% agarose with ethidium bromide (Sigma).

2.2.5. FISH analysis

No contamination by maternal cells was confirmed at passages 3-9 for *SRY* positive samples using XY FISH analysis. Cells were fixed on slides as previously described (Guillot, Gotherstrom et al. 2007), then digested with proteinase K before hybridization with human

specific centromeric mixture probe (CEP X/Y/18) according to manufacturer's protocol (Vysis). Nuclei were counterstained with DAPI and viewed on a fluorescence confocal laser scanning microscope Leica TCS SP5 (X600 PL APO oil objective). A total of 100 cells per group were analysed.

2.2.6. Analysis of H19 methylation

Genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen) and made free from RNA using RNase A (Invitrogen). The EpiTect Methyl qPCR assay (Qiagen) was used to digest DNA with methylation-sensitive and methylation-dependent enzymes to obtain fractions of unmethylated, partially methylated and hypermethylated DNA. The methylation status of *H19* was then quantified using qRT-PCR and specific primers (**Table 5**), data were then normalised to the total amount of DNA.

2.3. Protein detection methods

2.3.1. Protein Extraction and Western Blot

Total protein was extracted using RIPA buffer containing protease inhibitor cocktail and PMSF (Sigma). Protein concentrations were determined using the BCA protein assay (Thermo-scientific), with bovine serum albumin as the standard. Proteins were run on an 8% SDS-PAGE, transferred to nitrocellulose, blocked with milk and incubated with primary antibody COL1A2 (123kDa) (**Table 6**). Secondary HRP-linked anti-rabbit IgG (GE healthcare), followed by enhanced chemiluminescence (Thermo scientific) was used for detection (**Table 7**). Loading control used was β -actin (Santa Cruz).

2.3.2. Fluorescence immunostaining and confocal microscopy

Cells were fixed in 4% followed by 8% PFA in 125mM HEPES, and then permeabilized in 0.5% Triton X-100 in PBS. Cells were then rinsed in PBS, incubated in 20 μ M Glycine in PBS, blocked with PBS⁺ (PBS supplemented with 1% BSA, 0.2% gelatin, 0.1% casein; pH

7.6) and incubated with primary antibodies (**Table 6**). Cells were then washed in PBS+, incubated with secondary antibodies (**Table 7**), washed, then mounted in VectaShield labelled with DAPI (Vector Labs) and visualized immediately. Fluorescence confocal laser scanning microscopy images were collected on a Leica TCS SP5 (X400 PL APO oil objective) and transferred to Adobe Photoshop (Adobe Systems) (Guillot, Gotherstrom et al. 2007).

2.3.3. Flow cytometry

For intracellular FACS staining cells were detached, blocked with 1% bovine serum albumin (BSA; Sigma) in phosphate-buffered saline (PBS; Invitrogen) then fixed with 0.01% paraformaldehyde (PFA) (Sigma) and permeabilized with 0.5% Triton X-100 (Sigma). For cell surface and intracellular FACS staining detached cells were blocked with 1% BSA and incubated with specific anti-human primary antibodies (**Table 6**), either conjugated with PE, FITC or PE-Cy5 or unconjugated. For unconjugated antibodies, cells were subsequently washed with 1% BSA and incubated with secondary goat anti-murine IgM PE (Santa Cruz) (**Table 7**). All samples were analyzed in triplicate by FACScalibur flow cytometry (Becton Dickinson). Positive controls were hESC and negative controls were IgG or IgM primary antibody-specific isotype controls.

2.4. Animal experiments

2.4.1. Wound healing model

All experimental protocols complied with UK Home Office guidelines. C57/Bl6 mice were housed in individual ventilated cages (IVC) in 12:12-hour light dark cycle (21°C) with water and chow. After hair removal from the dorsal surface of mice, they were given a dermal excision wound of 4-mm full-thickness under anesthesia. Each mouse was given two dermal excisions on each dorsal side (Rajkumar, Shiwen et al. 2006). Stem cells (e-CSC and l-CSC, 10^6 cells in 30 μ l PBS) were applied to the left-side wounds and PBS alone applied to the right-side wounds. Seven days later, the mice were culled and the dermal tissues excised, cut

across the middle of the wound and fixed overnight in 10% neutral buffered formalin at 4°C and embedded in paraffin, sectioned and subjected to hematoxylin and eosin staining for histological analysis.

2.4.2. Breeding B6C3Fe a/a-Coll1a2^{oim}/Coll1a2^{oim} mice

All experimental protocols complied with UK Home Office guidelines. Heterozygous male and female (B6C3Fe a/a-Coll1a2^{oim}/Coll1a2^{oim}) mice (Jackson Laboratory) were housed in individual ventilated cages (IVC) in 12:12-hour light dark cycle (21°C) with water and chow. Offspring were genotyped by overnight enzyme extraction of DNA from an ear notch, followed by PCR using *oim* specific primers (**Table 5**) and sequencing of the *oim* fragment (Beckman). Homozygous and wild type colonies were subsequently established. Homozygous *oim* mice showed multiple fractures and brittle bones. Progeny were weaned at 30±1 day.

2.4.3. Neonatal transplantation in the *oim* model

All cells (e-CSC, l-CSC and hfMSC) were trypsinized, washed in PBS then injected intraperitoneal (10⁶ cells in 10 µl PBS) in homozygous *oim* neonates 3-4 days after birth. For SDF1 primed hfMSC, the cells were incubated with stromal-derived factor (SDF1) 30ng/ml in culture medium for 1 hour prior to transplantation. The mice were culled at 8 weeks of age for analysis. Controls were age-matched non-transplanted *oim* and wild type mice.

2.5. Tissue analysis methods

2.5.1. Three-point bending test

Three-point bending tests were performed on 8 week old unfractured frozen and thawed femurs in a materials testing machine (5866 Instron) at a loading rate of 50 µm/s on two supports 9 mm apart until fracture at mid-diaphysis. Force deflection curves were analyzed with a custom program (Matlab, MathWorks) to measure the bending stiffness (slope of the

linear elastic deformation), the yield force (limit between the elastic and plastic deformation), the ultimate force (maximum force sustained), the total work to fracture (total area under the curve) and the plastic work to fracture (area under the curve from the yield point to fracture).

2.5.2. Visualization of donor cells

Presence of human donor cells was visualized in 3 different parts of non-fractured tibia: epiphysis, growth plate and diaphysis as well as from fracture callus in transplanted 8 week old *oim* and compared to non-transplanted *oim* as a negative control. Dissected tibias were decalcified in 10% EDTA pH7.4 and subsequently embedded in paraffin. Five micron sections were cut, deparaffinised in histoclear and rehydrated. Slides were then incubated with peroxidase blocking reagent (Dako), further blocked with PBS+ and goat serum, then stained with human-specific rabbit monoclonal Vimentin (Abcam) primary antibody (**Table 6**). Slides were subsequently incubated with HRP-labelled anti-rabbit polymer followed by DAB+ substrate-chromogen (n=3 samples per group).

2.5.3. Quantifying donor cell engraftment

Femurs from transplanted 8 week old *oim* had the bone marrow flushed and were then dissected into callus if present (n=8), epiphysis (n=6) and diaphysis (n=6). Liver (n=6) was also used. RNA was extracted using TRIzol (Invitrogen) followed by cDNA synthesis with M-MLV reverse transcriptase (Promega). To calculate donor cell engraftment quantitative real time PCR (qRT-PCR) was performed using SYBR green dye and the ABI Prism 7700 Sequence Detection System (Applied Biosystem) with human specific and human-mouse unspecific β -Actin primers (**Table 5**). Human: mouse chimerism was estimated as the ratio of human β -Actin to total human and mouse β -Actin in the total cDNA sample to give the $2^{-\Delta Ct}$ value. Samples were considered positive with a human specific β -Actin Ct above 36 at a threshold of 0.13 ΔRn . Primer specificity was confirmed by absence of amplification of non-transplanted mouse cDNA (Guillot, Abass et al. 2008)

Presence of donor cells was estimated in non-fractured tibia bone marrow in transplanted 8 week old *oim*. Dissected tibias were decalcified, paraffin embedded and sectioned as above (2.5.2). Sections were then deparaffinised in histoclear and rehydrated. Heat-induced epitope retrieval was performed in a steamer (Dako), followed by incubation with peroxidase blocking reagent (Dako), followed by PBS+ with 1 in 20 goat serum. Donor cells were visualised in the bone marrow using human-specific mouse monoclonal Vimentin (Dako) primary antibody (**Table 6**) and incubation with HRP-labelled anti-mouse polymer followed by DAB+ substrate-chromogen. Positive cells were counted in the bone marrow (n=4 samples and n=4 sections for each group). Staining specificity was verified using non-transplanted negative controls.

2.5.1. Osteoblast gene expression by donor cells

QRT-PCR was performed using SYBR green dye (Qiagen) and the MJ-Opticon with human specific *Osteopontin* and *Osteocalcin* primers (**Table 5**). Results with a Ct below 36 were normalised to human β -actin to give the $2^{-\Delta Ct}$ value. Expression in 8 week old transplanted *oim* femurs (n=6) was compared to e-CSC (n=3) undifferentiated and grown in osteogenic permissive media for 2 weeks. Negative controls were non-transplanted *oim* (Guillot, Abass et al. 2008).

2.5.2. Western blot

Collagen was extracted from ground bone over 72hrs at 4°C in a lysis buffer of 6M guanidine HCl and 100mM Tris pH7.4 containing protease inhibitor cocktail. Proteins were precipitated with 10% TCA then resuspended in RIPA buffer containing PMSF (Sigma) and run on an 8% SDS-PAGE, transferred to nitrocellulose, blocked with milk and stained with COL1A2 (129kDa) (Abcam), then with secondary antibody HRP-linked anti-rabbit IgG (GE healthcare), followed by enhanced chemiluminescence (Thermo scientific). Loading control used was β -ACTIN (43kDa) (Millipore). Detection of COL1A2 in transplanted *oim* bones was confirmed using wild type positive controls and non-transplanted *oim* negative controls (n=4 per group).

2.5.3. X-ray

Tibias from 8 week-old mice (n=19 transplanted, n=20 *oim*, n=12 wild type) were fixed in formalin for 24 hours and stored in 70% ethanol prior to removal of soft tissues. Digital X-ray images were obtained at a 10µm pixel resolution using a Faxitron MX20 variable kV point projection X-ray source and digital image system (Qados, Cross Technologies plc, Sandhurst, Berkshire, UK). An X-ray image of a digital micrometer was used to calibrate ImageJ 1.41 software (<http://rsb.info.nih.gov/ij/>) prior to determination of cortical bone thickness and diameter at 5 locations along the mid shaft, and also to determine bone length. Relative bone mineral content (BMC) was determined by comparison with 1 mm diameter steel, aluminium and polyester standards included in each frame. 16 bit DICOM images were converted to 8 bit Tiff images using ImageJ and the image histogram stretched between the polyester (grey level 0) to steel (grey level 255) standards. Bone mineralization densities were represented by a pseudo colour scheme representing 16 equal intervals (Bassett, Boyde et al. 2010) [Results contributed by Dr Duncan Bassett].

2.5.4. Counting of fractures

Fractures in the femur, tibia and humerus of both body sides were assessed at 8 weeks of age after dissection and were delineated by callus formation (n=120 transplanted bones, n=78 *oim* control bones). The number of mice with at least one long bone fracture and the fracture incidence (number of fractured bones/total bones assessed) were calculated by two independent observers blinded to whether mice were transplanted or not.

2.5.5. Dynamic histomorphometry

All mice were calcein injected 10 days and 3 days before sacrifice with 20mg/kg of calcein (Sigma). Tibia (n=7 transplanted, n=6 wild type, n=5 *oim*) were then fixed in formalin for 24hrs and transferred to 70% ethanol, before being dehydrated in acetone for 48hrs, infiltrated over 6-9 days at -20°C and embedded in methylmethacrylate (MMA) (Chappard, Palle et al. 1987). Embedded samples were imaged on a Leica TCS SP5 confocal laser scanning microscope and analysed using ImageJ. Fluorescent images of calcein labels were

taken 500µm and 1000µm below the proximal growth plate of the trabecular and endocortical regions respectively. The amount of mineralizing surface per total bone surface (MS/BS; %), the daily mineral apposition rate (MAR; µm/day) and the bone formation rate (BFR; µm³/µm²/days) were calculated.

2.5.6. Static histomorphometry

MMA embedded samples were cut into eight micron sections and stained using the Leucognost AP kit (Merck), according to the manufacturer's instructions (n=6 transplanted, n=6 *oim*, n=5 wild type). Sections were analysed on a light microscope using the Osteomeasure system (OsteoMetrics Inc). Histomorphometric measurements of the % trabecular bone volume per total tissue volume (BV/TV) were performed on stained sections 500µm from the end of the hypertrophic zone of the growth plate in the secondary spongiosa. For growth plate analysis dissected tibia were decalcified, paraffin embedded and 5µm sections were cut and stained with alcian blue 8GX (2%), Weigert's haematoxylin and van Gieson, mounted and growth plate morphology analysed using Image J (n=14 transplanted *oim*, n=5 *oim*, n=5 wild type).

2.5.7. Osteogenesis PCR array

Total RNA was extracted from femoral epiphysis of 8 week old mice using the TRIzol (Invitrogen) protocol, followed by RNA clean up using the RNeasy Mini kit (Qiagen), then cDNA was synthesised using RT² First Strand Kit (Qiagen). The RT² Profiler mouse osteogenesis PCR array (Qiagen) was run and analysed according to the manufacturer's instructions (n=3 mice for each group). To verify results further quantitative real time PCR was performed with RT² qPCR Master Mix and primers (**Table 5**) and analysed with MJ-opticon. Data was normalised against 2 housekeeping genes (β-Actin and Hsp90ab1) and the 2^{-ΔCt} of each sample calculated (n=8 transplanted *oim*, n=5 *oim* controls).

2.6. Statistical analysis

Data were expressed as mean $\pm$ s.d (standard deviation) or mean $\pm$ s.e.m (standard error). Normally distributed data were analyzed by unpaired two-tailed Student's *t*-test or one-way ANOVA followed by a Tukey's post-hoc test. Data that did not have equal variance as confirmed by Bartlett's test was analysed Welch's *t*-test. Data that was non-parametric was analysed by Kruskal-Wallis or Mann-Whitney U test. $P < 0.05$ was considered significant. Two-tailed 2x2 Fisher exact was used for categorical comparisons. The frequency distributions of bone mineralization in 1024-pixel data sets were compared using the Kolomogorov-Smirnov test, where $D \geq 6.01$, $P < 0.05$; $D \geq 7.20$, $P < 0.01$; $D \geq 8.62$, $P < 0.001$. Chi-squared with Yates correction and to one degree of freedom was used to compare fracture incidence, where $\chi^2 \geq 3.84$, $P < 0.05$; $\chi^2 \geq 6.64$, $P < 0.01$; $\chi^2 \geq 10.83$, $P < 0.001$.

2.7. Primers

Table 5 – List of primer sequences.

List of primers used for RT-PCR and quantitative real time PCR and EpiTect Methyl qPCR assay.

Gene	Forward	Reverse	Accession no.
<i>Aggrecan (Mouse)</i>	5'-GTTGGTACTTCGCCTCCAG-3'	5'-GTCCTCCAAGCTCTGTGACC-3'	NM_007424
<i>β-ACTIN (Human)</i>	5'-CTGGAACGGTGAAGGTGACA-3'	5'-AAGGGACTTCCTGTAACAATGCA-3'	NM_001101
<i>β-ACTIN (Human-Mouse)</i>	5'-GCTCCTCCTGAGCGCAAGTA-3'	5'-GATGGAGGGGCCGACT-3'	NM_001101 NM_007393
<i>β-Actin (Mouse)</i>	330001 PPM02945A	Qiagen (http://www.qiagen.com/)	NM_007393
<i>BMP2 (Human)</i>	5'-TTCCACCATGAAGAATCTTTGGA-3'	5'-CCTGAAGCTCTGCTGAGGTGAT-3'	NM_001200
<i>BMP4 (Human)</i>	5'-GTGAGGAGCTTCCACCA CGA-3'	5'-ACTGGTCCCTGGGATGTT CTC-3'	NM_130850
<i>BSP (Human)</i>	5'-AAACGAAGAAAGCGAAGCA GAA-3'	5'-GCTGCCGTTGCCGTTTT-3'	NM_004967
<i>CK19 (Human)</i>	5'-GGCCACACGGAGCAGCT CCA-3'	5'-CGCCTGGATATGCGCCAGCT-3'	NM_002276
<i>CK3 (Human)</i>	5'-CGTACAGCTGCTGAGAATGA-3'	5'-CTGAGCGATATCCTCATACT-3'	NM_057088
<i>c-KIT (Human)</i>	5'-CACCGAAGGAGGCACTTA CAC-3'	5'-GGAATCCTGCTGCCACACA-3'	NM_000222
<i>C-MYC (Human)</i>	5'-TTGTACCTGCAGGATCTGAG-3'	5'-CTTGTTCTCCTCAGAGTCG-3'	NM_002467
<i>Col1α1 (Mouse)</i>	330001 PPM03845F	Qiagen	NM_007742
<i>Col2α1 (Mouse)</i>	5'-CCTCCGTCTACTGTCCAC TGA-3'	5'-ATTGGAGCCCTGGATGA GCA-3'	NM_001113515
<i>Col10α1 (Mouse)</i>	330001 PPM05135F	Qiagen	NM_009925
<i>Dmp1 (Mouse)</i>	330001 PPM04680E	Qiagen	NM_016779
<i>FRAGILIS (Human)</i>	5'-CTCAAGGAGGAGCAGGA AGT-3'	5'-TGAAGAGGGTGTGAAC AGG-3'	NM_006435
<i>GAPDH (Human)</i>	5'-TCTGCTCCTCCTGTTGACA-3'	5'-AAAAGCAGCCCTGGTGACC-3'	NM_002046

<i>GATA6</i> (Human)	5'-ACCACCTTATGGCGCAGA AAC-3'	5'-TTTTTCATAGCAAGTGGTCT GGG-3'	NM_005257
<i>H19</i> (Human)	Catalogue number: MePH28624-1B	Qiagen (http://www.qiagen.com)	NC_000011
<i>Hsp90ab1</i> (Mouse)	330001 PPM04803E	Qiagen	NM_008302
<i>Igf1</i> (Mouse)	330001 PPM03387E	Qiagen	NM_010512
<i>KLF4</i> (Human)	5'-CACATTAATGAGGCAGCC ACC-3'	5'-AAGTCGCTTCATGTGGGA GAG-3'	NM_004235
<i>NANOG</i> (Human)	5'-CCAACATCCTGAACCTCAGC TAC-3'	5'-GCCTTCTGCGTCACACCATT- 3'	NM_024865
<i>NANOS3</i> (Human)	TaqMan Hs00928455_s1*	Applied Biosystems (http://www.appliedbiosystems.com)	NM_0010986 22
<i>NESTIN</i> (Human)	5'-TCCAGGAACGGAAAATC AAG-3'	5'-GCCTCCTCATCCCCTACTTC- 3'	NM_006617
<i>OC</i> (Human)	5'-CCTCACACTCCTCGCCCT ATT-3'	5'-CCCTCCTGCTTGGACACAAA- 3'	NM_199173
<i>OCT4A</i> (Human)	5'-GTATTCAGCCAAACGACC ATC-3'	5'-CTGGTTCGCTTTCTCTTTCG- 3'	NM_002701
<i>OCT4A v1</i> (Human)	5'- CCGCCGTATGAGTTCTG TGG-3'	5'- AGAGTGGTGACGGAGAC AGG-3'	NM_002701
<i>OP</i> (Human)	5'-GCCGACCAAGGAAAACCTCA CTA-3'	5'-CAGAACTTCCAGAATCAGCC TGTT-3'	NM_0010400 58
<i>OSX</i> (Human)	5'-CCCCACCTCTTGCAACCA-3'	5'-GGCTCCACCACTCCCTTC TAG-3'	NM_152860
<i>OSX</i> (Mouse)	5'-AGGCACAAAGAAGCCATAC- 3'	5'-AATGAGTGAGGGAAGGGT-3'	NM_130458
<i>PAX6</i> (Human)	5'-AGATTCAGATGAGGCTC AAA-3'	5'-AATTGGTTGGTAGACACTGG- 3'	NM_000280
<i>PCE</i> (Human)	5'-TTCGCTGGGTCTGGGACTT-3'	5'-CATCCTCAGGGTCACCTG GTT-3'	NM_002593
<i>PHEX</i> (Mouse)	330001 PPM41830E	Qiagen	NM_011077
<i>PTHRP</i> (Mouse)	5'-GAACATCAGCTACTGCATG ACAAG-3'	5'-TCTGATTTCGGCTGTGTGG ATC-3'	NM_008970
<i>Runx2</i> (Mouse)	5'-CCGGCCACTTCGCTAACTT-3'	5'-TGGTGCTCGGATCTACAG GAA-3'	NM_009820
<i>SMAD3</i> (Mouse)	330001 PPM04461B	Qiagen	NM_016769
<i>SOX2</i> (Human)	5'- CACACTGCCCCCTCTCACA	5'- CATTTCCTCGTTTTTCTTT	NM_003106

	CAT-3'	GAA-3'	
<i>Sox9 (Mouse)</i>	330001 PPM05134C	Qiagen	NM_011448
<i>SRY (Human)</i>	5'-TGGCGATTAAGTCAAATT CGC-3'	5'-CCCCCTAGTACCCTGACAA TGTATT-3'	NM_003140
<i>SSEA1 (Human)</i>	5'-CACAACTGTTCCCGATTAC- 3'	5'-AGCCACAGTCCTTCCACTCT- 3'	NM_002033
<i>STELLA (Human)</i>	TaqMan Hs01931905_g1*	Applied Biosystems	NM_199286
<i>SYCP1 (Human)</i>	5'-ACAGCGAAAAGCCATTCA GGA-3'	5'-GCCTGGTTTCTTCCCGTTC ATA-3'	NM_003176
<i>VASA (Human)</i>	TaqMan Hs00987130_m1*	Applied Biosystems	NM_0011665 33

2.8. Antibodies

Table 6 – List of primary antibodies

List of primary antibodies used, the host species developed in, dilution used, supplier and application for either immuno-fluorescence (IF), flow cytometry (FC), immuno-histochemistry (IH) and western blot (WB).

Antibody	Host Species	Application	Dilution	Supplier
AFP	Mouse	IF	1:100	Abcam (http://www.abcam.com)
ALBUMIN	Mouse	IF	1:100	Abcam
B-ACTIN	Rabbit	WB	1:5000	Santa Cruz
B-TUBULIN	Mouse	IF	1:100	Chemicon (http://www.millipore.com)
B-TUBULIN	Mouse	WB	1:5000	Abcam
BLIMP1	Goat	IF	1:5	Santa Cruz (http://www.scbt.com)
CD105	Mouse	IF	1:50	BD Biosciences (http://www.bdbiosciences.com)
CD14	Mouse	IF	1:100	Dako Cytomation (http://www.dako.com)
CD24-FITC	Mouse	FC	1:10	Abcam
CD29	Mouse	IF	1:100	Abcam
CD34	Mouse	IF	1:100	Dako Cytomation
CD44	Mouse	IF	1:50	Abcam
CD45	Mouse	IF	1:100	Dako Cytomation
CD73	Mouse	IF	1:50	BD Biosciences
CD90	Mouse	IF	1:100	Abcam

C-KIT-PE	Mouse	FC	1:10	R&D Systems (http://www.rndsystems.com)
C-MYC-PE	Mouse	FC	1:20	R&D Systems
COL1A2	Rabbit	WB	1:1000	Abcam
CXCR4-PE	Mouse	FC	1:10	R&D Systems
DAZL	Goat	IF	1:10	Santa Cruz
FRAGILIS	Goat	IF	1:20	Santa Cruz
GATA4	Rabbit	IF	1:100	Abcam
HLAI	Mouse	IF	1:100	Dako Cytomation
HLAII	Mouse	IF	1:100	Dako Cytomation
KLF4	Mouse	IF	1:2	Stemgent (http://www.stemgent.com)
LAMININ	Rabbit	IF	1:100	Sigma (http://www.sigmaaldrich.com)
MAP2	Mouse	IF	1:100	Abcam
NANOG	Rabbit	IF	1:20	Abcam
NANOS 3	Goat	IF	1:20	Santa Cruz
NESTIN	Mouse	IF	1:50	Chemicon (http://www.millipore.com)
NMDAR1	Rabbit	IF	1:50	Abcam
OCT-4A	Mouse	IF	1:2	Santa Cruz
OCT-4A-PE	Mouse	FC	1:10	R&D Systems
PUM2	Goat	IF	1:20	Santa Cruz
REX1	Rabbit	IF	1:100	Stemgent
SOX2	Rabbit	IF	1:100	Abcam
SOX2-PE	Mouse	FC	1:10	R&D Systems
SSEA1	Mouse	IF	1:50	Chemicon
SSEA3	Rat	IF	1:50	Abcam
SSEA3 -PE	Mouse	FC	1:5	Dev. Studies Hybridoma Bank (http://dshb.biology.uiowa.edu)
SSEA4	Mouse	IF	1:50	Abcam
SSEA4-PE	Mouse	FC	1:10	R&D Systems
STELLA	Rabbit	IF	1:50	Santa Cruz
TNAP	Mouse	IF	1:50	Abcam
TRA-1-60	Mouse	IF, FC	1:10	Santa Cruz
TRA-1-81	Mouse	IF, FC	1:10	Santa Cruz
VASA (DDX4)	Rabbit	IF	1:50	Abcam
VIMENTIN	Mouse	IF, IH	1:100	Dako Cytomation
VIMENTIN	Rabbit	IH	1:150	Abcam

Table 7 – List of secondary antibodies

List of secondary antibodies used, the host species developed in, application for either immunofluorescence (IF), flow cytometry (FC) and western blot (WB), dilution used, supplier and conjugated fluorescent marker.

Antibody	Host Species	Application	Dilution	Conjugated marker	Supplier
IgG-anti-goat	Donkey	IF	1:100	FITC	Jackson ImmunoResearch Inc (http://www.jacksonimmuno.com)
IgG-anti-goat	Donkey	FC	1:100	FITC	Santa Cruz (http://www.scbt.com)
IgG-anti-goat	Donkey	WB	1:10000	HRP	Santa Cruz
IgG-anti-mouse	Donkey	IF	1:100	FITC	Jackson ImmunoResearch Inc
IgG-anti-mouse	Donkey	FC	1 in 100	Cy TM 3	Jackson ImmunoResearch Inc
IgG-anti-mouse	Donkey	WB	1 in 15000	HRP	Jackson ImmunoResearch Inc
IgM-anti-mouse	Goat	IF	1:100	FITC	Santa Cruz
IgM-anti-mouse	Donkey	FC	1:100	PE	Santa Cruz
IgG-anti-rabbit	Donkey	IF	1:1000	FITC	Jackson ImmunoResearch Inc
IgG-anti-rabbit	Donkey	WB	1:10000	HRP	Santa Cruz

CHAPTER 3 – Results

Human fetal chorionic stem cells

3.1. Chorionic stem cells have a phenotype intermediate of MSC and hESC

3.1.1. Introduction

The literature shows fetal and extra-embryonic stem cells express both markers of MSC and hESC and have a broadly multipotent differentiation potential, forming mesodermal lineages and some lineages from the endoderm and ectoderm germ layers (Abdulrazzak, Moschidou et al. 2010). It was therefore hypothesised that fetal and extra-embryonic stem cells present an intermediate phenotype between embryonic and adult stem cells with characteristics of both. Extra-embryonic stem cells derived from mid-trimester amniotic fluid have been extensively characterised and offer support for this hypothesis (De Coppi, Bartsch et al. 2007), whereas the phenotype of chorionic stem cells from early (e-CSC) and late (l-CSC) gestations has yet to be fully elucidated. Therefore I aimed to characterise the immunophenotype and differentiation potential of e-CSC and l-CSC to determine if they too presented an intermediate phenotype.

3.1.2. Results

Both early and late chorionic stem cells (e-CSC and l-CSC respectively) were isolated based on their plastic adherence from first trimester placental chorionic villous tissue and term placenta chorion respectively. Cells were cultured in DMEM supplemented with 10 % FBS and maintained at sub-confluence by passaging with Trypsin-EDTA. Both populations were c-KIT⁺ (**Fig. 6A**), as reported for AFSC (Moschidou, Drews et al. 2012; Moschidou, Mukherjee et al. 2012), grew in monolayers and showed a spindle shaped cytoplasm morphology (**Fig. 6B**). Therefore the chorionic stem cells at the morphological level were similar to the previously described mid-trimester AFSC.

The fetal origin of the cells was confirmed by the presence of the *SRY* gene in male samples (**Fig. 6C**). Fluorescence *in situ* hybridisation (FISH) analysis showed that all nuclei in male samples contained the X and Y chromosome in both e-CSC and l-CSC populations, identifying cells as being of fetal origin, with no contaminating maternal cells (**Fig. 6D**).

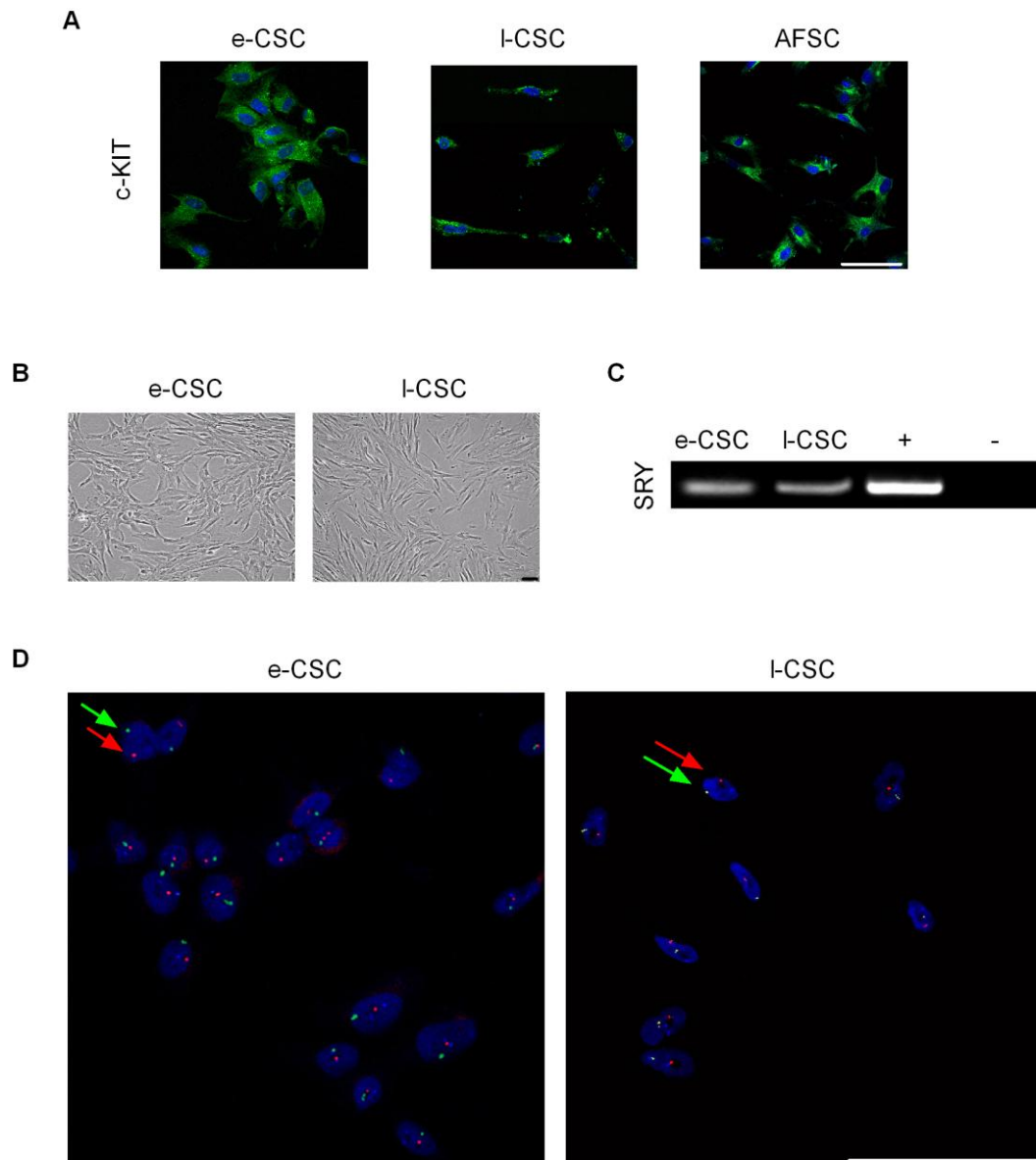


Figure 6 - e-CSC and l-CSC are of fetal origin.

(a) Confocal immuno-fluorescence for c-KIT in early and late gestation chorionic stem cells (e-CSC and l-CSC respectively). Positive control amniotic fluid stem cells (AFSC) shown. Nuclei stained with DAPI (blue). $n=4$ per group. (b) Cell morphology at passage 4-5. (c) PCR for Y chromosome specific SRY. Positive (male cells) and negative (female cells) controls shown. (d) FISH analysis for X (FITC) and Y (Texas Red) chromosomes indicated with green and red arrows respectively. 100 cells were counted ($n = 1$). All scale bars 100 μ m.

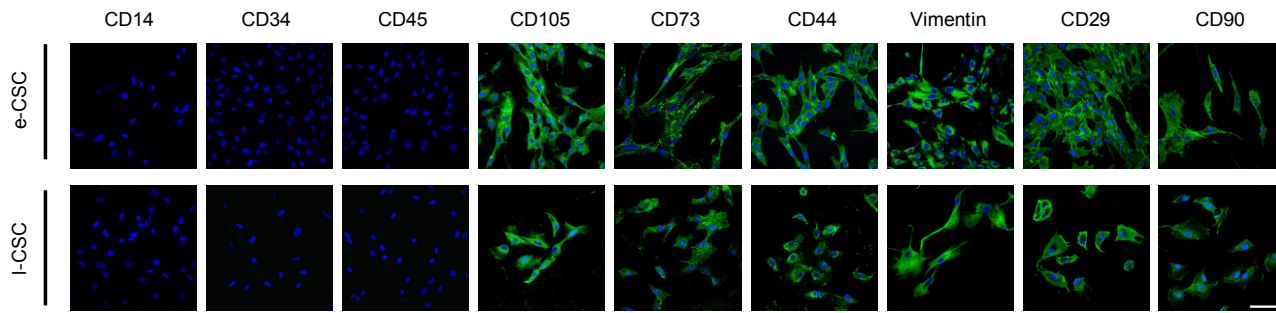


Figure 7 - e-CSC and l-CSC have a similar immunophenotype.

Confocal immuno-fluorescence for endothelial marker (CD14), hematopoietic markers (CD34 and CD45), MSC-associated markers (CD105, CD73 and CD44), matrix protein (vimentin) and markers found in pluripotent cells as well as MSC (CD29 and CD90) stained with FITC (green). Nuclei stained with DAPI (blue). Scale bar 100 μ m. n = 4 per group.

Both e-CSC and l-CSC share a common morphology as well as immunophenotype with mesenchymal stem cells (MSC) (Dominici, Le Blanc et al. 2006), immunophenotype of bone marrow hfMSC is given in Appendix 1A. This is shown through exclusion of hematopoietic and endothelial cells in the e-CSC and l-CSC populations, as demonstrated by absence of *CD14*, expressed on monocytes and macrophages, *CD34*, which marks hematopoietic progenitors and endothelial cells and *CD45*, a pan-leukocyte marker (Dominici, Le Blanc et al. 2006). The positive control for *CD14*, *CD34* and *CD45* were human umbilical vein endothelial cells (HUVEC), which stained positive for all three markers as shown in Appendix 1B. In addition both e-CSC and l-CSC populations showed expression of MSC-associated markers *CD105*, *CD73*, *CD44* and the matrix protein VIMENTIN as well as *CD29* and *CD90*, although *CD29* and *CD90* are also found in human embryonic stem cells (hESC) (Carpenter, Rosler et al. 2004; Lian, Lye et al. 2007) (**Fig.7**). The positive control used was bone marrow hfMSC, which showed positive expression for all six markers as shown in Appendix 1A [Confocal immuno-fluorescence of bone marrow hfMSC was carried out with assistance from Dr. Dafni Moschidou]. No quantification or flow cytometry analysis was carried out on these markers since confocal immuno-fluorescence indicated all cells in the population were positive and therefore have an MSC phenotype.

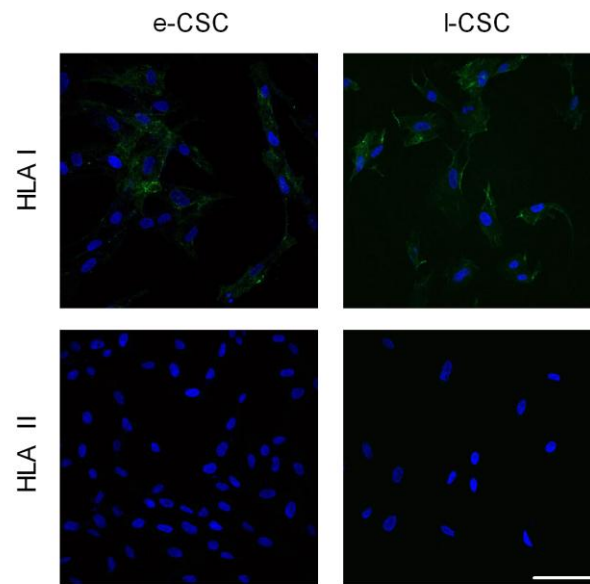


Figure 8 – cells have low expression of HLA antigens

Confocal immuno-fluorescence for expression of human leukocyte antigens; HLA I and HLA II in e-CSC and l-CSC shown by FITC (green). Nuclei stained with DAPI (blue). Scale bar 100 μ m. n = 4 per group.

Similar to hfMSC (Gotherstrom, Ringden et al. 2004) both e-CSC and l-CSC present low levels of intracellular human leukocyte antigen I (HLA-I i.e. HLA-ABC) and no expression of HLA II (HLA-DP, DQ, DR) (**Fig.8**). Low/null expression of these major histocompatibility (MHC) antigens suggests e-CSC and l-CSC may have low immunogenicity and may subsequently have potential for allogeneic clinical use (Le Blanc, Tammik et al. 2003). Positive controls used for the MHC antigens were HUVECs that had been induced to express HLA I and II using IFN- γ , shown in Appendix 1C.

However, in contrast to MSC, pluripotency markers typical of undifferentiated hESC were expressed by a subset of cells in both the e-CSC and l-CSC populations (**Fig.9A**). In particular an OCT4A specific antibody was used to prevent false positive detection of the OCT4B isoform commonly found in other cell types (Cauffman, Van de Velde et al. 2005; Lee, Kim et al. 2006; Atlasi, Mowla et al. 2008). OCT4A expression was further verified by the nuclear localisation of the protein in the cells, whilst OCT4B is known to be cytoplasmic

(Cauffman, Van de Velde et al. 2005). Both chorionic stem cell populations were also heterogeneous for the expression of a subset of proteins involved in stemness and pluripotency (Skottman, Mikkola et al. 2005; Adewumi, Aflatoonian et al. 2007) including SOX2, KLF4, NANOG, REX1, c-MYC, SSEA4, SSEA3, TRA-1-60, and TRA-1-81 (**Fig.9A**). The expression of these proteins in undifferentiated hESC [from Dr. Wei Cui] was also confirmed (**Fig.9B**).

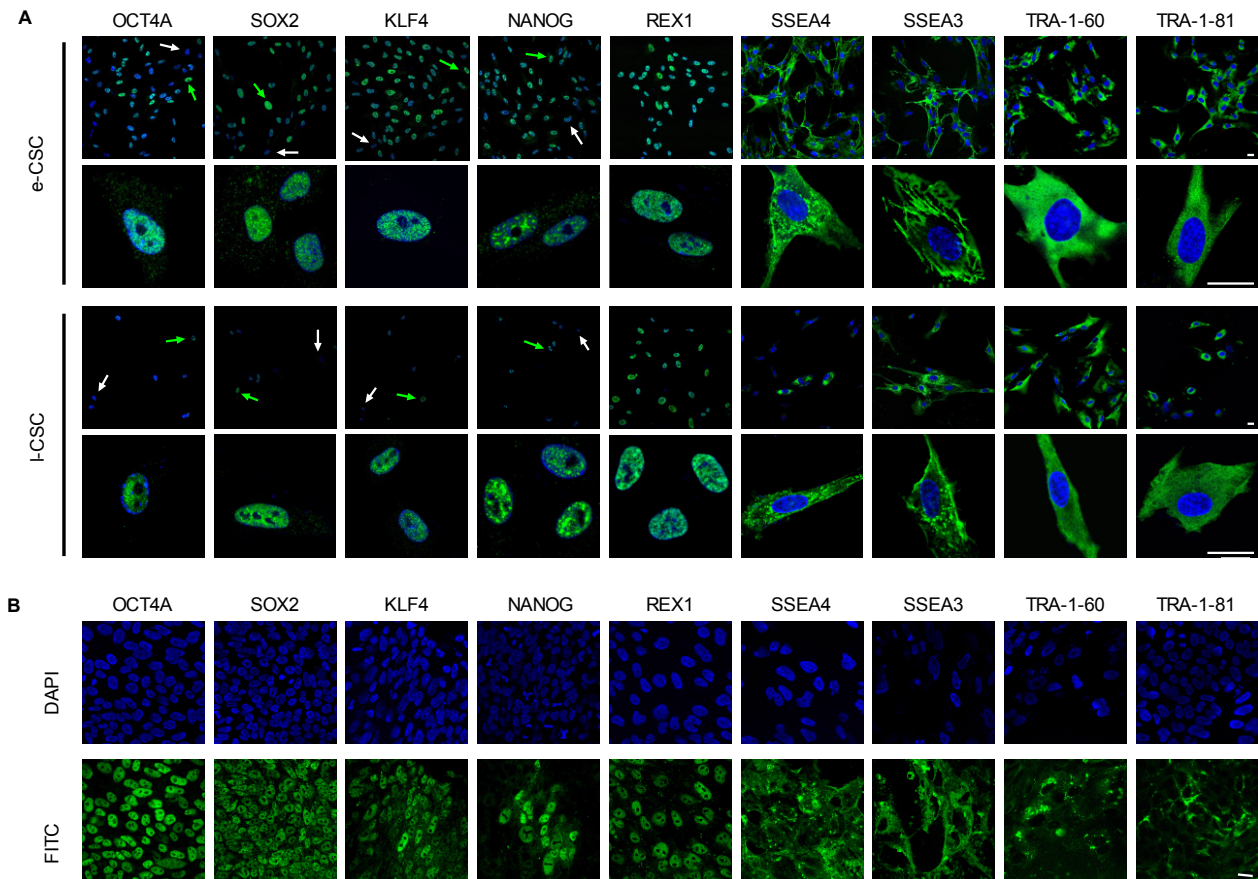


Figure 9 - e-CSC and l-CSC contain a subpopulation expressing hESC markers.

(a) Representative confocal immunofluorescence images for OCT4, SOX2, KLF4, NANOG, REX1, SSEA4, SSEA3, TRA-1-60 and TRA-1-81 stained with FITC (green). Positive cells indicated with green arrow, negative cells with white arrow. Nuclei stained with DAPI (blue). (b) Positive controls (hES cell line H9) OCT4A, SOX2, KLF4, NANOG, REX1, SSEA4, SSEA3, TRA-1-60, and TRA-1-81. All genes stained with FITC (green). Nuclei stained with DAPI (blue). All scale bars 25 μ m. $n = 4$ per CSC group. $n = 1$ hES cell sample.

In parallel with my findings our group also showed using flow cytometry that the subset of cells positive for pluripotency genes was similar sized in the e-CSC and l-CSC populations. For example CD24, a marker of undifferentiated hESC, not expressed in MSC (Assou, Le Carrou et al. 2007; Lian, Lye et al. 2007), was found in $34.2\% \pm 3.5$ s.e.m and $36.0\% \pm 10.0$ of the e-CSC and l-CSC respectively. As well as CD24, a similar sized subpopulation of cells were positive for OCT4A ($63.1\% \pm 5.1$ s.e.m vs. $70.2\% \pm 6.6$), SOX2 ($62.2\% \pm 4.2$ s.e.m vs. $66.1\% \pm 3.3$), c-MYC ($52.3\% \pm 11.2$ vs. $61.6\% \pm 11.4$), SSEA4 ($83.1\% \pm 8.2$ vs. $69.6\% \pm 22.3$), SSEA3 ($63.7\% \pm 6.6$ vs. $66.1\% \pm 3.3$), TRA-1-60 ($59.4\% \pm 10.6$ vs. $62.8\% \pm 12.1$), TRA-1-81 ($61.8\% \pm 3.2$ vs. $62.8\% \pm 12.1$) in e-CSC and l-CSC respectively [Data published in (Jones, Moschidou et al. 2012) from Dr. Dafni Moschidou].

An MSC is defined as a multipotent cell that can differentiate to the specialised cells of the mesodermal germ layer (bone, fat and cartilage) (Dominici, Le Blanc et al. 2006). Both e-CSC and l-CSC differentiated to bone as shown by alizarin red staining of calcium deposition and von Kossa staining of mineralisation after cells were grown in osteogenic permissive media (**Fig.10**). The chorionic stem cells also differentiated to fat, evident from lipid droplet formation after growth in adipogenic permissive media (**Fig.11**), indicating an MSC phenotype.

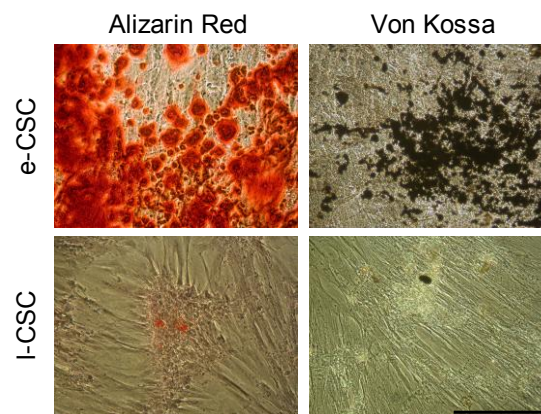


Figure 10 - Osteogenic differentiation of e-CSC and l-CSC.

Alizarin red staining (calcium deposits) and von Kossa staining (mineralisation) of cells grown in osteogenic permissive media for 2 weeks. Scale bar 100 μ m. n = 3 per group.

In addition to differentiation to mesodermal lineages, both e-CSC and l-CSC were also capable of differentiation into the ectodermal lineage, indicating the cells to be broadly multipotent. After 5 days of neuronal differentiation e-CSC and l-CSC changed morphology and expressed the neuronal markers β -TUBULIN, the microtubule-associated protein 2 (MAP2) and the NMDA receptor NR1 (**Fig.12**), indicating that both cell populations have the potential to differentiate into neural-like cells.

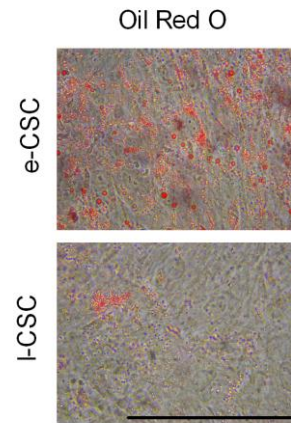


Figure 11 - Adipogenic differentiation of CSC.

Oil Red O staining (lipid droplets) of e-CSC and l-CSC grown in adipogenic permissive media for 3 weeks. Scale bar 100 μ m. n = 4 per group.

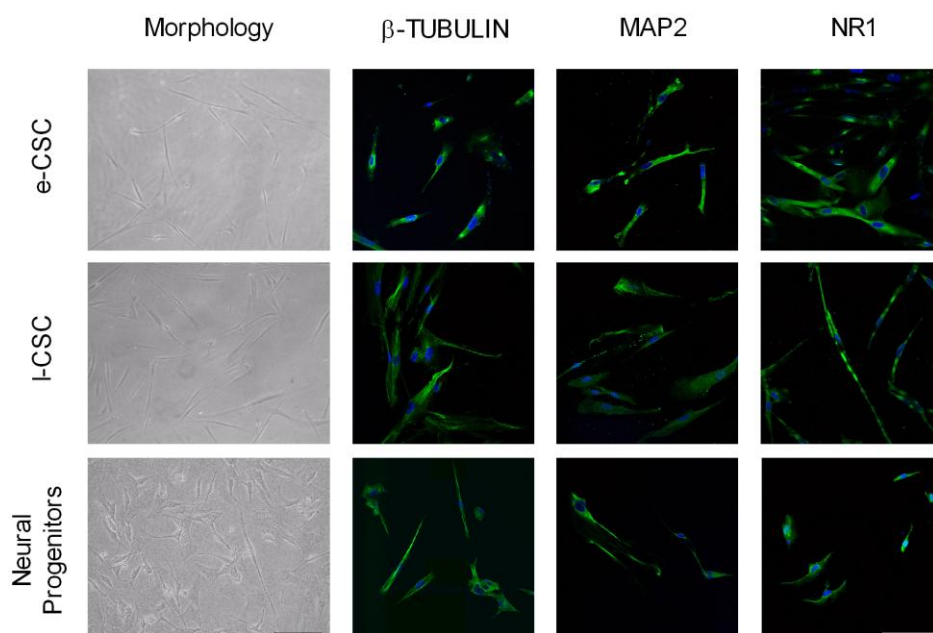


Figure 12 - Neurogenic differentiation of e-CSC and l-CSC.

Morphology and expression of neuronal markers β -TUBULIN, MAP2 and NMDA receptor NR1 shown with FITC (green) of e-CSC and l-CSC after 5 days neurogenic differentiation. Nuclei stained with DAPI (blue). All scale bars 100 μ m. n = 2 per group.

However, differentiation to the endodermal lineage was not found as culture of e-CSC and l-CSC in hepatic-permissive medium over 21 days failed to induce expression of the hepatic markers ALBUMIN and alpha-fetoprotein (AFP) as visualized by confocal immunostaining (**Fig.13A**). Further confirmation of the absence of functional hepatic cells was shown by the lack of urea in the culture medium (**Fig.13B**).

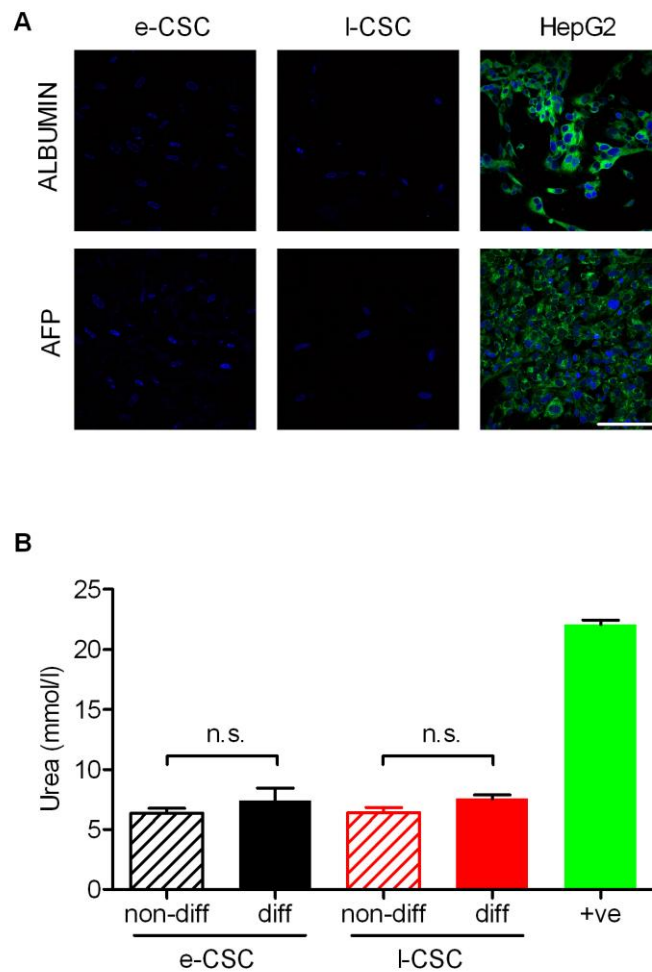


Figure 13 - Growth of e-CSC and l-CSC in hepatogenic differentiation media.

(a) ALBUMIN and AFP immuno-fluorescence of e-CSC and l-CSC grown 3 weeks in hepatogenic permissive media. Positive control is HepG2 cells [from Wei Cui]. All genes stained with FITC (green) and nuclei with DAPI (blue). Scale bars 100 μ m. $n = 3$ per CSC group. (b) Presence of urea (mmol/l) in hepatogenic differentiation media (diff) of e-CSC and l-CSC. Positive (urea; according to the manufacturer's instructions) and negative controls; non-differentiated (non-diff) e-CSC and l-CSC shown. Data. n.s. (not significant), Student's t test. Mean $\pm$ s.e.m. $n = 4$ per CSC group.

3.1.3. Discussion

Here I have shown e-CSC and l-CSC present an intermediate immunophenotype between hESC and adult BM-MSC, expressing MSC markers and containing a sub-fraction of cells that express pluripotency genes. Both populations also have a broadly multipotent differentiation capacity, forming tissues of the ectodermal as well as mesodermal lineages, but not the endodermal lineage.

My findings verify previous research that demonstrated, albeit qualitatively, the mesodermal differentiation potential of e-CSC (Portmann-Lanz, Schoeberlein et al. 2006; Poloni, Rosini et al. 2008) and l-CSC (Li, Zhang et al. 2005; Yen, Huang et al. 2005; Soncini, Vertua et al. 2007; Barlow, Brooke et al. 2008; Brooke, Rossetti et al. 2009) to the osteogenic and adipogenic lineages. I also show neurogenic differentiation of e-CSC and l-CSC, verifying previous reports that they can differentiate beyond the mesodermal lineages to neural progenitors and oligodendrocytes (Portmann-Lanz, Schoeberlein et al. 2006; Poloni, Rosini et al. 2008; Portmann-Lanz, Baumann et al. 2010; Portmann-Lanz, Schoeberlein et al. 2010). The potential of term placenta derived stem cells to treat neurological diseases has recently been investigated and shows the cells could differentiate to a neural phenotype under niche conditions (Martini, Jeremias Tda et al. 2013) and were able to cross the blood-brain barrier to engraft in models of cerebral artery occlusion after intravenous transplantation (Kholodenko, Yarygin et al. 2012). Similarly, a recent study using first trimester placenta stem cells has shown their formation to neural progenitors that can engraft in the brain of an animal model with hypoxia-ischemia and improve motor activity (Park, Koh et al. 2011).

I found neither e-CSC nor l-CSC differentiated to hepatocytes, which is in contrast to others that have shown stem cells isolated from both early and term placental tissue can form cells of the endodermal lineage including hepatocytes (Shin, Lee et al. 2010; Kim, Shin et al. 2011; Lee, Jung et al. 2012) as well as cardiomyocytes (Schmidt, Mol et al. 2006; Okamoto, Miyoshi et al. 2007; Weber, Zeisberger et al. 2011), vascular endothelial cells (Nur Fariha, Chua et al. 2012) and pancreatic cells (Kadam, Muthyala et al. 2010; Susman, Soritau et al. 2010). The discrepancies in findings may be the result of different culture conditions and methods used, but it should also be noted that a number of studies, mainly those on term

placental stem cells, may be confounded by contamination with or overgrowth of maternal/decidual cells (In 't Anker, Scherjon et al. 2004). This was verified in some by characterising the cells (Soncini, Vertua et al. 2007; Barlow, Brooke et al. 2008; Brooke, Rossetti et al. 2009; Semenov, Koestenbauer et al. 2010; Rus Ciuca, Soritau et al. 2011; Jaramillo-Ferrada, Wolvetang et al. 2012) and not tested for in others (Yen, Huang et al. 2005; Miao, Jin et al. 2006; Portmann-Lanz, Schoeberlein et al. 2006; Spitalieri, Cortese et al. 2009; Castrechini, Murthi et al. 2010; Bacenkova, Rosocha et al. 2011; Fariha, Chua et al. 2011; Kim, Shin et al. 2011). Here the presence of the male specific SRY gene was used to show CSC isolated from male samples were of fetal as opposed to maternal origin. We further confirmed with FISH that the male CSC populations did not contain a mixture of fetal and maternal cells and were therefore not contaminated with maternal cells.

The expression of key pluripotency factors in both the e-CSC and l-CSC population indicates that although these cells share many features with MSC they also share some characteristics found in pluripotent hESC and may therefore have an intermediate phenotype. I have referred to the cells as chorionic stem cells (CSC) and not as MSC to avoid confusion. The endogenous expression of pluripotency markers in addition to MSC markers has also been shown for amniotic fluid stem cells (De Coppi, Bartsch et al. 2007; Moschidou, Mukherjee et al. 2012; Moschidou, Mukherjee et al. 2012), as well as amnion derived MSC (Miki, Mitamura et al. 2007; Izumi, Pazin et al. 2009), but the data is limited for the chorionic stem cells with studies only showing expression of a few pluripotency associated genes in e-CSC (Poloni, Rosini et al. 2008; Spitalieri, Cortese et al. 2009; Sung, Hong et al. 2010; Arakawa, Aoki et al. 2012) and l-CSC (Yen, Huang et al. 2005; Semenov, Koestenbauer et al. 2010; Bacenkova, Rosocha et al. 2011; Fariha, Chua et al. 2011; Kim, Shin et al. 2011; Rus Ciuca, Soritau et al. 2011; Koo, Park et al. 2012). Here I show [in combination with flow cytometry data from Dr. Dafni Moschidou] that a panel of pluripotency markers are expressed by a sub-fraction of both CSC populations. This sub-fraction was similar sized in both e-CSC and l-CSC, which suggests the proportion of cells expressing pluripotency genes is maintained throughout gestation. However, whether the expression levels were maintained throughout gestation is investigated next.

3.2. Phenotype features of e-CSC are more primitive than l-CSC

3.2.1. Introduction

Fetal stem cells from various tissues such as bone marrow, liver and blood as well as from extra-embryonic tissues including amniotic fluid, amnion, chorion and Wharton's jelly appear to have a more primitive phenotype than adult BM-MSC, which may be due to the younger age of the tissues they were isolated from (Abdulrazzak, Moschidou et al. 2010). The comparison of first trimester fetal BM-MSC to neonatal UC-MSC (Zhang, Teoh et al. 2009) and adult BM-MSC (Guillot, Gotherstrom et al. 2007) has shown fetal stem cells have an earlier state of stemness (e.g. smaller size, faster proliferation and greater differentiation potency), which may be advantageous for use in regenerative medicine.

Studies using placenta derived stem cells have shown both first trimester (Poloni, Rosini et al. 2008; Meraviglia, Vecellio et al. 2012) and term stem cells (Barlow, Brooke et al. 2008; Jaramillo-Ferrada, Wolvetang et al. 2012) have greater expansion potential than adult BM-MSC. However, a direct comparison of their phenotypes has yet to be carried out. I hypothesised that the chorionic stem cells isolated early in pregnancy would present a more primitive phenotype than those isolated at term. Accordingly my aim was to compare the phenotype of e-CSC and l-CSC when cultured *in vitro* in terms of cell size, growth kinetics, differentiation potential and pluripotency gene expression.

3.2.2. Results

Cell size was measured in equivalent passage cells (4-5) (i.e. a similar number of population doublings) to prevent senescence and subsequent cell enlargement confounding results. On average e-CSC were smaller in size than l-CSC ($16.5 \mu\text{m} \pm 0.1 \text{ s.e.m}$ vs. $24.0 \mu\text{m} \pm 0.1$, $P < 0.01$) (**Fig.14A and 14B**). Kinetic analysis revealed that e-CSC grew faster than l-CSC (population doubling time $48.6 \text{ h} \pm 3.7 \text{ s.e.m}$ vs. $120.7 \text{ h} \pm 27.2$, $P < 0.05$) (**Fig.15A**) and subsequently generated greater cell numbers when grown beyond confluence ($5.0 \text{ PD} \pm 0.6 \text{ s.e.m}$ vs. $2.3 \text{ PD} \pm 1.3$ over 288 hours, $P < 0.05$) (**Fig.15B**). In combination with our previous findings that e-CSC also senesced later than l-CSC, growing respectively to 17 passages compared to 11 passages without reduced growth (Jones, Moschidou et al. 2012), these results show e-CSC has greater expansion potential than l-CSC.

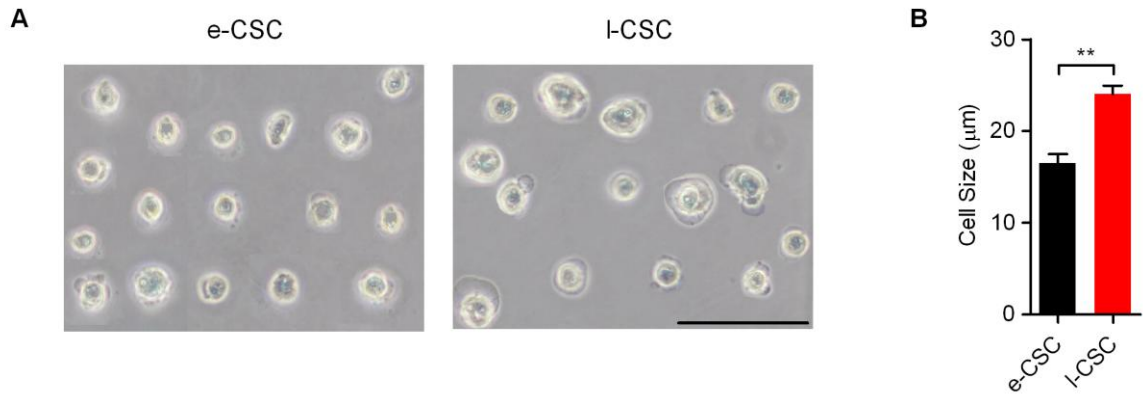


Figure 14 - e-CSC are smaller in size than l-CSC.

(a) Image of cells in suspension used for cell size analysis. Scale bar 100 μm. (b) Average cell size in μm of e-CSC (black) and l-CSC (red) when in suspension. ** $P < 0.01$, Student's t test, $n = 3$ different samples per cell group. Mean $\pm$ s.e.m.

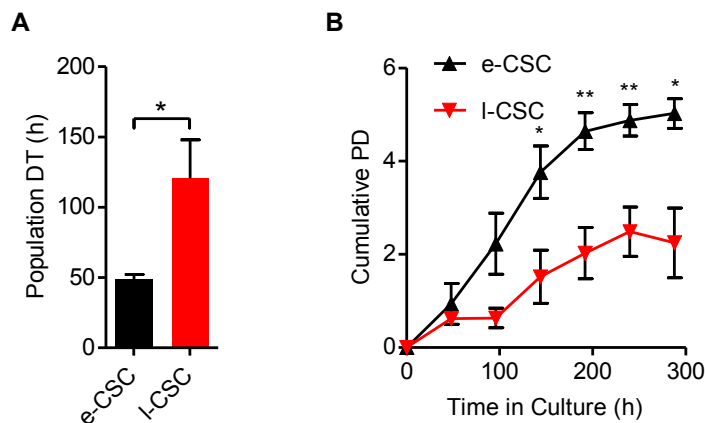


Figure 15 - e-CSC grow faster than l-CSC.

(a) Average growth rate in hours taken for cell population to double during exponential growth phase; i.e. population doubling time (DT). e-CSC (black), l-CSC (red). (b) Cell kinetics measured by average cumulative population doublings of e-CSC (▲) and l-CSC (▼) when seeded at low density and grown beyond confluence for 288 hours. * $P < 0.05$; ** $P < 0.01$, Student's t test, $n = 3$ different samples per cell group. Mean $\pm$ s.e.m.

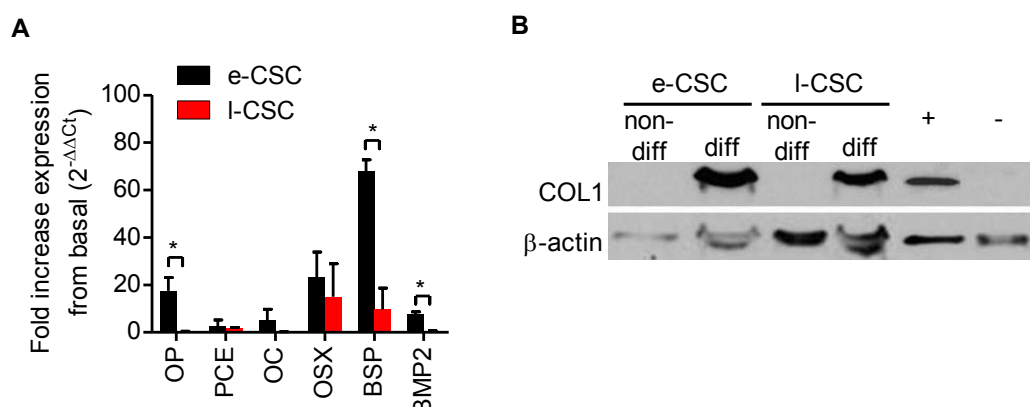


Figure 16 - e-CSC have greater osteogenic differentiation than l-CSC.

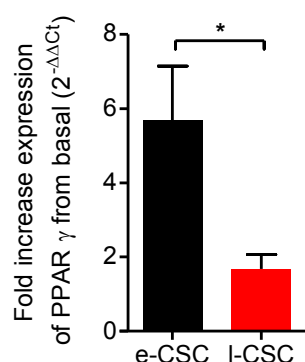
(a) Quantitative real time PCR of fold increased expression of osteogenic genes after osteogenic differentiation; Osteopontin (OP), Procollagen endopeptidase enhancer (PCE), Osteocalcin (OC), Osterix (OSX), Bone sialo-protein-II (BSP) and bone morphogenic protein-2 (BMP2). Expression normalised to GAPDH and basal expression levels of differentiation genes (i.e. $2^{-\Delta\Delta C_t}$). e-CSC (black) and l-CSC (red). Data. * $P < 0.05$, Student's *t* test. Mean $\pm$ s.e.m. $n = 3$ per cell group. (b) Western blot of COL1 for e-CSC and l-CSC not differentiated (non-diff) and differentiated to bone for 2 weeks (diff). Positive control (AFSC differentiated to bone for 4 weeks) and negative control shown. Loading control is β -actin.

Qualitatively the osteogenic differentiation of e-CSC over 14 days of culture in osteogenic permissive conditions appeared greater than l-CSC, due to more intense alizarin red and von Kossa staining (**Fig.8**). I subsequently used quantitative real time PCR to semi-quantify the upregulation of late osteogenic genes and bone matrix related genes in e-CSC and l-CSC after 2 weeks *in vitro* differentiation. Relative expression of each gene was normalized by its expression in non-differentiating growth medium (basal level) after normalization to *Gapdh* ($2^{-\Delta\Delta C_t}$). Results showed only differentiated e-CSC expressed *Osteocalcin* (OC; 5.2 fold upregulation $\pm$ 4.6 s.e.m) and differentiated e-CSC compared to l-CSC had higher expression of *Osteopontin* (OP; 17.5 fold upregulation $\pm$ 5.6 vs. 0.3 fold upregulation $\pm$ 0.3, $P < 0.05$), *Bone sialo-protein-II* (BSP; 67.8 fold upregulation $\pm$ 5.0 vs. 9.7 fold upregulation $\pm$ 11.1, $P < 0.05$) and *Bone morphogenic protein-2* (BMP2; 7.5 fold upregulation $\pm$ 1.2 vs. 0.5 fold upregulation $\pm$ 0.3, $P < 0.01$) (**Fig.16A**). At the protein level, type I collagen (COL1A2) was

absent in non-differentiated cells, but present in differentiated e-CSC and l-CSC samples, with e-CSC showing 3.2 times more protein (**Fig.16B**). This shows e-CSC has a greater osteogenic potency than l-CSC.

In addition to greater osteogenic differentiation e-CSC also produced considerably more Oil Red O-positive lipid vacuoles than l-CSC when cultured in adipogenic permissive medium for three weeks (**Fig.11**). Quantification of adipocyte differentiation regulator peroxisome proliferator-activated receptor gamma (*PPAR* γ) using real time PCR analysis confirmed the higher adipogenic differentiation potential of e-CSC as expression was greater in differentiated e-CSC than l-CSC (5.7 fold upregulation $\pm$ 1.4 s.e.m vs. 1.7 fold upregulation $\pm$ 0.4 s.e.m, $P < 0.05$), after normalization to *Gapdh* and basal cell expression (**Fig.17**). The upregulation of *PPAR* γ in l-CSC was typical of previous reports for this cell type (Kim, Park et al. 2012).

Figure 17 - e-CSC have greater adipogenic differentiation than l-CSC.



Quantitative real time PCR of fold increased expression of adipocyte differentiation regulator peroxisome proliferator-activated receptor gamma (*PPAR* γ) after 3 weeks of adipogenic differentiation. Expression normalised to *GAPDH* and basal expression levels of differentiation genes (i.e. $2^{-\Delta\Delta C_t}$). e-CSC (black) and l-CSC (red). Data. * $P < 0.05$, Student's *t* test. Mean $\pm$ s.e.m. $n = 3$ per cell group.

At the protein level OCT4A was expressed in both e-CSC and l-CSC (**Fig.9A**), however pseudogenes not involved in maintaining pluripotency are found in the *Oct4A* isoforms and may have resulted in a false positive result (Pesce and Scholer 2000; Lee, Kim et al. 2006; Atlasi, Mowla et al. 2008; Ryan, Pettit et al. 2011). To avoid pseudogene detection RT-PCR was performed using specific primers for *Oct4A* variant 1 previously reported to be negative in e-CSC (Ryan, Pettit et al. 2011), and results showed expression in e-CSC, but not l-CSC, indicating a more primitive phenotype of e-CSC (**Fig.18A**). However, both e-CSC and l-CSC expressed the other *Oct4A* variants when primers that also detected *Oct4A* variant 2 and 3 were used (**Fig.18B**), explaining the detection of OCT4A protein in both cell populations.

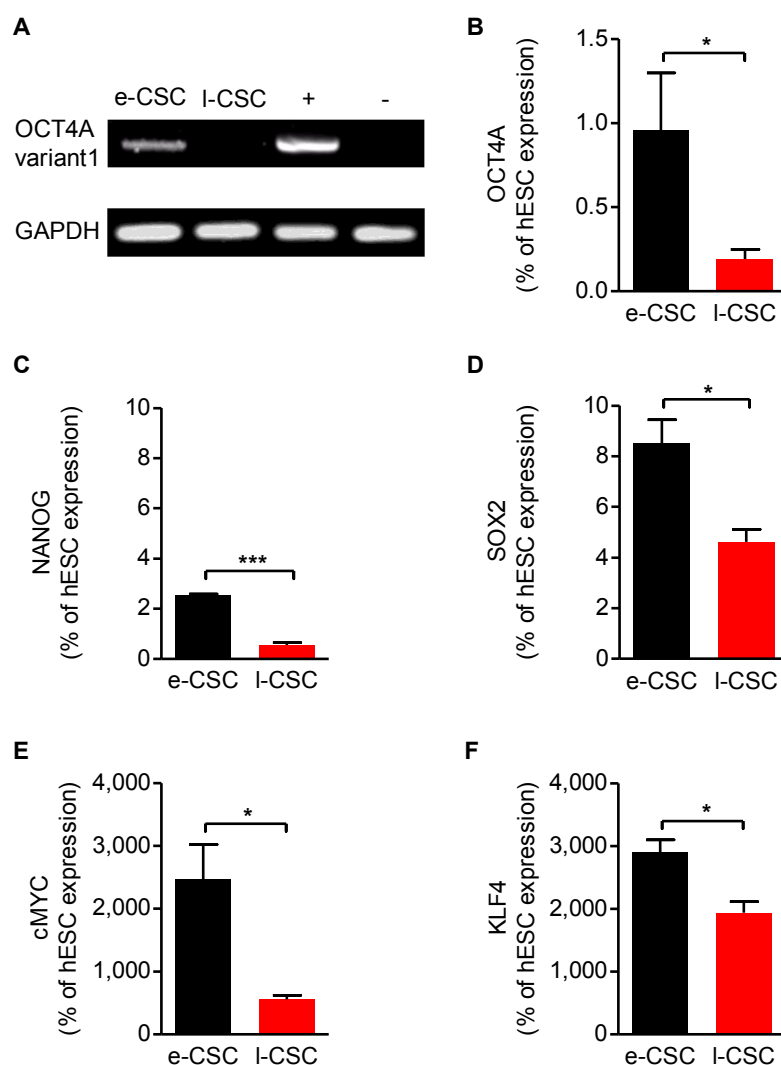


Figure 18 - e-CSC express higher levels of pluripotency genes than l-CSC.

(a) RT-PCR for *Oct4A* variant 1. *Gapdh* and positive and negative controls shown. (b-f) Quantitative real time PCR for (b) *Oct4A* all variants, (c) *Nanog*, (d) *Sox2*, (e) *c-Myc* and (f) *Klf4* for e-CSC (black) and l-CSC (red) as percentage expression of human embryonic stem cells (hESC). * $P < 0.05$; *** $P < 0.001$, Student's *t* test. Mean $\pm$ s.e.m. $n = 4$ per cell group.

Transcript levels of the other reprogramming factors *Sox2*, *c-Myc*, *Klf4* as well as *Nanog* were subsequently quantified and showed expression levels were considerably lower relative to hESC for *Nanog* (2.5 % $\pm$ 0.1 and 0.6 % $\pm$ 0.1; **Fig.18C**) and *Sox2* (8.5 % $\pm$ 1.0 s.e.m and 4.6 % $\pm$ 0.5; **Fig.18D**) However, expression levels were markedly higher in both e-CSC and

l-CSC relative to those found in hESC for *c-Myc* (2,472 % $\pm$ 785.9 vs. 556.4 % $\pm$ 106.7, respectively; **Fig.18E**) and *Klf4* (2,906 % $\pm$ 277.3 vs. 1,940 % $\pm$ 305.8, respectively; **Fig.18F**). When we compared e-CSC to l-CSC, all five pluripotency markers were expressed at greater relative levels in e-CSC; *Oct4A* ($P < 0.05$), *Sox2* ($P < 0.05$), *c-Myc* ($P < 0.05$), *Klf4* ($P < 0.05$) and *Nanog* ($P < 0.001$), indicating a e-CSC may have a more primitive phenotype.

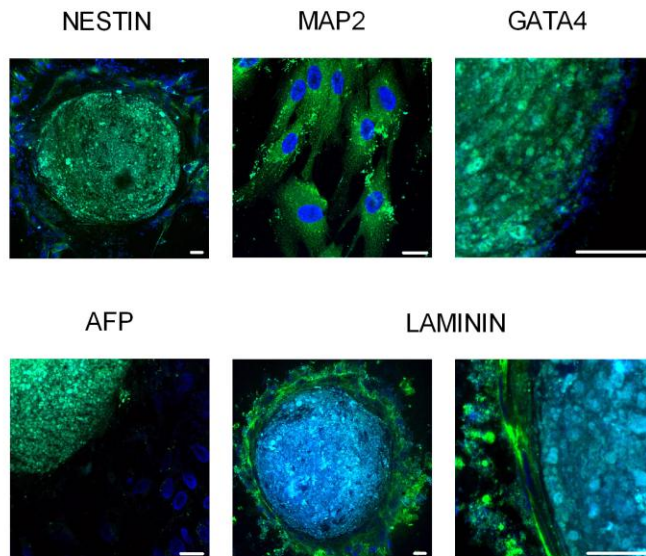


Figure 19 - e-CSC uniquely form embryoid bodies (EBs) with tri-lineage differentiation.

Confocal immuno-fluorescence of e-CSC EBs for expression of NESTIN, MAP2, GATA4, AFP and LAMININ stained with FITC (green). Nuclei stained with DAPI (blue). Scale bars 25 μ m. n = 1

in l-CSC. In contrast e-CSC formed EBs with a complex and compact morphology and a defined peripheral lining after 12 days of differentiation [Data published in (Jones, Moschidou et al. 2012) from Dr. Dafni Moschidou and Aya Harada]. I showed the expression of differentiation markers representative of all three germ layers using confocal microscopy for NESTIN and MAP2 (ectoderm), GATA4 (mesoderm), AFP (endoderm) and LAMININ, which was expressed in the peripheral lining of the EBs (**Fig.19**). Teratoma formation was not tested for in these cells.

To investigate if endogenous expression of pluripotency markers translates to an ability to differentiate beyond mesodermal lineages, I [in combination with Dr Dafni Moschidou and Aya Harada] tested the capacity of e-CSC and l-CSC to form embryoid bodies (EBs) *in vitro* with differentiation to cells from the three germ layers. We showed that only e-CSC, but not l-CSC, formed EBs when grown in EB-permissive media, which may be linked to lack of *Oct4A variant 1* expression

3.2.3. Discussion

Here I show ontological differences between chorionic stem cells isolated at different gestational ages with a decline in primitive characteristics seen in l-CSC compared to e-CSC. This suggests that there is an evolution of the stem cell phenotype during pregnancy, with an increase in cell size and a decrease in kinetics and differentiation potency paralleling a down regulation of expression levels of early “stemness” genes.

My findings support previous work, which showed bone marrow hfMSC in contrast to their adult counterparts were smaller, grew faster and senesced later, despite sharing a common immunophenotype with expression of mesenchymal, adhesion and lineage markers in the whole population (Guillot, Gotherstrom et al. 2007). Others have previously reported that early placental MSC or e-CSC have increased proliferative potential when compared to adult bone marrow MSC (Poloni, Rosini et al. 2008; Meraviglia, Vecellio et al. 2012) due to the maintenance of long, stable telomeres without telomerase activity (Poloni, Maurizi et al. 2011). Telomere length was also shown to be maintained in l-CSC without telomerase (Nazarov, Lee et al. 2012). Our data indicates that human fetal chorionic stem cells retain their immunophenotype with age, but lose associated primitive characteristics, not only from fetal to adult, but also from fetal to neonatal.

The absence of *Oct4A variant 1* expression in l-CSC at the transcript level and downregulation of other pluripotency markers may be pivotal to the decline of primitive characteristics seen in these cells when compared to their early gestational counterparts. *Oct4A variant 1* transcript is expressed in the nuclei of undifferentiated hESC, EC cells and PGC and is responsible for maintaining “stemness”, whilst the other pseudogenes are found in the cytoplasm and have no known function (Pesce and Scholer 2000; Lee, Kim et al. 2006; Atlasi, Mowla et al. 2008). In particular the lack of *Oct4A variant 1* in l-CSC may explain the inability of l-CSC to form embryoid bodies (EBs) as *Oct4A variant 1* is critical to retain pluripotentiality (Atlasi, Mowla et al. 2008; Ryan, Pettit et al. 2011). From a technical view, OCT4A was detected at the protein level in the nuclei of both e-CSC and l-CSC using OCT4A-specific antibodies; this is likely to reflect lack of specificity of the available antibody for the *variant 1* isoform, potentially also binding to *variants 2* and *3* (Ryan, Pettit et al. 2011). This was confirmed through qRT-PCR analysis that showed expression of *Oct4A* in

both e-CSC and l-CSC when primers that amplified all 3 of the *Oct4A* variants were used. Therefore the cell populations differ in expression of *Oct4A variant 1* only.

Although *Oct4A variant 1* was not detectable in l-CSC, other pluripotency associated markers were found in both e-CSC and l-CSC populations at both the transcript and protein level. Significantly both cell populations contained a similar proportion of cells expressing these markers, however, expression of key pluripotency markers (*Oct4A*, *Klf4*, *Sox2*, *c-Myc* and *Nanog*) decreased in l-CSC compared to e-CSC. This indicates a downregulation of expression with increased gestational age rather than a reduced number of cells expressing these early developmental markers. A similar result has been shown in amniotic epithelium stem cells, whereby expression of *NANOG* and *SOX2* was higher in first trimester amnion than term, although levels of *OCT4* were unchanged (Izumi, Pazin et al. 2009). While there is little literature on this in human chorionic stem cells (Sung, Hong et al. 2010), work to date on whole chorion tissues indicates a down regulation of telomerase activity over gestation, supporting the decline of primitive stem cell characteristics with age in this tissue (Kyo, Takakura et al. 1997).

Despite both e-CSC and l-CSC expressing early developmental markers and differentiating beyond the mesodermal lineages to neural progenitors, only e-CSC formed EBs, which was validated with typical morphology and expression of markers from the three germ layers. The lack of EB formation in the l-CSC indicates a reduced potency of these cells compared to the e-CSC and may be due to the absence of *Oct4A variant 1* in the l-CSC or may be a result of sub-optimal culture conditions for this cell type. Recent work from our group has shown that similar to e-CSC, first trimester amniotic fluid stem cells (AFSC) grown in MSC media can form EBs with differentiation to some lineages of all three germ layers (Moschidou, Mukherjee et al. 2012), whereas mid trimester AFSC do not have the capacity to form EBs under the same conditions (Moschidou, Mukherjee et al. 2012). This loss of potency with increased gestational age was attributed to the first trimester AFSC being phenotypically more primitive than their mid trimester counterparts, with expression of *Nanog*, *Sox2*, *Klf4*, *Ssea3*, *Tra-1-60* and *Tra-1-81* (Moschidou, Mukherjee et al. 2012; Moschidou, Mukherjee et al. 2012), which reflects what I have shown with the CSC. In addition single cell analysis showed first trimester AFSC contained cells that were smaller in size than the mid trimester

AFSC and this subfraction of small cells highly expressed *Ssea3*, *Tra-1-60* and *Tra-1-81* (Moschidou, Drews et al. 2012). These results indicated expression of pluripotency markers may decrease with gestation due to either the loss of the high expressing subfraction of primitive cells with gestational age or the increase in size and decrease in primitive characteristics of this subfraction of cells. These results together with my data offer strong support that there is a decline in stemness with increased gestational age. Investigation into the heterogeneity of the CSC populations is now needed at a single cell level, to address how the molecular signature of the populations change with fetal development and if a primitive subfraction of cells is present in the parental populations.

Semi-quantitative analysis of the adipogenic and osteogenic differentiation of e-CSC and l-CSC compared the upregulation of lineage specific genes and protein and confirmed that l-CSC had a reduced adipogenic and osteogenic potency. These findings parallel previous findings that, despite not comparing the same tissue type, found MSC from fetal bone marrow had higher osteogenic potency than term umbilical cord blood (Zhang, Teoh et al. 2009). Likewise results from our group have shown the osteogenic potency of fetal BM-MSC was higher than adult BM-MSC (Guillot, Gotherstrom et al. 2007) and others have shown greater differentiation potency of first trimester placenta derived stem cells compared to term decidua derived MSC along ectoderm lineages (Park, Koh et al. 2013). However, one study reported a restricted plasticity of e-CSC, with less efficient adipocyte and osteocyte differentiation than adult bone marrow MSC and instead had preference towards endothelial differentiation (Meraviglia, Vecellio et al. 2012). This difference is likely to reflect different methods of culture and differentiation that may have inadvertently reduced the differentiation potency of the e-CSC, potentially by not maintaining cells at sub-confluence.

3.3. Chorionic stem cells may share a developmental origin with PGC

3.3.1. Introduction

Primordial germ cells (PGC) are the most primitive cell to be specified during development and are found at the beginning of gastrulation where they reside in extra-embryonic mesoderm prior to migration along the genital ridge, upon which they differentiate to mature germ cells in the gonads (Shin, Zuba-Surma et al. 2009). Kucia *et al.* hypothesised that cells isolated from adult and fetal tissues that have pluripotency characteristics may share an origin with PGC (Kucia, Machalinski et al. 2006; Ratajczak, Machalinski et al. 2007). Investigation of very small embryonic-like (VSEL) cells, isolated from cord blood (Kucia, Wu et al. 2007), adult bone marrow (Hua, Pan et al. 2009; Hua, Yu et al. 2009) as well as mouse fetal liver (Shin, Liu et al. 2010), has revealed similarities to PGC in morphology, expression of pluripotency genes and methylation status and were therefore proposed to have a PGC origin. Since I have shown expression of pluripotency markers in the chorionic stem cells I aimed to investigate if e-CSC and l-CSC might also share a developmental origin with PGC.

3.3.2. Results

At the transcript level both e-CSC and l-CSC expressed markers found in PGC (**Fig.20**) as has been previously shown for first trimester amniotic fluid stem cells (AFSC) (Moschidou, Mukherjee et al. 2012). These included *c-kit*, a regulator of PGC survival and growth (De Miguel, Cheng et al. 2002; Hoyer, Byskov et al. 2005), which is expressed in PGC, neural crest stem cells and haematopoietic stem cells during embryogenesis (Motohashi, Aoki et al. 2007). As well as other markers which commonly define PGC: *Stella*, which is thought to maintain pluripotentiality (Bowles, Teasdale et al. 2003; Payer, Saitou et al. 2003), *Fragilis* and *Nanos3*, which have roles in PGC migration (Tsuda, Sasaoka et al. 2003; Tanaka, Yamaguchi et al. 2005) as well as *SSEA1*, an early marker found in PGC and human embryonic germ (hEG) cells but not hESC (Henderson, Draper et al. 2002; Park, Kim et al. 2004). *Vasa* is expressed by PGC in the gonadal ridge and also by post-migratory PGC (Castrillon, Quade et al. 2000), *Vasa* was only detected in the e-CSC and not l-CSC populations at the transcript level (**Fig.20**). These results suggest both populations may contain cells of pre-migratory PGC origin, whilst e-CSC may also contain cells of post-migratory PGC origin. Positive controls for PGC markers are shown in **Fig.20** /Appendix 2.

Confocal immunofluorescence confirmed the expression of PGC markers at the protein level for STELLA, FRAGILIS, NANOS3 and SSEA1 and also demonstrated expression of DAZL and PUM2, which co-localise in hEG cells (Moore, Jaruzelska et al. 2003) (**Fig.21**). The presence of PGC proteins was confirmed by comparison to first trimester AFSC (shown in

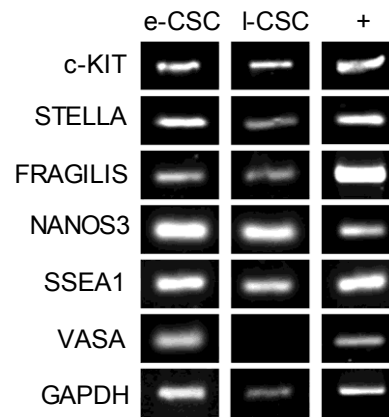


Figure 21 - e-CSC and l-CSC express PGC genes.

Gel electrophoresis band following RT-PCR for expression of PGC genes in e-CSC and l-CSC. + shows positive controls used for each gene: first trimester AFSC was control for Stella, Fragilis, Nanos3, Sseal and Gapdh loading control; second trimester AFSC was control for c-kit; mouse testes was control for Vasa (for loading controls see Appendix2A and 2B). n = 3 per cell group.

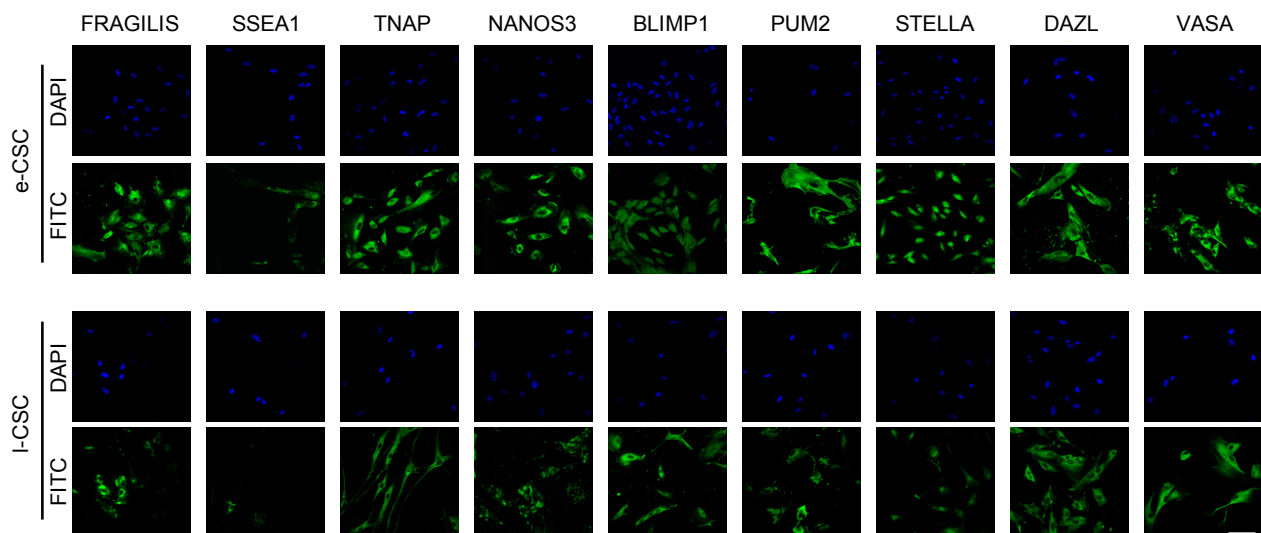


Figure 20 - e-CSC and l-CSC express PGC proteins.

Representative confocal immuno-fluorescence for protein expression of FRAGILIS, SSEA1, TNAP, NANOS3, BLIMP1, PUM2, STELLA, DAZL and VASA stained with FITC (green). Nuclei stained with DAPI (blue). Scale bar 100 μ m. For positive control see Appendix 2C. n = 4 per cell group.

Appendix 2C), which have high expression of a number of PGC markers and have been compared to the seminoma cell line TCam-2, which have characteristics of fetal germ cells (Nettersheim, Gillis et al. 2011; Moschidou, Mukherjee et al. 2012).

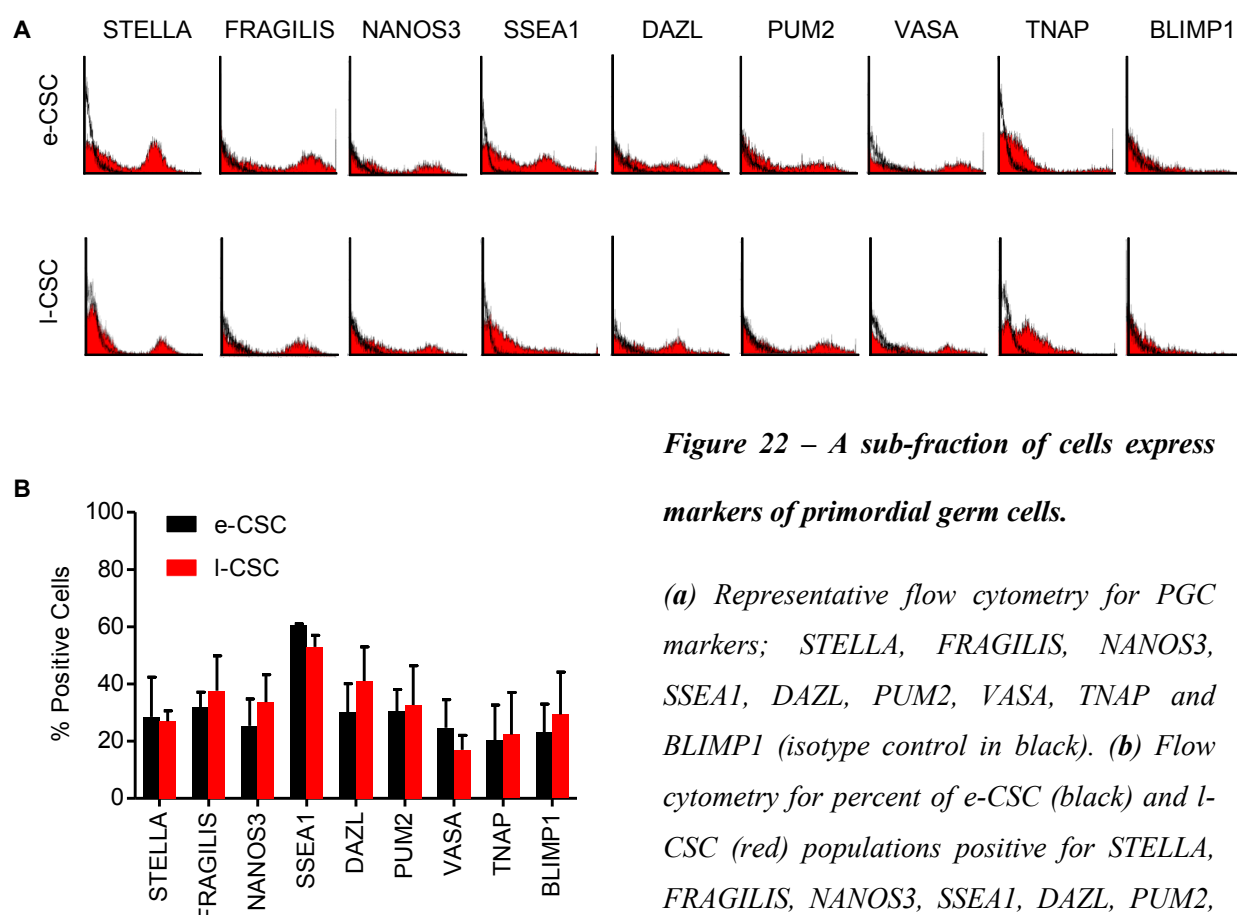


Figure 22 – A sub-fraction of cells express markers of primordial germ cells.

(a) Representative flow cytometry for PGC markers; STELLA, FRAGILIS, NANOS3, SSEA1, DAZL, PUM2, VASA, TNAP and BLIMP1 (isotype control in black). (b) Flow cytometry for percent of e-CSC (black) and l-CSC (red) populations positive for STELLA, FRAGILIS, NANOS3, SSEA1, DAZL, PUM2, VASA, TNAP and BLIMP1. For positive control see Appendix 2D. $n = 3$ per cell group.

Flow cytometry showed there was a similar sized subpopulation of cells in both e-CSC and l-CSC respectively positive for STELLA ($29\% \pm 14$ and $27\% \pm 4$), FRAGILIS ($32\% \pm 5$ and $38\% \pm 12$), NANOS3 ($25\% \pm 9$ and $34\% \pm 10$), SSEA1 ($61\% \pm 1$ and $53\% \pm 4$), as well as DAZL ($30\% \pm 10$ and $41\% \pm 12$) and PUM2 ($30\% \pm 8$ and $33\% \pm 14$) (**Fig.22A** and **22B**). Whereas only a small proportion of cells expressed VASA, specific to the germ cell lineage (Castrillon, Quade et al. 2000), in both e-CSC and l-CSC ($25\% \pm 10$ and $17\% \pm 5$ respectively) with RT-PCR failing to detect *Vasa* transcripts in the l-CSC population (**Fig.20**). Also only a small proportion of e-CSC and l-CSC respectively expressed TNAP,

used to denote early PGC (MacGregor, Zambrowicz et al. 1995; McLaren 2003) ($20\% \pm 12$ and $22\% \pm 14$) and BLIMP1, not found in hEG cells (Durcova-Hills, Tang et al. 2008), but a key marker in PGC that prevents dedifferentiation into pluripotent stem cells (Ohinata, Payer et al. 2005) ($13\% \pm 1$ and $15\% \pm 1$) (**Fig.22A** and **22B**). Positive controls for flow cytometry were first trimester AFSC, which express the PGC markers in a large sub-population of cells (Moschidou, Mukherjee et al. 2012), these are shown in Appendix 2D. Down-regulation of *Blimp1* in e-CSC and l-CSC may be responsible for the high expression levels of its targets *c-Myc* and *Klf4*, which were at least five times higher than expressed by hESC (**Fig.18E** and **18F**).

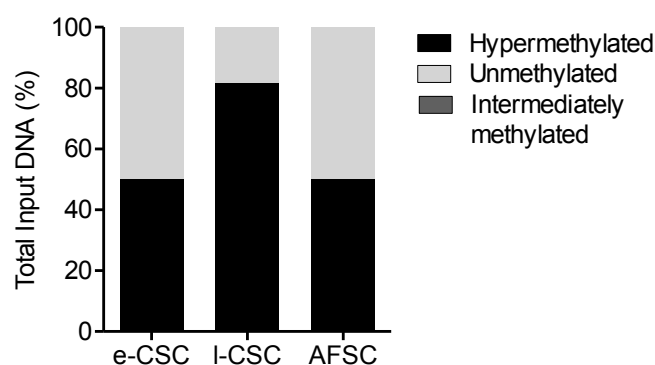


Figure 23 - Partial methylation of H19 in e-CSC and l-CSC.

DNA methylation status of imprinted gene *H19* as percent total input DNA hypermethylated (black), unmethylated (light grey) and intermediately methylated (dark grey). Reference control is first trimester AFSC. $n = 3$ per cell group.

PGC reside in extra-embryonic tissues prior to migration along the genital ridge where they undergo epigenetic reprogramming including erasure of methylation on *H19* (Hajkova, Erhardt et al. 2002; Arnaud 2010). To determine to which developmental stage of PGC e-CSC and l-CSC are most similar, we investigated the methylation status of *H19*. We found *H19* was both hypermethylated and unmethylated in both e-CSC ($50\% \pm 0$ hypermethylated and $50\% \pm 0$ unmethylated, $n=3$) and l-CSC ($70\% \pm 8$ hypermethylated and $30\% \pm 8$ unmethylated, $n=3$) (**Fig.23**), which was similar to the *H19* methylation status of first

trimester AFSC, which was used as a reference control since bisulfite sequencing shows *H19* methylation at a 1:1 ratio of methylated and unmethylated regions (Moschidou, Mukherjee et al. 2012). These results may indicate that e-CSC and l-CSC contain cells of PGC origin which have been retained in placental tissue prior to the start of genital ridge migration, at a time when imprint erasure has not yet started.

3.3.3. Discussion

This work offers support for Kucia *et al.*'s hypothesis that cells isolated from adult and fetal tissues that have pluripotency characteristics may share an origin with PGC (Kucia, Machalinski et al. 2006; Ratajczak, Machalinski et al. 2007). This has been shown for murine fetal liver and adult bone marrow derived VSEL cells (Shin, Liu et al. 2010), and may also be true for umbilical cord blood VSEL cells (Kucia, Halasa et al. 2007) as well as human fetal bone marrow and lung stem cells (Hua, Pan et al. 2009; Hua, Yu et al. 2009) and potentially hESC (Zwaka and Thomson 2005). During fetal development PGC reside at the junction between embryonic and extra-embryonic tissues, then migrate through the primitive streak to give rise to extra-embryonic mesoderm (Donovan 1998). Erasure of *H19* methylation occurs during PGC migration. As we have demonstrated e-CSC and l-CSC maintained both methylated and unmethylated regions on *H19* and expressed a wide array of markers associated with pre-migratory PGCs (OCT4, c-KIT, STELLA, FRAGILIS, NANOS3) (Hayashi, de Sousa Lopes et al. 2007; Saiti and Lacham-Kaplan 2007). Therefore the cells may originate from PGCs deposited in the extra-embryonic tissues before migration and hence before methylation was erased.

Notwithstanding this, we also showed that e-CSC and l-CSC contain a sub-fraction of cells expressing post-migratory PGC markers (VASA, DAZL), which demonstrates the heterogeneity of the chorionic stem cell populations (Hayashi, de Sousa Lopes et al. 2007; Saiti and Lacham-Kaplan 2007). Placental stem cells are known to reside in a vascular niche (Castrechini, Murthi et al. 2010), therefore these cells may have been deposited later in PGC development following their mobilisation into the peripheral blood as shown for VSELs (Ratajczak, Liu et al. 2011). Further investigation is needed, potentially using an *in vivo* model, to precisely trace the lineage of PGCs and determine if this hypothesis is true.

The size of the subpopulation positive for PGC markers in e-CSC and l-CSC remained fairly consistent, which may result from the surrounding tissue niche maintaining a fairly stable, albeit heterogeneous, population of cells. In contrast the comparison of first and second trimester AFSC, showed distinct populations of cells (Moschidou, Drews et al. 2012); with the majority of first trimester AFSC expressing all the PGC markers (Moschidou, Mukherjee et al. 2012), whereas only a small fraction of the second trimester AFSC were positive for the PGC markers, in particular expression of STELLA and VASA was decreased (Moschidou, Mukherjee et al. 2012). The loss of the PGC-expressing subfraction in AFSC with increased gestational age may be due to the amniotic fluid niche being more dynamic than the placenta and subsequently unable to maintain the primitive subpopulation of cells.

CHAPTER 4 – Results

Therapeutic benefits of e-CSC in a model of osteogenesis imperfecta

4.1. Greater tissue repair capacity of e-CSC compared to l-CSC

4.1.1. Introduction

In the first chapter I have shown *in vitro* that e-CSC has advantages for regenerative medicine in terms of differentiation potency and growth kinetics compared to l-CSC due to a more primitive phenotype. Others have shown a greater *in vitro* osteogenic capacity of first trimester bone marrow hfMSC compared to neonatal UC-MSC, translated to a more robust mineralization of cellular scaffolds *in vivo*, an advantage for use in tissue engineering (Zhang, Teoh et al. 2009). I subsequently aimed to test if the superior *in vitro* phenotype of e-CSC compared to l-CSC translated to higher tissue repair capacity *in vivo* using two different murine models.

Firstly I used a mouse model of skin wound repair where it was known that the addition of stem cells promoted healing of dermal wounds and the rate of tissue repair could be measured (Direkze, Forbes et al. 2003; Fathke, Wilson et al. 2004; Ishii, Sangai et al. 2005; Mori, Bellini et al. 2005). Secondly I used the mouse model of type III OI (*oim*) (Chipman, Sweet et al. 1993) that has a deletion in the *Col1a2* gene resulting in bone fragility, which can be quantified using the 3-point bending test. This model has previously shown marked therapeutic improvements following transplantation of first trimester blood hfMSC (Guillot, Abass et al. 2008; Vanleene, Saldanha et al. 2011).

4.1.2. Results

To compare the tissue repair capacity of e-CSC and l-CSC, a skin wound mouse model was used, which consisted of wild type mice given 4 full thickness dermal excisions on their dorsum. Skin wounds were treated with local application of either e-CSC or l-CSC in PBS or PBS alone [Animal experiments carried out with the help of George Bou-Gharios at the Kennedy Institute of Rheumatology, University of Oxford]. Morphological analysis of H&E sections of the wound beds seven days later showed that the healing process for the e-CSC-treated wounds was more advanced (i.e. more closely resembled the normal skin morphology) than for either PBS or l-CSC-treated wounds (**Fig.24A**). This indicated e-CSC promoted greater tissue repair than l-CSC. It was evident by a lower number of infiltrated cells in the e-CSC-treated wounds compared to PBS-treated wounds (53 cells $\pm$ 3 s.e.m vs. 66

cells ± 4 , respectively, calculated in a $100 \mu\text{m}^2$ surface, $P < 0.05$). In contrast, l-CSC-treated wounds did not significantly differ from the controls (70 cells ± 6) (**Fig.24B**). Similarly, the epithelial thickness of the wound beds was thinner for the wounds covered with e-CSC compared to controls ($46 \mu\text{m} \pm 7$ s.e.m vs. $76 \mu\text{m} \pm 5$, respectively, $P < 0.01$), whilst l-CSC-treated wounds did not significantly differ from the controls ($81 \mu\text{m} \pm 11$) (**Fig.24C**).

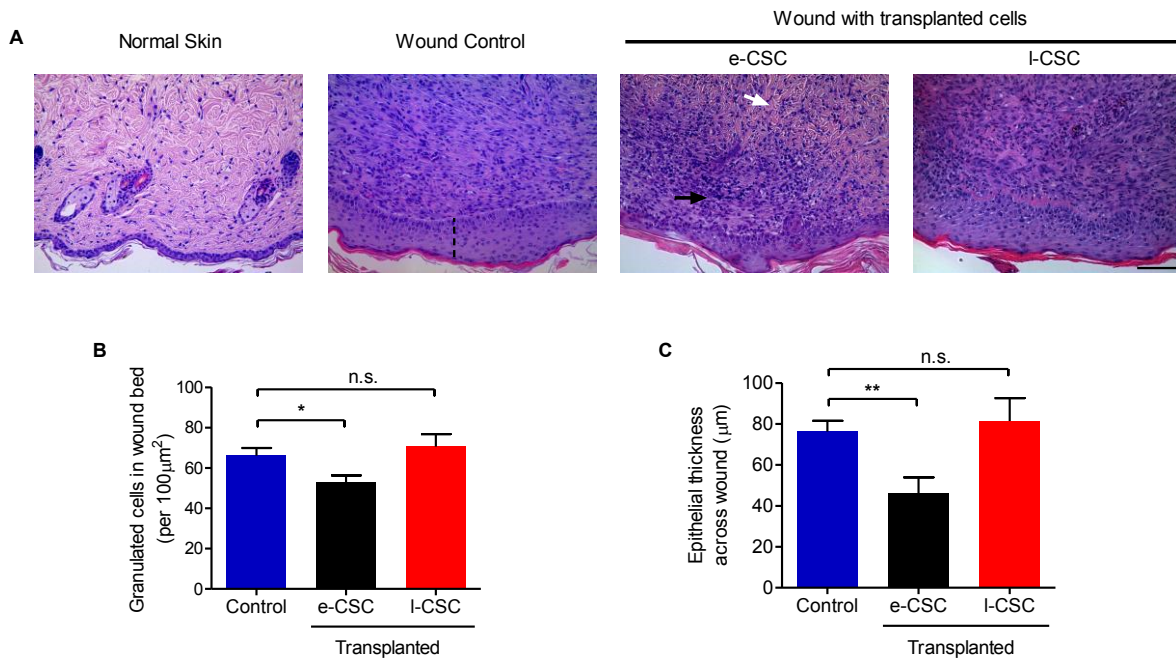


Figure 24 - e-CSC have better tissue repair capacity in vivo than l-CSC.

(a) Cross-section of normal skin and skin wounds 7 days after 4mm dermal biopsy in mice transplanted with e-CSC or l-CSC or PBS (Control); stained with haematoxylin and eosin. Indicated is collagen (white arrow), granulated cells (black arrow) and epithelial thickness (dashed line). In the same model of wound repair, (b) number of granulated cells in the wound bed (per $100 \mu\text{m}^2$) and (c) epithelial thickness across wound in μm . Data. * $P < 0.05$; ** $P < 0.01$, n.s. (not significant), Student's *t* test. Mean $\pm$ s.e.m. $n = 4$ cell samples per cell source. $n = 8$ mice per group.

Secondly the capacity of e-CSC and l-CSC to improve bone pathology in a mouse model of osteogenesis imperfecta (*oim* mice) was compared by measuring the mechanical properties of the bone. *Oim* mice have significantly lower overall bone quality (i.e. total work) than wild type mice ($0.90 \text{ mJ} \pm 0.09$ s.e.m for non-transplanted *oim* mice vs. $7.91 \text{ mJ} \pm 0.61$ for wild

type mice, $P < 0.0001$) and lower bone plasticity (i.e. work from yield to fracture) than wild type mice ($0.62 \text{ mJ} \pm 0.08 \text{ s.e.m}$ for non-transplanted *oim* mice vs. $7.91 \text{ mJ} \pm 0.61$ for wild type mice, $P < 0.0001$). E-CSC and I-CSC (10^6 cells in PBS) were transplanted i.p. into *oim* neonates ($n = 15$ per transplanted group and $n = 30$ for non-transplanted *oim*) and culled 8 weeks later for analysis. Compared to non-transplanted control *oim*, transplantation of e-CSC led to an improvement of overall bone quality ($1.86 \text{ mJ} \pm 0.42$, $P < 0.05$) (**Fig.25A**) and bone plasticity ($1.54 \text{ mJ} \pm 0.40$, $P < 0.01$) (**Fig.25B**), whilst *oim* mice transplanted with I-CSC did not show any significant improvements ($1.16 \text{ mJ} \pm 0.14$ for total work, and $0.89 \text{ mJ} \pm 0.12$ for work from yield to fracture) (**Fig.25A** and **25B** respectively). This suggests under these conditions of transplantation e-CSC have the capacity to improve the bone pathology of *oim* mice, whilst I-CSC do not. However, more samples would be required to determine if there is a significant difference between the two transplantation groups. [Three-point bending experiments carried out with the help of Maximilien Vanleene in the Department of Bioengineering, Imperial College London].

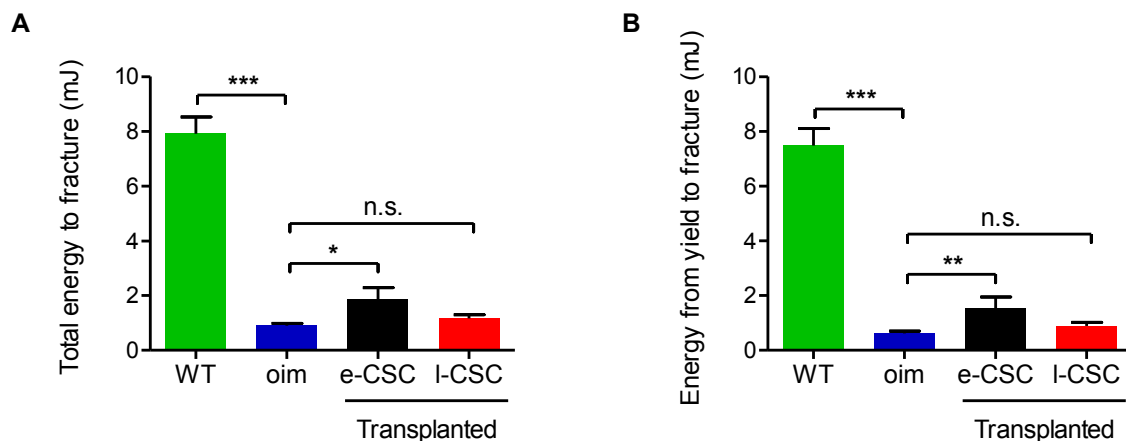


Figure 25 - e-CSC improve *oim* bone mechanical properties more than I-CSC.

(a) Mechanical 3-point bending data in a mouse model of bone brittleness (*oim*) for overall bone quality shown by total energy input in millijoules to fracture and for (b) femoral plasticity shown by energy input in millijoules from yield to fracture. Results shown per mouse for wild type (WT; green), non-transplanted *oim* (*oim*; blue) and *oim* transplanted with e-CSC (e-CSC; black) and I-CSC (I-CSC; red). $n = 17$ wild type, $n = 30$ non-transplanted *oim*, $n = 15$ per transplanted group..

Human donor cell engraftment was visualised by DAB staining on paraffin-embedded tibial sections of 8 week old *oim* using human specific *Vimentin*. Both cell populations had similar engraftment in the bone marrow; ranging from 1.7-3.9% for e-CSC transplanted *oim* and 1.6-3.6% for l-CSC transplanted *oim* with average engraftments of 2.6% and 2.4% respectively (**Fig.26**). This was verified by quantitative real time PCR (qRT-PCR), which quantified the ratio of human specific β -actin to total human and mouse β -actin expressed. Results showed no significant difference in engraftment of human cells between e-CSC and l-CSC transplanted *oim* in the bone marrow as well as the bone (**Fig.27A**).

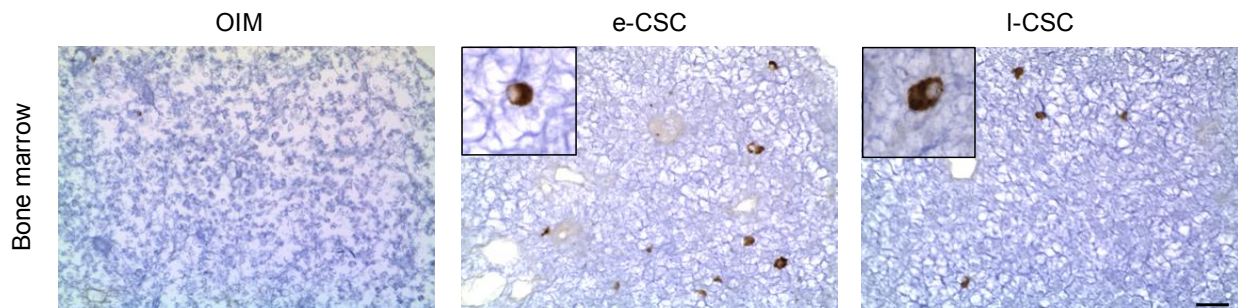


Figure 26 - Transplanted e-CSC and l-CSC engraft in the bone marrow of *oim*.

*Visualisation of human donor cells with DAB staining (brown) of human specific vimentin in the tibial bone marrow of *oim* neonatally transplanted with e-CSC (e-CSC) and l-CSC (l-CSC), compared to age matched non-transplanted *oim* controls (OIM). Zoomed in bone marrow cell shown. Nuclei counter stained with Haematoxylin (blue). Scale bar 10 μ m. n = 4 per group.*

Therefore differences in the bone parameters of transplanted *oim* are likely to result from differences in the intrinsic donor cell properties. Flow cytometry showed a higher proportion of e-CSC expressed STRO-1, a marker associated with early osteoprogenitors (Gronthos, Zannettino et al. 1999; Tare, Khan et al. 2009), than l-CSC (77.4% vs. 32.8%) (**Fig.27B**). Greater numbers of osteoprogenitors corresponded to greater expression of human specific osteogenic genes after transplantation in the bones of *oim* transplanted with e-CSC compared to l-CSC; *Bgn* $0.25 \cdot 2^{-\Delta Ct} \pm 0.07$ s.e.m vs. $0.07 \cdot 2^{-\Delta Ct} \pm 0.03$, $P < 0.05$ respectively; *Phex* $0.47 \cdot 2^{-\Delta Ct} \pm 0.12$ s.e.m vs. $0.08 \cdot 2^{-\Delta Ct} \pm 0.03$, $P < 0.01$ respectively (**Fig.27C**). *Bsp*, a gene responsible for synthesis of structural proteins of the bone matrix was not different between the two transplanted groups.

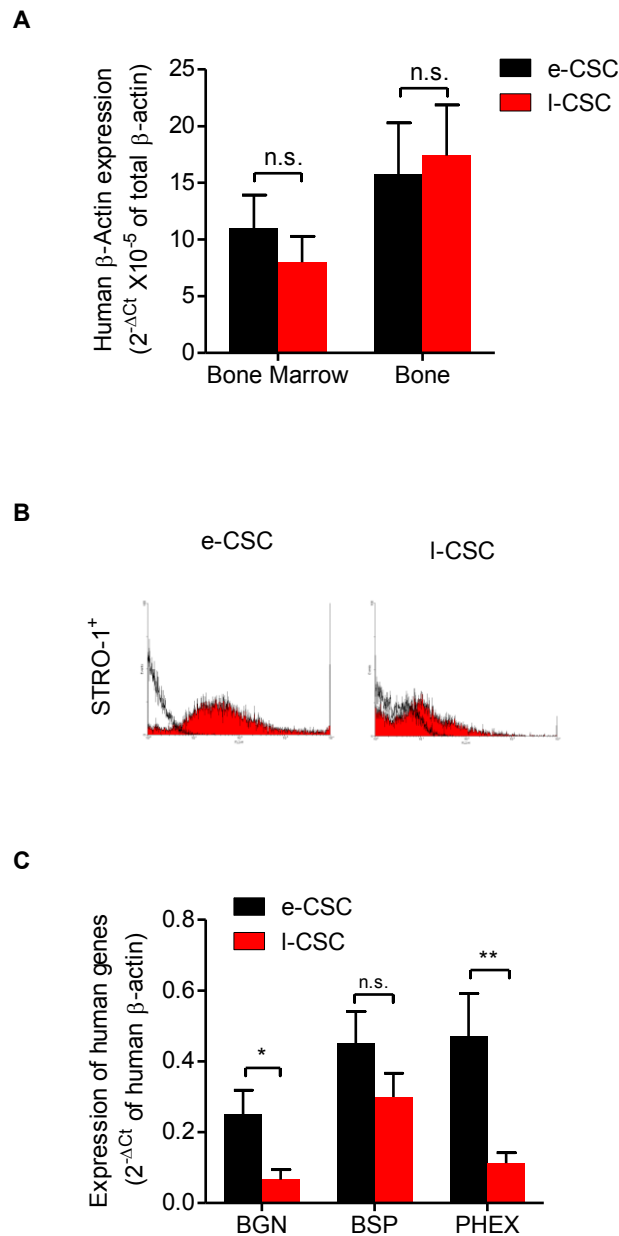


Figure 27 – Transplanted e-CSC have higher osteogenic potency than l-CSC.

(a) Donor cell engraftment in bone marrow and bone; $2^{-\Delta Ct}$ of human specific β -actin normalised to human-mouse non-specific β -Actin in oim mice transplanted with e-CSC (black) or l-CSC (red). $n = 8$ mice per group. (b) Flow cytometry for the proportion of osteoprogenitors (STRO-1⁺ cells; red) in the e-CSC and l-CSC populations before transplantation ($n = 1$ per group) (isotype control in black). (c) Expression of human ossification genes; Biglycan (BGN), Bone sialo-protein II (BSP) and Phosphate regulating endopeptidase homolog (PHEX) in transplanted oim, normalised to expression of human specific β -actin ($2^{-\Delta Ct}$). Data. n.s. (not significant), * $P < 0.05$; ** $P < 0.01$, Student's t test. Mean $\pm$ s.e.m. $n = 8$ mice per group for BGN and PHEX, $n = 12$ mice analysed per group for BSP.

4.1.3. Discussion

I showed that compared to l-CSC, e-CSC had accelerated tissue repair in dermal excision skin wounds and resulted in an improvement in the bone quality and plasticity of bones in *oim* mice. This demonstrates *in vivo* that e-CSC have a greater tissue repair capacity than l-CSC and suggests the more primitive characteristics of these cells are advantageous for use in regenerative medicine.

Accelerated dermal wound healing was evident after just 7 days in e-CSC transplanted mice, but not in the l-CSC transplanted mice, with production of new collagen and reduced epithelial thickness at the wound site. The advancement of tissue repair may be in response to paracrine signals from the e-CSC, which potentially produced more anti-inflammatory cytokines, growth factors or proangiogenic molecules than l-CSC, to enhance repair by the endogenous cells (Ankrum and Karp 2010). Healing of skin wounds has been shown to accelerate in response to increased production of type I collagen (Direkze, Forbes et al. 2003; Fathke, Wilson et al. 2004; Ishii, Sangai et al. 2005; Mori, Bellini et al. 2005), primarily produced from resident cells indicating a paracrine effect of transplanted cells may be beneficial (Higashiyama, Nakao et al. 2011). However, adult bone marrow derived cells have also been shown to differentiate into dermal cells following transplantation that directly contributed to tissue repair (Badiavas, Abedi et al. 2003; Kataoka, Medina et al. 2003). Therefore the mechanisms of tissue repair of skin wounds has yet to be elucidated, but may be a combination of direct donor cell differentiation and paracrine action on the host cells.

The transplantation of e-CSC in the osteogenesis imperfecta model (*oim*) resulted in improved *oim* bone plasticity compared to controls, not seen for l-CSC transplanted *oim*. The improvement in bone plasticity was in line with previous studies which transplanted first trimester blood hfMSC prenatally in *oim* mice (Guillot, Abass et al. 2008; Vanleene, Saldanha et al. 2011). The change in bone mechanics seen in e-CSC transplanted *oim* and not l-CSC transplanted mice corresponded with greater expression of human specific osteogenic differentiation genes, despite a similar level of donor cell engraftment, indicating e-CSC had higher osteogenic differentiation *in vivo*. This offers support for my findings *in vitro* that demonstrated a greater osteogenic potency of e-CSC compared to l-CSC and matches the

findings of Zhang *et al.* that showed higher osteogenic differentiation *in vitro* and *in vivo* of bone marrow hfMSC compared to neonatal UC-MSC (Zhang, Teoh et al. 2009).

The osteoblast differentiation of transplanted cells in the *oim* model and subsequent improvements in disease pathology has been previously shown for adult bone marrow derived cells and blood hfMSC (Wang, Li et al. 2006; Li, Wang et al. 2007; Guillot, Abass et al. 2008; Vanleene, Saldanha et al. 2011). The greater osteogenic potency of e-CSC compared to l-CSC may be due to a higher number of early osteoprogenitors within the population. Previous research, albeit with hematopoietic stem cells (HSCs), has shown correlations with engraftment levels and number of progenitor cells transplanted (Glass, Uharek et al. 1997). I hypothesised that pre-selection of STRO-1+ cells could equalise the therapeutic effects seen between e-CSC and l-CSC. However, preliminary tests showed separation using magnetic beads yielded low numbers of STRO-1+ cells and expansion of the positive fraction after separation was not possible as the cell population reverted back to its original heterogeneous state containing both STRO1+ and STRO1- cells. Therefore I focused on the effect of e-CSC cells in the *oim* model as they contained a higher endogenous fraction of osteoprogenitors as well as advantages for regenerative medicine including higher population expansion *in vitro* and a greater capacity for tissue repair *in vivo*.

4.2. Transplanted e-CSC engraft and differentiate to osteoblasts

4.2.1. Introduction

Our group has previously shown that *in utero* transplantation of first-trimester blood hfMSC in *oim* resulted in donor cells preferentially engrafting at sites of active bone formation and remodelling, as well as at sites of fracture healing (Guillot, Abass et al. 2008). More recently, examining the cellular mechanisms and mechanical modifications underlying the improvement of the *oim* phenotype, our group showed that donor cells differentiated into functional mature osteoblasts, expressing mature osteoblast genes and producing the normal Collagen type 1 alpha 2 (COL1A2) chain protein, which is absent in non-transplanted *oim* mice. This was associated with reduced bone brittleness, indicating that the normal collagen produced from donor cells prevented long bone fracture occurrence by improving the mechanical integrity of the bone at the molecular and whole bone level (Vanleene, Saldanha et al. 2011).

In chapter 4.1 I showed e-CSC and l-CSC engrafted in the bone and bone marrow and expressed some human specific osteogenic genes. Here I have focused on investigating the fate of transplanted e-CSC, in terms of in which part of the bone the cells homed to and if e-CSC differentiated to functional osteoblasts that expressed late osteoblast markers and produced the COL1A2 protein.

4.2.2. Results

Tibia were analysed using DAB staining of human specific Vimentin 8 weeks after e-CSC were transplanted and donor cells were localized in the articular cartilage of the epiphysis, the primary spongiosa of the growth plate, the periosteum of the diaphysis and in the primary spongiosa of fracture calluses (**Fig.28**). This shows transplanted cells migrated to the bone and survived there 8 weeks after transplantation.

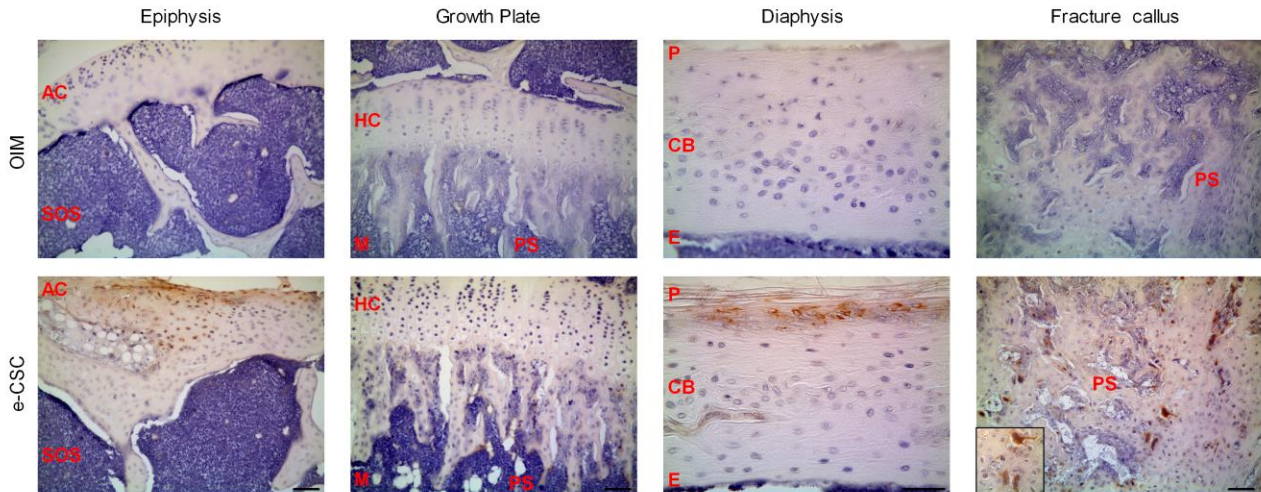


Figure 28 - Transplanted e-CSC engraft in oim bone.

Visualisation of human donor cells with DAB staining (brown) of human specific vimentin in the tibial epiphysis, growth plate, diaphysis and fracture callus of oim neonatally transplanted with e-CSC (e-CSC), compared to age-matched non-transplanted oim controls (OIM). Zoomed in human MSC in the fracture callus shown. Nuclei counter-stained with Haematoxylin (blue). Scale bars all 100 μ m. AC; articular cartilage, SOS; secondary ossification site, HC; hyaline cartilage, M; metaphysis, PS; primary spongiosa, P; periosteum, CB; cortical bone, E; endosteum. $n = 3$ per group.

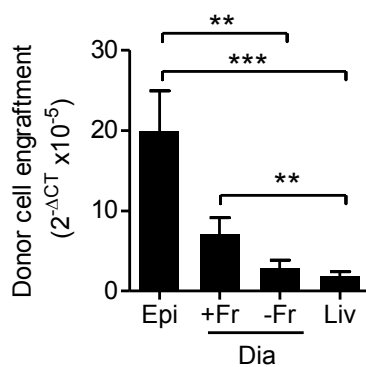


Figure 29 - Donor e-CSC localize to where bone formation is most active.

Quantitative real time PCR of donor cell engraftment calculated as the $2^{-\Delta CT}$ of human specific β -actin normalised to human-mouse non-specific β -actin in the femoral epiphysis (Epi), diaphysis (Dia); with (+Fr) and without fracture callus (-Fr), and in the liver (Liv). ** $P < 0.01$, *** $P < 0.001$; One-way ANOVA followed by Tukey's post hoc test. Error bars are s.e.m. $n = 6$ for Epi, -Fr, Liv. $n = 9$ +Fr.

Quantitative real time PCR (qRT-PCR) showed that donor cell engraftment in transplanted oim was highest in the epiphysis, the site of active bone formation. This was 7.1 fold ($P < 0.05$) higher than in the non-fractured diaphysis where bone formation is less active, and

11.7 fold ($P<0.05$) higher than engraftment in the liver (**Fig.29**). Donor cells also preferentially homed to sites of bone repair, where engraftment was 4 fold ($P<0.05$) higher than engraftment in the liver. Engraftment in fractured and non-fractured diaphysis was not significantly different, but interestingly more mice were positive for human cDNA in the diaphysis if a fracture callus was present; 90% compared to 60%.

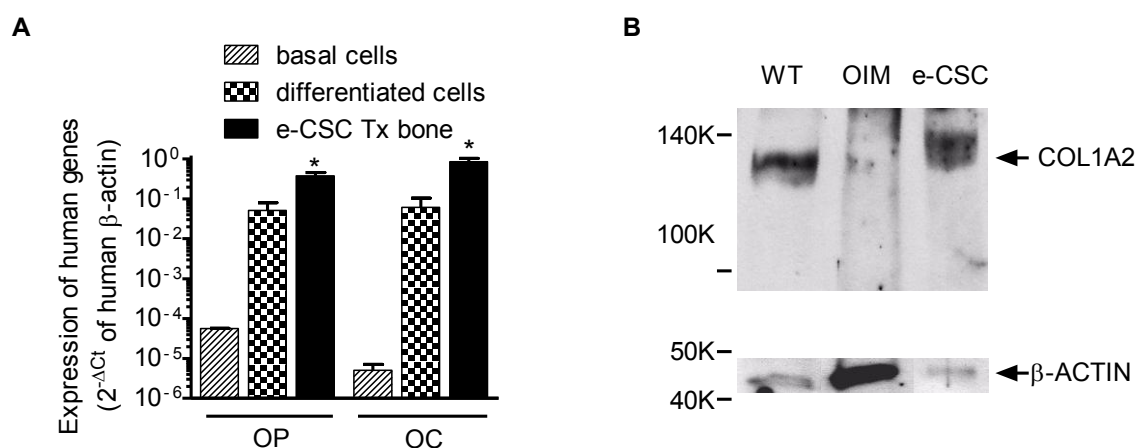


Figure 30 - Donor e-CSC differentiate to functional osteoblasts in vivo.

(a) Quantitative real time PCR of expression of human specific *Osteopontin* (OP) and *Osteocalcin* (OC) normalised to human specific β -actin ($2^{-\Delta C_t}$) in the femurs of e-CSC transplanted oim (e-CSC Tx bone, $n = 6$; black) and compared to the basal expression level of e-CSC (basal cells, $n = 3$; stripes) and expression level of cells grown in osteogenic permissive media for 2 weeks (differentiated cells, $n = 3$; checks). * $P<0.05$; One-way ANOVA followed by Tukey's post hoc test. Error bars are s.e.m. (b) Western blot of COL1A2 protein in the femurs of e-CSC transplanted oim (e-CSC) compared to age matched wild type (WT) control and non-transplanted oim (OIM). Loading control is β -ACTIN. $n = 4$ per group.

To determine if transplanted cells underwent osteogenic differentiation, *in vivo* expression using qRT-PCR was determined for human specific *Osteopontin* (OP); a major interfacial extracellular matrix protein found in bone and secreted by osteoblasts (McKee and Nanci 1996) and also for *Osteocalcin* (OC); an osteoblast specific gene (Lian, Stein et al. 1989) with an important role in osteoblast differentiation (Ryoo, Hoffmann et al. 1997). Results showed expression of human OP and OC in the transplanted mouse bones ($0.38 \cdot 2^{-\Delta C_t} \pm 0.08$ s.e.m and $0.85 \cdot 2^{-\Delta C_t} \pm 0.18$, respectively), which was greater than expression in e-CSC after

growth in osteogenic permissive media for 2 weeks ($0.05 \cdot 2^{-\Delta Ct} \pm 0.03$, $P < 0.05$ and $0.06 \cdot 2^{-\Delta Ct} \pm 0.04$, $P < 0.05$, respectively) and greater than the low/null basal expression level of the undifferentiated cells ($P < 0.05$) (**Fig.30A**). Western blot of the COL1A2 protein, missing in *oim* (Chipman, Sweet et al. 1993), showed a substantial amount of COL1A2 in transplanted *oim* (**Fig.30B**). This confirmed osteogenic differentiation of e-CSC to functional osteoblasts.

4.2.3. Discussion

I showed donor e-CSC preferentially engrafted at sites of active bone formation and repair, and differentiated to functional osteoblasts, shown by expression of late osteoblast genes and production of the COL1A2 protein. Osteoblast differentiation of blood hfMSC and bone marrow MSC after transplantation in *oim* has been previously shown by production of the missing COL1A2 protein (Li, Wang et al. 2007; Vanleene, Saldanha et al. 2011) and expression of osteoblast markers (Wang, Li et al. 2006; Guillot, Abass et al. 2008). Production of normal COL1A2 by blood hfMSC was associated with improvements in the mechanical and biochemical matrix properties of the *oim* bone, including an increase in lamella bone and some improvement in maturation of matrix apatite crystals, which led to reduced bone brittleness and 85% decreased femoral fracture incidence (Vanleene, Saldanha et al. 2011). I have verified that e-CSC, a source of clinically translatable stem cells, engrafted and differentiated *in vivo* to osteoblasts that contributed to collagen production. Whether these mice also had an improvement in therapeutic outcome was investigated next.

4.3. Transplantation of e-CSC in *oim* improves disease pathology

4.3.1. Introduction

OI, or brittle bone disease, is a debilitating inherited skeletal dysplasia characterised by multiple fractures and osteopenia and has a range of severities across the 11 known types depending on the causative mutation in collagen type I or genes involved in its biosynthesis (Stoss and Freisinger 1993; Cohen-Solal, Zylberberg et al. 1994; Dalglish 1997; Van Dijk, Pals et al. 2010). The current 'gold standard' treatment of OI is bisphosphonates, which temporarily improves bone strength by inhibiting bone resorption, but does not significantly improve growth or bone pain beyond a year (Letocha, Cintas et al. 2005) and does not reduce fracture incidence long term (Antoniazzi, Monti et al. 2010; Ward, Rauch et al. 2011). A number of studies have shown the engraftment and differentiation of transplanted cells in rodent models of OI, but have failed to investigate if the cell therapy also had therapeutic benefits (Pereira, O'Hara et al. 1998; Wang, Li et al. 2006; Li, Wang et al. 2007). Recent work from our lab has shown transplantation of hfMSC from first-trimester blood in *oim* mice led to a striking two-third reduction in long bone fractures as well as improvements in bone pathology and mechanical properties (Guillot, Abass et al. 2008; Vanleene, Saldanha et al. 2011). However, the collection of fetal blood is ethically and technically challenging (Chan, Kumar et al. 2008), therefore I aimed to test if the transplantation of e-CSC in *oim* could also improve the OI disease pathology (i.e. the manifestation of the disorder).

4.3.2. Results

To assess the therapeutic benefits of e-CSC when transplanted in *oim* mice, the fracture incidence compared to non-transplanted *oim* was calculated. Eight weeks after e-CSC were transplanted 11/20 *oim* mice (55%) had no long bone (femur, tibia and humerus) fractures whereas all non-transplanted *oim* mice ($n = 13$) had at least one long bone fracture or more (**Fig.31A**). The fracture incidence in long bones, calculated as the number of fractured tibia, femur and humeri over the total number of bones, was significantly reduced from 29.5% (23/78 total bones) in non-transplanted *oim* to 10.0% (12/120 total bones) in e-CSC transplanted *oim*. This corresponded to a 66% decrease in fracture rate ($X^2=21$, $P<0.001$) (**Fig.31B**). Parallel work in the lab found tail fractures were also reduced by 29.2% ($X^2=33.8$, $P<0.001$) in e-CSC transplanted *oim* compared to *oim* controls, suggesting bones throughout

the *oim* mice were more resistant to fractures after transplantation [Publication in progress. Data from Bhalraj Singh Kalirai].

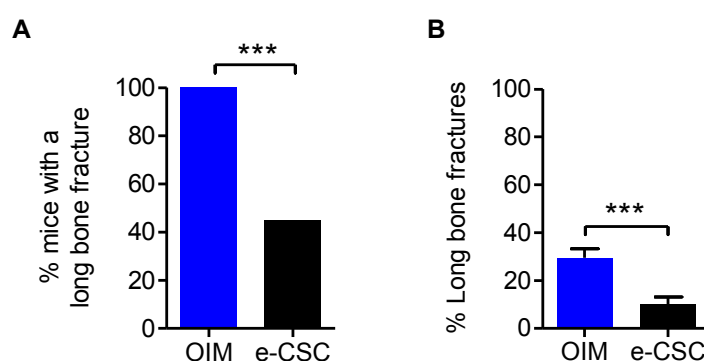


Figure 31 - e-CSC transplanted *oim* show reduced long bone fractures.

(a) Percentage of mice with at least one long bone fracture. (b) Fracture rate; total proportion of fractured femur, tibia and humerus over total number of bones per mouse. Data. *** $P < 0.001$ Fisher's exact test or Chi squared test respectively. Error bars are s.e.m. $n = 13$ *oim*, $n = 20$ transplanted mice.

The transplanted *oim* bones had improved bone plasticity and overall quality compared to *oim* controls (Fig.25) due to an increased post-yield strain as shown by the representative 3-point bending load-displacement curve (Fig.32A). Further investigation of the load-displacement data showed that the bones of e-CSC transplanted *oim* were not only more plastic, but also more ductile due to a reduction in stiffness in the pre-yield region and an increase in maximum deflection before fracture (Fig.32A). Bone stiffness of e-CSC transplanted *oim* was reduced by an average of 16% compared to non-transplanted *oim* femurs ($29.7 \text{ N/mm} \pm 2.1 \text{ s.e.m}$ vs. $35.3 \text{ N/mm} \pm 1.7 \text{ s.e.m}$ respectively, $P < 0.05$) (Fig.32B), while the maximum deflection was increased in transplanted compared to control *oim* by an average of 24% ($0.26 \text{ mm} \pm 0.02 \text{ s.e.m}$ vs. $0.21 \text{ mm} \pm 0.01$, $P < 0.05$ respectively) (Fig.32C). However, the maximal load sustained by transplanted and non-transplanted *oim* femurs prior to fracture was similar ($4.5 \text{ N} \pm 0.3$ vs. $4.8 \text{ N} \pm 0.3$) (Fig.32D). In contrast, wild type compared to *oim* bones were stiffer ($73.6 \text{ N/mm} \pm 2.0$, $P < 0.001$) (Fig.32B), with greater maximum deflection ($0.88 \text{ mm} \pm 0.07$, $P < 0.001$) (Fig.32C) and sustained higher loads before fracture ($14.0 \text{ N} \pm 0.5$, $P < 0.001$) (Fig.32D). Thus the material properties of bones from

transplanted *oim* were not intermediate between the properties of wild type and *oim* bone. Instead bones from transplanted *oim* were of similar strength to non-transplanted *oim*, but displayed greater plasticity and ductility which may explain their reduced fracture susceptibility. [Three-point bending experiments carried out with the help of Maximilien Vanleene in the Department of Bioengineering, Imperial College London].

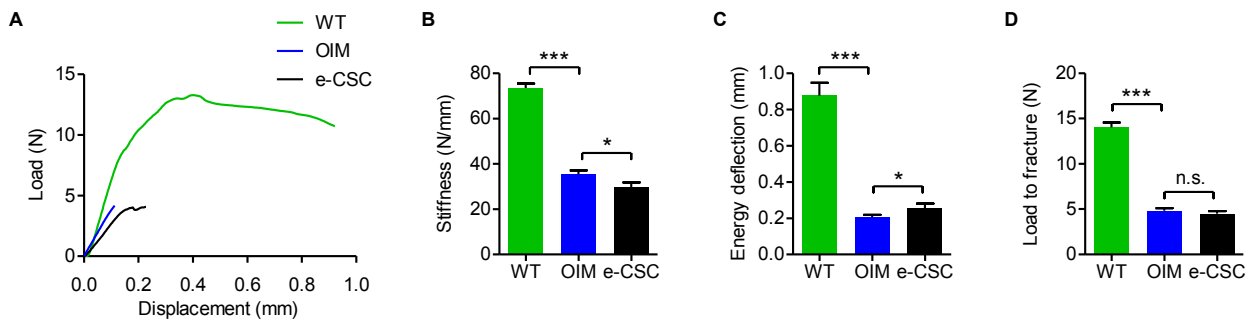


Figure 32 - e-CSC transplanted *oim* bones have greater ductility

(a) Three-point bending load (N) displacement (mm) curves shown up to the critical fracture point. (b) Bending stiffness of femurs (slope of the linear elastic deformation; N/mm). (c) Total energy deflected during bending (energy displacement extension to the point of fracture; mm). (d) Load to fracture (maximum force sustained by femur prior to fracture; N). All mice were 8 weeks old and either wild type (WT; green), non-transplanted *oim* (OIM; blue) or e-CSC transplanted *oim* (e-CSC; black). Data. n.s. not significant, * $P < 0.05$, *** $P < 0.001$; Student's *t*-test. Error bars are s.e.m. $n = 14$ WT, $n = 34$ *oim*, $n = 22$ transplanted mice.

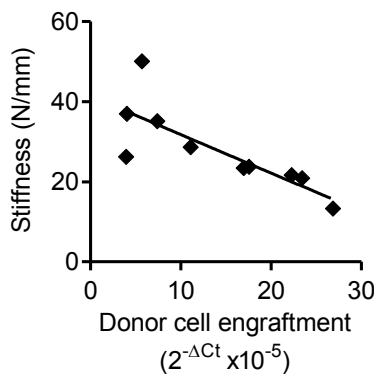


Figure 33 - Engraftment levels correlate with reduced *oim* bone stiffness.

Correlation per mouse of donor cell engraftment ($2^{-\Delta C_t}$) in the femoral epiphysis against the stiffness (N/mm) of the femur calculated from the three point bending test. Linear line of best fit given. $n = 10$ transplanted *oim*.

Engraftment in the femoral epiphysis was inversely correlated with bone stiffness ($R^2=0.64$, $y = -0.96x + 41.43$, $P < 0.01$ deviation from zero), indicating that bone flexibility increases with increasing numbers of donor cells (**Fig.33**).

Tibial length was unaffected by transplantation being similar in transplanted *oim* compared to control *oim* ($15.1\text{mm} \pm 0.1$ and $15.2\text{mm} \pm 0.1$ respectively), with both being shorter than wild type tibia ($17.0\text{mm} \pm 0.1$, $P < 0.001$) (**Fig.34A**). The diameter of the tibia at the mid-diaphysis was also similar in transplanted and control *oim* ($1.01\text{ mm} \pm 0.01$ and 1.05mm respectively) but less than in wild types ($1.28\text{ mm} \pm 0.02$, $P < 0.001$) (**Fig.34B**). Cortical bone thickness was less in *oim* than wild type ($17.9\% \pm 0.5$ and $20.4\% \pm 0.3$, $P < 0.001$ respectively), and was not increased in e-CSC transplanted *oim* ($17.2\% \pm 0.5$ s.e.m) (**Fig.34C**). [X-ray microradiography carried out with the help of J.H. Duncan Bassett in the Department of Medicine, Imperial College London].

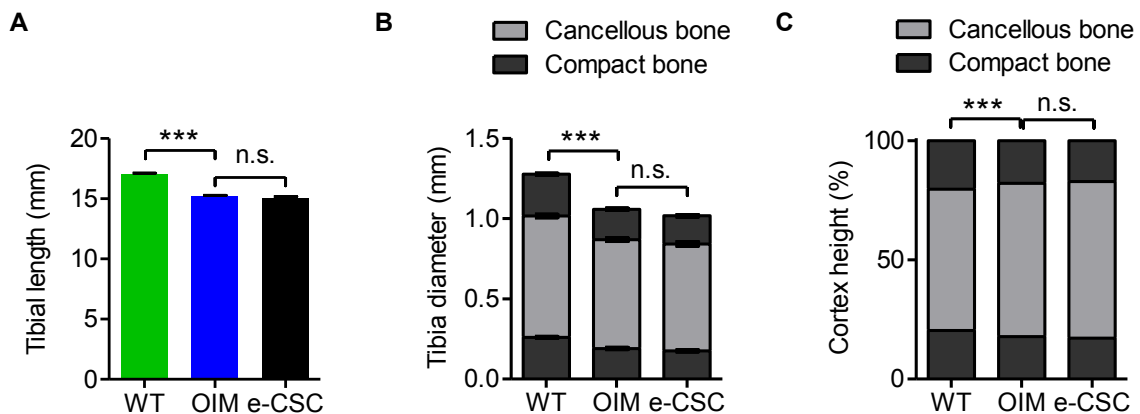


Figure 34 - Bone length and cortex thickness are not changed by transplantation.

(a) Tibia length (mm) of 8 week old wild type (WT; green), non-transplanted *oim* (OIM; blue) and e-CSC transplanted *oim* (e-CSC; black). (B) Tibia diameter (mm) at the mid shaft for each group across all osseous tissues including cancellous bone (light grey) and compact cortical bone (dark grey). (C) Height of tibial cortex (compact bone) at the mid shaft shown as a percentage of all osseous tissues in the tibia. Data. n.s. not significant, *** $P < 0.001$; Student's *t*-test. Error bars are s.e.m. $n = 12$ WT, $n = 20$ *oim*, $n = 19$ transplanted mice.

Cortico-endosteal bone formation rate (BFR) was greater in wild type mice compared to *oim* ($2.4 \mu\text{m}^3/\mu\text{m}^2/\text{day} \pm 0.21 \text{ s.e.m}$ vs. $1.54 \mu\text{m}^3/\mu\text{m}^2/\text{day} \pm 0.23$, $P < 0.05$ respectively) (**Fig.35A**). This difference resulted from an increased mineral apposition rate (MAR) at the cortico-endosteal interface ($2.6 \mu\text{m}/\text{day} \pm 0.3$ vs. $1.7 \mu\text{m}/\text{day} \pm 0.3$, $P < 0.05$ respectively) (**Fig.35B**), because there was no difference in mineralizing surface ($92\% \pm 2$ vs. $90\% \pm 2$, respectively) (**Fig.35C**). The MAR and mineralizing surface, however, did not differ between transplanted and non-transplanted *oim* mice (**Fig.35A, 35B and 35C**).

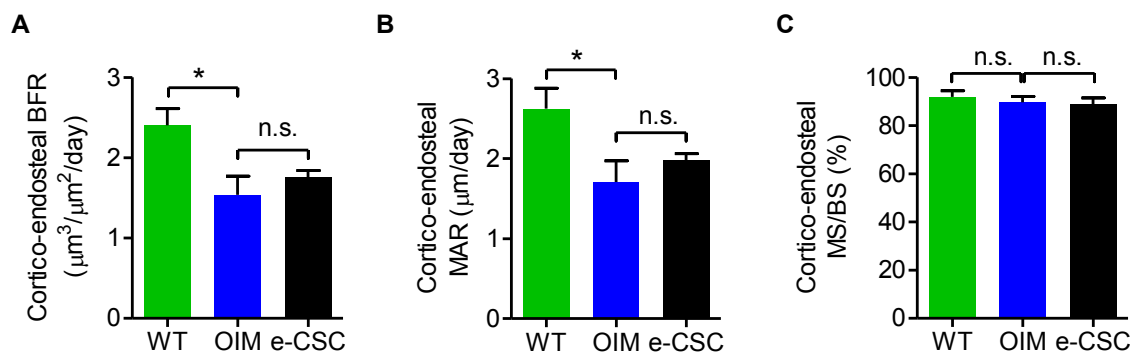


Figure 35 – Cortico-endosteal bone formation not affected by transplantation.

(a) Bone formation rate (BFR; $\mu\text{m}^3/\mu\text{m}^2/\text{days}$) for the endosteal cortex calculated from dual calcein labelling in 8 week old wild type (WT; green), non-transplanted *oim* (OIM; blue) and e-CSC transplanted *oim* (e-CSC; black). (b) Mineral apposition rate (MAR) in μm per day for the endosteal region of the tibial cortex. (c) Percentage mineralizing surface per total bone surface (MS/BS) for the cortico-endosteal. Data. n.s. not significant, * $P < 0.05$; Student's *t*-test. Error bars are s.e.m. $n = 6$ WT, $n = 5$ *oim*, $n = 7$ transplanted mice.

Total bone mineral content (BMC) of combined trabecular and cortical bone compartments did not differ between transplanted and non-transplanted *oim* mice, both of which BMC was reduced compared to wild-type ($P < 0.001$) (**Fig.36A** and **Fig.36B**). [X-ray microradiography and BMC analysis carried out with the help of J.H. Duncan Bassett]. Trabecular bone volume (bone volume/ total volume; BV/TV), however, was increased in transplanted compared to non-transplanted *oim* mice (4.1% of total bone volume ± 0.6 vs. $2.0\% \pm 0.4$ respectively, $P < 0.05$), but remained lower than in wild type mice ($11.9\% \pm 0.9$) (**Fig.36C**). [BV/TV data generated with Hassan Abdulrazzak].

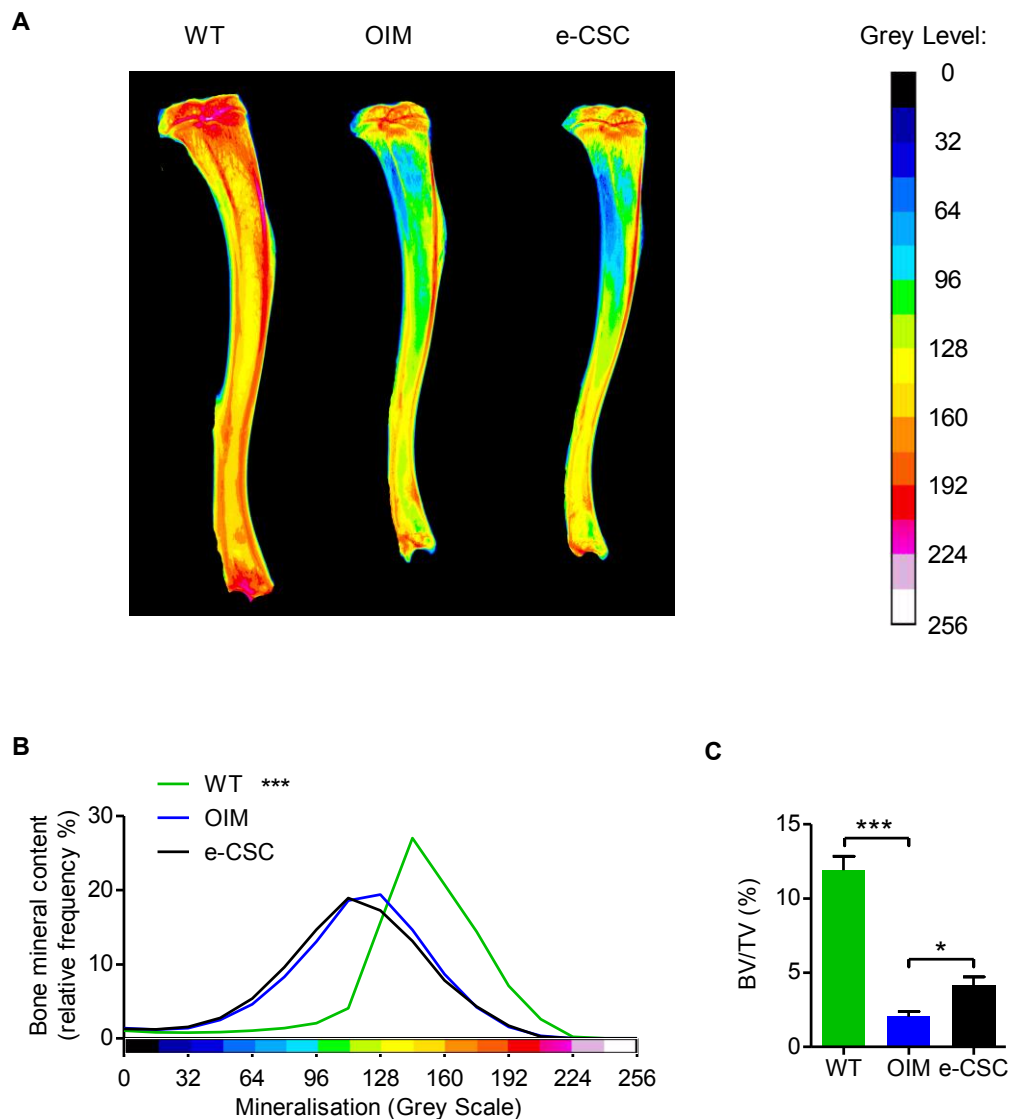


Figure 36 - Transplantation increased trabecular bone, but not bone mineral content.

(a) Digital X-ray images of tibia from 8 week old wild type (WT), non-transplanted oim (OIM) and oim transplanted with e-CSC (e-CSC), where grey level has been pseudocoloured according to a 16-colour palette; ranging from black (low mineralization density) to white (high mineralization density). (b) Bone mineral content shown as relative frequency (%) across 16 equal intervals of mineralisation density (0 grey scale: no mineralisation; 256 grey scale: maximum mineralisation). Data. *** $P < 0.001$; Kolomogorov-Smirnov test. $n = 12$ WT, $n = 20$ oim, $n = 19$ transplanted mice. (c) Percentage of trabecular bone volume in the tibial metaphysis calculated over the total bone volume. Data. * $P < 0.05$, *** $P < 0.001$; Student's t -test or. Error bars are s.e.m. $n = 5$ WT, $n = 6$ oim, $n = 6$ transplanted mice.

BFR in the trabecular bone was not significantly different between transplanted and non-transplanted *oim* ($0.58 \mu\text{m}^3/\mu\text{m}^2/\text{day} \pm 0.03$ s.e.m vs. $0.52 \mu\text{m}^3/\mu\text{m}^2/\text{day} \pm 0.03$ respectively) with wild type having a faster BFR than *oim* ($1.10 \mu\text{m}^3/\mu\text{m}^2/\text{day} \pm 0.1$ s.e.m, $P < 0.001$) (**Fig.37A**). Transplanted and control *oim* also had a similar trabecular MAR ($0.86 \mu\text{m}/\text{day} \pm 0.04$ s.e.m vs. $0.89 \mu\text{m}/\text{day} \pm 0.04$, $P < 0.05$ respectively), whilst wild type had a greater MAR than *oim* ($1.62 \mu\text{m}/\text{day} \pm 0.09$ s.e.m, $P < 0.001$) (**Fig.37B**). MS/BS did not differ between transplanted *oim*, non-transplanted *oim* and wild type mice (**Fig.37C**).

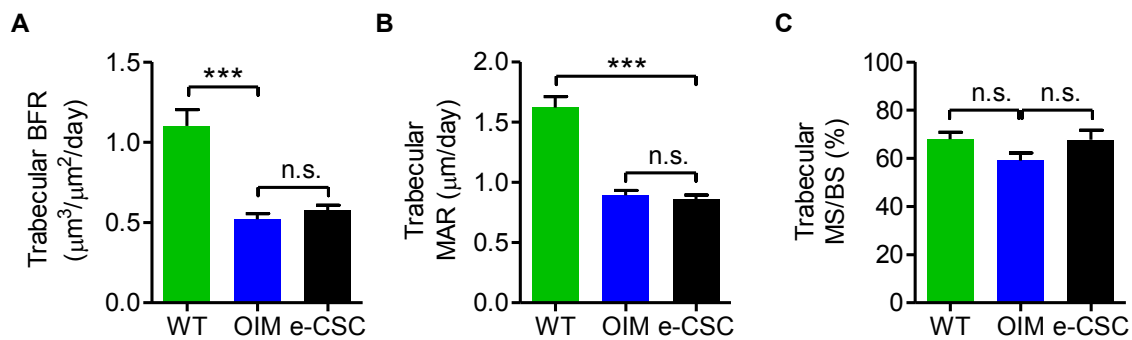


Figure 37 - Trabecular bone formation was not affected by e-CSC transplantation.

(a) Bone formation rate (BFR; $\mu\text{m}^3/\mu\text{m}^2/\text{days}$) for trabecular bone calculated from dual calcein labelling. (b) The MAR of the tibial trabecular bone. (c) MS/BS percentage for the tibial trabecular bone. Data. n.s. not significant, *** $P < 0.001$; Student's *t*-test. Error bars are s.e.m. $n = 6$ WT, $n = 5$ *oim*, $n = 7$ transplanted mice.

4.3.3. Discussion

Therapeutic benefits of e-CSC in the *oim* mice were evident from a two-third decrease in long bone fracture incidence compared to non-transplanted controls, following a single neonatal infusion of e-CSC. Fracture reduction in e-CSC transplanted mice was attributed to an increase in bone plasticity as well as greater bone ductility compared to non-transplanted controls. Changes to the bone mechanical properties of transplanted *oim* were most likely mediated by the exogenous cells since higher engraftment levels in bones correlated with decreased bone stiffness. Fracture reduction was comparable to that seen in *oim* transplanted with blood hfMSC (Guillot, Cook et al. 2008; Vanleene, Saldanha et al. 2011), although the

reduction in stiffness of e-CSC transplanted *oim* highlights a difference from the previous study whereby bone parameters of blood hfMSC transplanted mice were intermediate between wild type and *oim*.

The trabecular bone volume (BV/TV) of *oim* is lower than wild type mice due to the impaired osteoblast differentiation of *oim* (Kalajzic, Terzic et al. 2002; Li, Jiang et al. 2010), which results in a high numbers of preosteoblasts that support greater osteoclast bone resorption (Li, Jiang et al. 2010). *Oim* mice transplanted with e-CSC had a higher BV/TV than non-transplanted *oim*, despite bone formation rate remaining the same, which could be due to differentiation of exogenous cells to normal osteoblasts that better regulate bone remodelling.

BMC measured at the whole bone scale did not increase in *oim* after transplantation of e-CSC, despite changes in bone mechanical properties. Others have shown the importance of the collagen matrix organization on bone mechanical properties (Gupta, Schratter et al. 2005; Zebaze, Jones et al. 2011) and recently we have shown using nanoindentation that compared to wild type mice, *oim* have greater mineralization of a poorly organized matrix (Vanleene, Porter et al. 2012). Therefore exogenous cells may have affected the bone mineralization or improved organization of bone matrix collagen fibres in the *oim* bones at the microscopic matrix scale, potentially in response to production of normal COL1A2.

4.4. Transplantation of e-CSC upregulates endogenous ossification genes

4.4.1. Introduction

I have shown, along with others (Wang, Li et al. 2006; Li, Wang et al. 2007; Guillot, Abass et al. 2008; Vanleene, Saldanha et al. 2011), evidence in support of a direct contribution to tissue repair by donor cells via their osteogenic differentiation. However, transplantation may also affect the activity of endogenous cells via paracrine action (Dominici, Le Blanc et al. 2006; Phinney and Prockop 2007). Paracrine effects have been attributed to the release of various factors from the transplanted MSC, such as growth factors, anti-apoptotic factors, anti-inflammatory cytokines or pro-angiogenic molecules, but may also arise from cell-cell contacts that activate cell signalling or allow exchange of cytoplasmic components (Ankrum and Karp 2010). Evidence of a paracrine mechanism of action was recently reported in *oim* mice transplanted with adult BM-MSC, which had increased endogenous chondrocyte proliferation that led to increased lumbar vertebrae length (Otsuru, Gordon et al. 2012). I therefore aimed to investigate if transplantation of e-CSC affected endogenous osteogenesis or chondrogenesis activity in the *oim* mice.

4.4.2. Results

The mouse osteogenesis array (SABiosciences) was used to analyse changes in endogenous osteogenesis gene expression. The array results showed the femoral epiphysis of e-CSC transplanted mice compared to non-transplanted *oim* controls had a global increase in expression of genes involved in intramembranous ossification (**Fig.38**). For example, transplanted mice compared to *oim* controls had a 2.8 fold ($P<0.05$) increase in *Phex* and a 2.9 fold ($P<0.05$) increase in *Dmpl*; genes co-expressed by osteoblasts and osteocytes that regulate bone mineralization via FGFR signalling pathways (Lorenz-Depiereux, Bastepe et al. 2006; Martin, Liu et al. 2011). In addition, *Bgn*, which has a role in osteoblast differentiation and mineralisation of the matrix (Wang, Harimoto et al. 2010), and *Serpinh1*, which acts as a molecular chaperone in collagen biosynthesis (Christiansen, Schwarze et al. 2010) were upregulated in transplanted *oim* (2.6 fold, $P<0.05$, and 3.5 fold, $P<0.05$ respectively). This corresponded to higher expression of extracellular matrix proteins in transplanted mice, including a 3.2 fold increase in *Colla1* ($P<0.01$), involved in fibril formation of the abundant collagen type I (Stacey, Bateman et al. 1988; Dalglish 1997) (**Fig.38**).

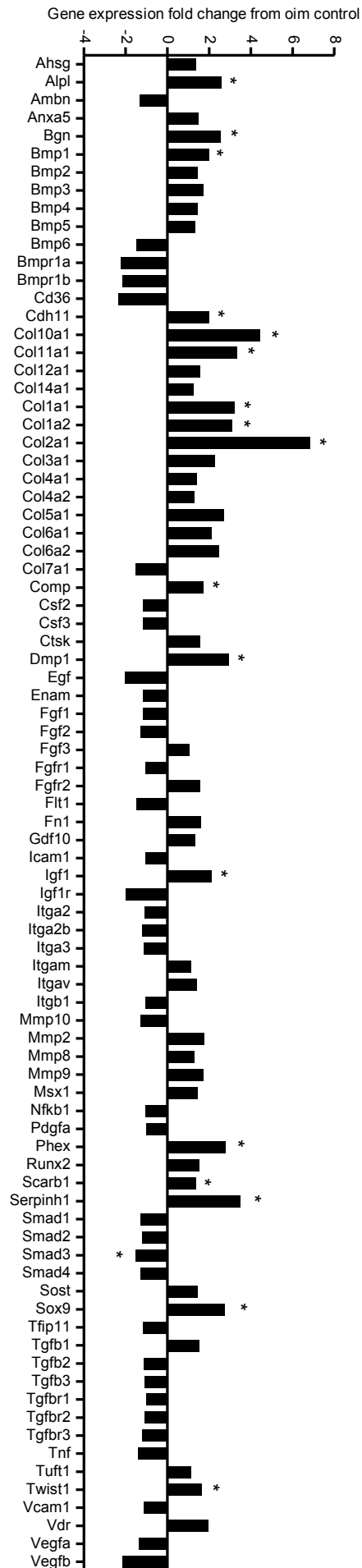


Figure 38 – Changes to endogenous osteogenesis and chondrogenesis.

*Fold changes in gene expression in the femoral epiphysis of oim transplanted with e-CSC when compared to non-transplanted oim controls, generated from a mouse osteogenesis PCR array (SABiosciences). Data. * $P < 0.05$. $n = 3$ per group.*

Array results were confirmed by qRT-PCR and showed *Runx2* expression, essential for differentiating mesenchymal stem cells to osteoblasts (Ducy, Zhang et al. 1997; Komori, Yagi et al. 1997; Otto, Thornell et al. 1997), was also increased in transplanted mice compared to non-transplanted *oim* ($2.1 \times 10^{-2} 2^{-\Delta Ct} \pm 0.2 \times 10^{-2}$ vs. $1.3 \times 10^{-2} 2^{-\Delta Ct} \pm 0.3 \times 10^{-2}$ respectively, $P < 0.05$) (**Fig.39**). However, the downstream transcription factor *Osterix*, required for osteoblast differentiation (Karsenty and Wagner 2002) was found to be similar in both e-CSC transplanted *oim* non-transplanted controls ($3.7 \times 10^{-2} 2^{-\Delta Ct} \pm 0.5 \times 10^{-2}$ vs. $3.3 \times 10^{-2} 2^{-\Delta Ct} \pm 0.7 \times 10^{-2}$ respectively). Whereas *Igf1*, which regulates both osteoblasts (Giustina, Mazziotti et al. 2008) and osteoclastogenesis via induction of *RANK-L* synthesis (Mochizuki, Hakeda et al. 1992; Niu and Rosen 2005) and is reported to increase linear bone

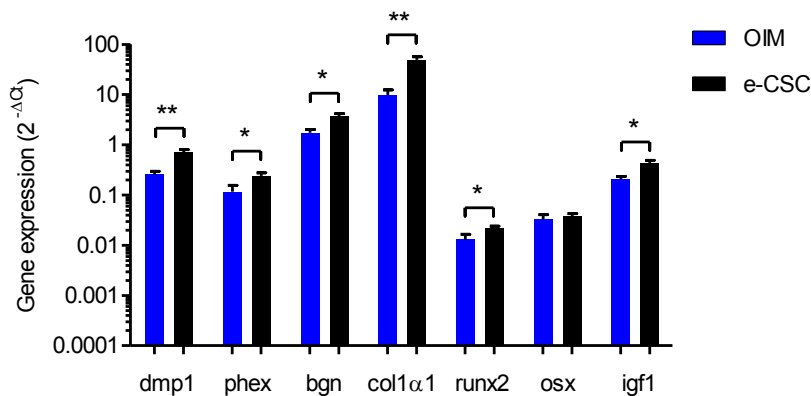


Figure 39 – Transplantation increases endogenous intramembranous ossification genes

Expression of genes involved in intramembranous ossification; *Dmp1*, *Phex*, *Biglycan* (*Bgn*), *Colla1*, *Runx2*, *Osterix* (*Osx*) and *Igf1*. Results are given as $2^{-\Delta Ct}$ normalised to mouse β -actin and *Hsp90ab1* for non-transplanted *oim* (OIM; blue) and *oim* transplanted with e-CSC (e-CSC; black). Data. * $P < 0.05$, ** $P < 0.01$; Student's *t*-test. Error bars are s.e.m. $n = 5$ *oim*, $n = 8$ transplanted mice.

growth (Yakar, Rosen et al. 2002) was upregulated in transplanted mice ($0.43 2^{-\Delta Ct} \pm 0.05$ for transplanted *oim* vs. $0.21 2^{-\Delta Ct} \pm 0.02$ for *oim* controls, $P < 0.05$). This suggests transplanted e-CSC may have an effect on the systemic hormones such as GH or PTH that regulate local production of *Igf1* in bone (Giustina, Mazziotti et al. 2008).

These results show that transplantation of e-CSC affects the levels of endogenous ossification genes. The expression of these genes correlated with their co-activators and trans-activation

targets as expected when mice were analysed on an individual basis. This included the co-activators *Dmp1* and *Phex* which were closely associated ($R^2=0.91$, $P<0.001$) (**Fig.40A**), as were the ECM genes *Col1a1* and *Bgn* ($R^2=0.93$, $P<0.001$) (**Fig.40B**). I also show a strong correlation between *Igf1* and *Bgn* expression ($R^2=0.89$, $P<0.001$), highlighting the importance of *Igf1* in regulating genes activated during intramembraneous ossification (**Fig.40C**).

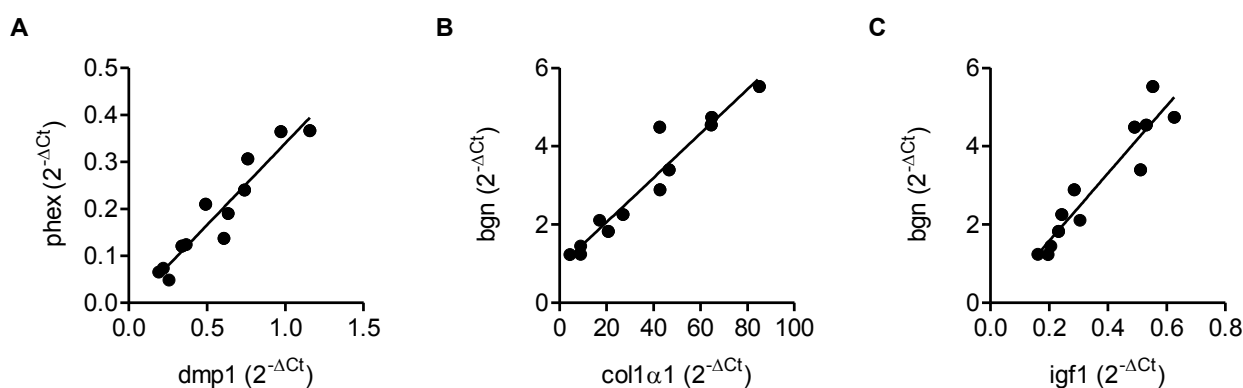


Figure 40 - Endogenous intramembraneous genes have linked expressions.

Correlation of gene expression per mouse (given as $2^{-\Delta Ct}$ normalised to mouse β -actin and Hsp90ab1) of (a) *Dmp1* and *Phex*, (b) *Col1a1* and *Bgn* and (c) *Igf1* and *Bgn*. Linear line of best fit given.

The PCR array also showed upregulation of genes involved in early stage endochondral ossification (**Fig.38**). These included a 2.7 fold increase of *Sox9* expression ($P<0.01$), 1.6 fold increase of *Twist1* ($P<0.05$), 6.8 fold increase of *Col2a1* ($P<0.01$) and 3.3 fold increase of *Col1a1* ($P<0.05$). In addition late hypertrophic chondrocyte differentiation genes were upregulated by 4.4 fold for *Col10a1* ($P<0.05$), 2.9 fold for *Dmp1* ($P<0.05$) and 2.6 fold for *Alkaline phosphatase* ($P<0.05$) (James, Stanton et al. 2010), whilst chondrocyte assembly gene *Comp* was also upregulated by 1.7 fold ($P<0.05$) (Haleem-Smith, Calderon et al. 2011) (**Fig.38**).

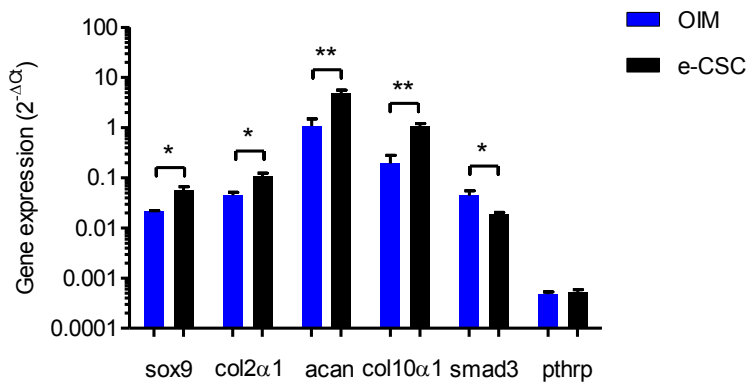


Figure 41 – Transplantation upregulates endogenous endochondral ossification genes.

Expression of genes involved in endochondral ossification; *Sox9*, *Col2α1*, *Aggrecan (Acan)*, *Col10α1*, *Smad3* and *Pthrp*. Results are given as $2^{-\Delta C_t}$ normalised to mouse β -actin and *Hsp90ab1* for non-transplanted *oim* (OIM; blue) and *oim* transplanted with *e-CSC* (*e-CSC*; black). * $P < 0.05$, ** $P < 0.01$; Student's *t*-test. Error bars are s.e.m. $n = 5$ *oim*, $n = 8$ transplanted mice.

The array results were again confirmed by qRT-PCR for the key transcription factor of chondrogenesis *Sox9* ($5.6 \times 10^{-2} 2^{-\Delta C_t} \pm 0.1 \times 10^{-2}$ s.e.m for transplanted *oim* vs. $2.1 \times 10^{-2} 2^{-\Delta C_t} \pm 0.01 \times 10^{-2}$ for *oim* controls, $P < 0.05$) (Bi, Deng et al. 1999). The subsequent upregulation in transplanted *oim* compared to *oim* controls was also confirmed for the *Sox9* transactivation target *Col2α1* (Bell, Leung et al. 1997; Lefebvre, Huang et al. 1997) ($0.11 2^{-\Delta C_t} \pm 0.01$ vs. $0.04 2^{-\Delta C_t} \pm 0.01$

respectively, $P < 0.05$) and key cartilage matrix component *Aggrecan* (Muir 1995; Tsuji, Shimada et al. 2000) ($4.8 2^{-\Delta C_t} \pm 0.8$ vs. $1.1 2^{-\Delta C_t} \pm 0.4$ respectively, $P < 0.01$), found in proliferating chondrocytes. However, expression of the *Sox9* target *Pthrp*, which inhibits chondrocyte maturation (Huang, Zhou et al. 2000; Huang, Chung et al. 2001; Amano, Hata et al. 2009) was similar in transplanted and non-transplanted *oim* ($5.2 \times 10^{-4} 2^{-\Delta C_t} \pm 0.7 \times 10^{-4}$ s.e.m vs. $4.8 \times 10^{-4} 2^{-\Delta C_t} \pm 0.5 \times 10^{-4}$, respectively). Importantly *Smad3*, which has been reported to inhibit maturation of chondrocytes through mediating *TGF-β* signalling (Ferguson, Schwarz et al. 2000) was downregulated in *e-CSC* transplanted mice compared to *oim* controls ($1.8 \times 10^{-2} 2^{-\Delta C_t} \pm 0.1 \times 10^{-2}$ vs. $4.4 \times 10^{-2} 2^{-\Delta C_t} \pm 1.1 \times 10^{-2}$ respectively, $P < 0.05$). This corresponded to increased expression in transplanted mice compared to non-transplanted *oim*, of chondrocyte maturation regulator *Col10α1* (Arias, Nakamura et al. 1997) ($1.0 2^{-\Delta C_t} \pm 0.2$ vs. $0.2 2^{-\Delta C_t} \pm 0.1$ respectively, $P < 0.01$) (**Fig.41**).

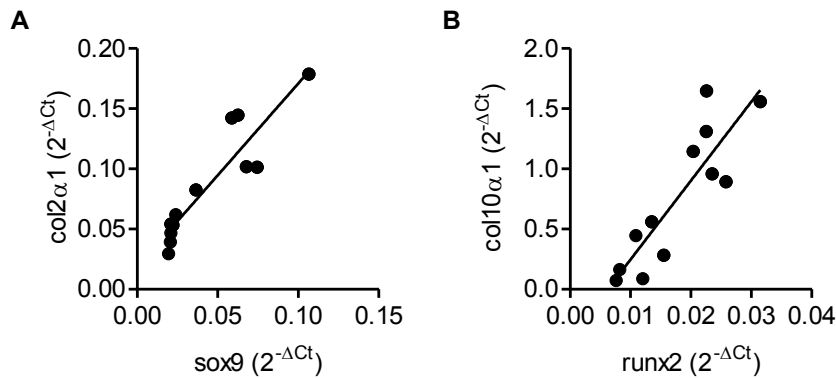


Figure 42 - Chondrogenesis genes have linked expressions.

Correlation of gene expression per mouse (given as $2^{-\Delta C_t}$ normalised to mouse β -actin and *Hsp90ab1*) of (a) *Sox9* and *Col2α1* and (b) *Col10α1* and *Runx2*. Linear line of best fit given.

As expected *Sox9* correlated positively with its transactivation target *Col2α1* ($R^2=0.84$, $P<0.001$) (**Fig.42A**) (Zhao, Eberspaecher et al. 1997). Likewise *Runx2* expression was closely linked with the expression of *Col10α1* ($R^2=0.76$, $P<0.001$), which is known to be transactivated by *Runx2* during chondrocyte maturation as well as osteoblast differentiation, (Zheng, Zhou et al. 2003) (**Fig.42B**).

Analysis of growth plate height confirmed previous findings (Guillot, Abass et al. 2008) that *oim* have larger growth plates than wild type mice ($153\mu\text{m} \pm 7$ s.e.m vs. $137\mu\text{m} \pm 9$ respectively, $P<0.05$). Interestingly, e-CSC transplanted *oim* had a growth plate that was substantially wider ($184\mu\text{m} \pm 6$) than the *oim* controls ($P<0.05$), primarily the result of a larger hypertrophic zone ($84\mu\text{m} \pm 4$ for e-CSC transplanted *oim* vs. $60\mu\text{m} \pm 7$ for *oim* controls, $P<0.01$) (**Fig.43A** and **Fig.43B**). When the relative proportions of the growth plate zones were calculated, the hypertrophic zone formed a larger proportion of the total growth plate in the transplanted mice than non-transplanted ($46.4\% \pm 1.1$ vs. $39.5\% \pm 3.2$ respectively, $P<0.05$), in which the hypertrophic zone was similar to wild type mice ($41.7\% \pm 1.2$) (**Fig.43C**). This finding was consistent with gene expression studies that showed transplanted *oim* had a 10:1 ratio of late hypertrophic chondrocyte marker *Col10α1* to proliferating chondrocyte marker *Col2α1*, whereas in non-transplanted *oim* the ratio was 4:1 ($P<0.05$).

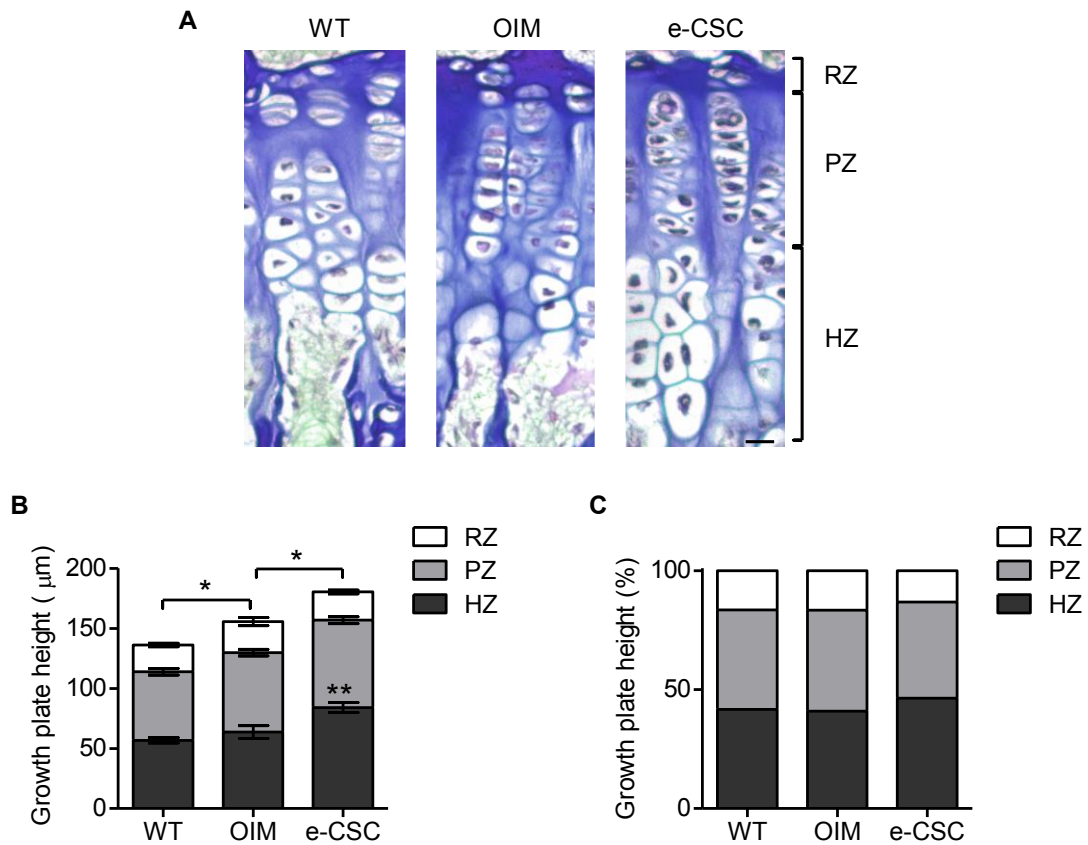


Figure 43 – Transplanted oim have more hypertrophic chondrocytes.

(a) Tibial growth plate architecture of 8 week old wild type (WT), non-transplanted oim (OIM) and transplanted oim (e-CSC). Chondrocyte matrix is stained blue and contains a reserve zone (RZ) of cells undergoing clonal expansion, a proliferative zone (PZ) containing columns of proliferating chondrocytes, and a hypertrophic zone (HZ) of differentiating chondrocytes. Scale bar is 20µm. $n = 3$ per group. (b) Average widths of the tibial growth plate RZ (white), PZ (light grey) and HZ (dark grey). Significance shown for total growth plate and HZ height. Data. * $P < 0.05$, ** $P < 0.01$; Student's t -test. Error bars are s.e.m. (c) Ratio of zones (RZ, PZ, and HZ) within the growth plate. $n = 5$ WT, $n = 5$ oim, $n = 14$ transplanted mice.

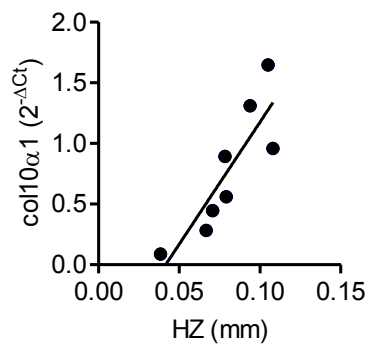


Figure 44 - Hypertrophic zone size corresponds to endogenous *Col10α1* expression.

Correlation of gene expression (given as $2^{-\Delta C_t}$ normalised to mouse β -actin and *Hsp90ab1*) of *Col10α1* and width of the hypertrophic zone (HZ) of the growth plate. Linear line of best fit given.

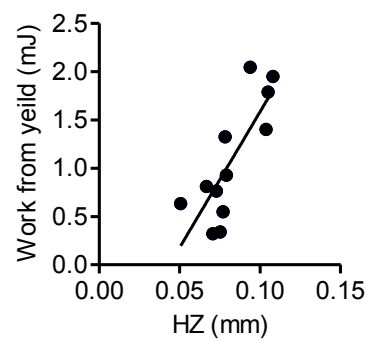


Figure 45 - Changes in chondrogenesis correlate with changes in bone plasticity.

Correlation of bone plasticity (total energy input from yield to fracture in millijoules) and width of the hypertrophic zone (HZ) of the growth plate. Linear line of best fit given.

As expected, there was a positive correlation ($R^2=0.75$, $P<0.01$) between *Col10α1* expression and the size of the hypertrophic zone of chondrocytes in the growth plate, shown per mouse (**Fig.44**). Importantly the size of the hypertrophic zone also positively correlated with increased bone plasticity in transplanted *oim* ($R^2=0.64$, $P<0.01$) (**Fig.45**). This suggests changes to the mechanical properties of the bone may be due to the paracrine effect of transplanted cells on endogenous chondrogenesis in addition to the direct differentiation of donor cells.

4.4.3. Discussion

I have shown changes in the expression of endogenous endochondral ossification genes in e-CSC transplanted *oim*, which corresponded with increased chondrocyte differentiation and may in part be responsible for the increased plasticity of the transplanted *oim* bones. Therefore my findings suggest e-CSC transplantation may have a paracrine effect on endogenous endochondral ossification. I have shown upregulation in transplanted *oim* of endogenous chondrogenesis genes including chondrogenesis regulator *Sox9* (Bi, Deng et al. 1999) and *Runx2*, implicated in chondrocyte maturation through *Col10α1* transactivation

(Zheng, Zhou et al. 2003). Whilst expression of chondrocyte maturation inhibitor *Smad3* (Ferguson, Schwarz et al. 2000) was downregulated in transplanted mice. These changes were associated with a larger zone of hypertrophic chondrocytes within the growth plate and more plastic bones. However, the physiological significance is questionable as there was no change in linear growth in the e-CSC transplanted *oim* compared to non-transplanted animals.

The larger growth plate of e-CSC transplanted *oim* compared to *oim* controls is in contrast to previous data with first trimester blood hfMSC that instead showed normalisation of growth plate height in prenatally transplanted *oim* compared to controls (Guillot, Abass et al. 2008). This may suggest different mechanisms of action between different transplanted cell sources. For example, recent work by Horwitz *et al.* in *oim* mice suggested different sources of cells contributed through different mechanisms when used in cell therapy, with non-adherent bone marrow cells differentiating to osteoblasts that produced normal collagen, whilst bone marrow MSC increased lumbar vertebrae length via paracrine mechanisms on chondrocyte proliferation at the growth plate, possibly through release of soluble growth factors (Otsuru, Gordon et al. 2012).

Transplanted *oim* also had upregulation of endogenous genes associated with osteoblast differentiation, including *Dmpl*, *Phex* and *Bgn* (Lorenz-Depiereux, Bastepe et al. 2006; Wang, Harimoto et al. 2010; Martin, Liu et al. 2011). *Oim* have lower BV/TV than wild type mice due to an impaired osteoblast differentiation (Kalajzic, Terzic et al. 2002; Li, Jiang et al. 2010). Therefore the increased BV/TV found in the transplanted *oim* may result from an indirect effect of the transplanted e-CSC on endogenous osteoblast differentiation. Others have also shown an effect of transplantation on endogenous osteoblast activity, for example transplantation of osteogenic differentiated MSC in SCID mice resulted in increased bone being produced by host cells (Zhou, Fan et al. 2012), and endogenous osteoblast numbers and bone formation was increased after transplantation of term placental stem cells in a SCID-rab mouse model of medullary myeloma-associated bone loss (Li, Ling et al. 2011).

From these findings I show that e-CSC has potential to treat OI and I hypothesise that e-CSC function in the *oim* model via a combination of direct osteoblast differentiation and indirect regulation of endogenous osteogenesis and chondrogenesis.

4.5. Improved donor cell chemotaxis translates to therapeutic benefits

4.5.1. Introduction

The clinical effectiveness of fetal cell therapy is challenged by low levels of engraftment in target organs. For example, I found engraftment of e-CSC and l-CSC reached a maximum of 3.9% and 3.6% respectively in *oim* bone marrow, which is similar to the maximum of 5% engraftment of blood hfMSC in *oim* (Guillot, Abass et al. 2008; Vanleene, Saldanha et al. 2011). The importance of the SDF1-CXCR4 chemotaxis axis has been shown for engraftment of hematopoietic stem cells (Tocci, Roberts et al. 2003) and for MSC migration to sites of tissue injury (Kucia, Jankowski et al. 2004; Son, Marquez-Curtis et al. 2006; Granero-Molto, Weis et al. 2009). Studies have shown adult MSC lack expression of CXCR4 (Von Luttichau, Notohamiprodjo et al. 2005), although this finding is not consistent (Ponte, Marais et al. 2007). Data from our group has shown all blood hfMSC express intracellular CXCR4, which is externalised when exposed to SDF1. Results showed increased *in vitro* migration of blood hfMSC to SDF1 as well as to *oim* bone and bone marrow when cells were first primed with SDF1. This was verified *in vivo* and showed SDF1 primed blood hfMSC had higher levels of bone engraftment compared to the non-primed cells [Data from Dr. Moschidou et al. published in (Jones, Moschidou et al. 2012)]. I subsequently investigated if the increased engraftment of donor cells in *oim* resulted in a better therapeutic outcome.

4.5.2. Results

By 8 weeks of age all control *oim* (16/16) had at least one long bone fracture (tibia, femur, humerus). Whereas only 27.8% (5/18 mice, $P < 0.001$) of 8 week old *oim* transplanted neonatally with human fetal MSC (hfMSC) from first trimester blood had a fracture. When cells were primed with SDF1 prior to transplantation, the percentage of mice with fractures was lower still, with only 18.2% (4/22 mice, $P < 0.001$ compared to *oim* controls) having a fracture (**Fig.46A**). The fracture incidence (number of fractured bones/total bones) of *oim* transplanted with SDF1 primed blood hfMSC was reduced compared to control *oim* (4.5% fractures vs. 27.1%, respectively, $\chi^2=32.8$, $P < 0.001$) and was lower than *oim* transplanted with unprimed cells (8.3% fractures, $\chi^2=18.3$, $P < 0.001$ compared to *oim* controls), although significance was not reached (**Fig.46B**). This corresponded to a 69% and 83% decrease in fracture rate in *oim* transplanted with unprimed and primed blood hfMSC respectively.

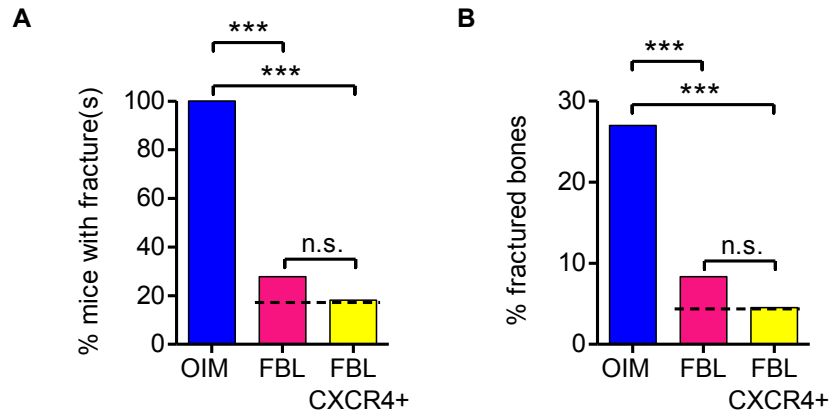


Figure 46 - Enhanced engraftment of FBL CXCR4+ has therapeutic benefits.

(a) Percentage of 8 week old oim mice with at least one long bone fracture following transplantation of human fetal blood MSC (FBL; pink) or human fetal blood MSC primed with SDF1, 30ng/ml for 1 hour (FBL CXCR4+; yellow) or not transplanted (OIM; blue). (b) Fracture rate; total proportion of fractured femur, tibia and humerus over total number of bones per mouse. Data. n.s. not significant, *** $P < 0.001$; Fisher's exact test or Chi squared test respectively. $n = 16$ oim, $n = 18$ FBL transplanted mice. $n = 22$ FBL CXCR4+ transplanted mice.

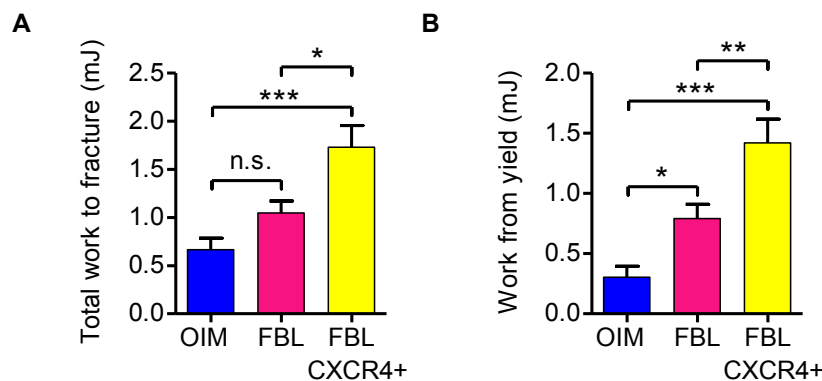


Figure 47 - Enhanced engraftment of FBL CXCR4+ improves bone mechanics.

(a) Mechanical 3-point bending data for overall bone quality shown by total energy input in millijoules to fracture and for (b) femoral plasticity shown by energy input in millijoules from yield to fracture. Results shown per mouse for non-transplanted oim (OIM; blue) and oim transplanted with human fetal blood MSC (FBL; pink) or human fetal blood MSC primed with SDF1, 30ng/ml for 1 hour (FBL CXCR4+; yellow). Data. n.s. not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; One-way ANOVA followed by Tukey's multiple comparison post hoc test. Error bars are s.e.m. $n = 15$ oim, $n = 15$ FBL transplanted mice. $n = 14$ FBL CXCR4+ transplanted mice.

At the bone mechanical level the total energy required to fracture determined with the 3-point bending test was used to measure the overall quality of the bone. Findings showed that *oim* transplanted with SDF1 primed hfMSC had increased bone quality compared to *oim* transplanted with unprimed cells ($1.7\text{mJ} \pm 0.2 \text{ s.e.m}$ vs. $1.0\text{mJ} \pm 0.1$, $P < 0.05$ respectively) and compared to *oim* controls ($0.7\text{mJ} \pm 0.1$, $P < 0.001$), indicating mice transplanted with primed hfMSC were more resistant to fracture (**Fig.47A**). To assess bone plasticity the post-yield strain was measured. Results showed increased bone plasticity of *oim* treated with SDF1 primed cells compared to *oim* transplanted with unprimed blood hfMSC ($1.4\text{mJ} \pm 0.2$ vs. $0.8\text{mJ} \pm 0.1$, $P < 0.01$ respectively) (**Fig.47B**). Both primed and unprimed groups had improved bone plasticity compared to the non-transplanted *oim* controls ($0.3\text{mJ} \pm 0.1$, $P < 0.001$ and $P < 0.05$ respectively). [Three-point bending experiments carried out with the help of Maximilien Vanleene in the Department of Bioengineering, Imperial College London]. These results show the improved chemotaxis of donor cells to *oim* bone translated to a greater improvement in the *oim* disease pathology.

4.5.3. Discussion

Our lab has developed a strategy to improve the therapeutic benefits of fetal cell therapy by increasing the migration of transplanted cells to damaged tissues by first priming donor cells with SDF1. Transplanted primed blood hfMSC had increased CXCR4 expression and were able to enhance the bone quality and plasticity of *oim* significantly more than unprimed cells, due to increased migration of donor cells to the target tissue. The improved bone parameters resulted in lower fracture rates compared to control *oim* and a trend towards less fractures compared to *oim* transplanted with unprimed cells. This demonstrates the importance of the SDF1-CXCR4 axis in the chemotactic response of blood hfMSC to bone and may be applicable to other cell types such as the e-CSC. These findings are in line with others that showed migration of MSC to fractures was CXCR4-dependent (Granero-Molto, Weis et al. 2009), whilst bone formation by MSC was inhibited if SDF1 is blocked (Kitaori, Ito et al. 2009)al 2009). Finally Lein *et al.* transduced bone marrow MSC with CXCR4 to increase cell surface expression and showed improved bone mass, strength and stiffness over non-transduced cells when transplanted in a model of osteoporosis (Lien, Chih-Yuan Ho et al. 2009).

Low engraftment levels is one of the main challenges of stem cell therapy, for example I found only 2-4% engraftment of e-CSC in the bone marrow. I showed e-CSC engraftment in *oim* bone directly correlated with improvements in bone mechanical parameters. Stem cells isolated from the fetal part of term chorionic villi have shown migration in response to SDF-1, PDGF, HGF and monocyte chemotactic protein-1 (MCP-1) (Abumaree, Al Jumah et al. 2012). Therefore the next step to enhance the therapeutic effects of e-CSC transplantation would be to apply the same principles as with the blood hfMSC and prime the cells with SDF1 then test if the migratory response is increased.

CHAPTER 5 – Discussion, Conclusions and Future Work

5.1. Chorionic stem cells are candidates for cell therapy

Identifying a safe, suitable and readily obtainable supply of therapeutic cells is a major challenge in regenerative medicine. My work characterises and compares two potential candidates; first trimester and term human placental chorionic stem cells (e-CSC and l-CSC respectively), available from discarded tissues after chorionic villous sampling /termination of pregnancy during the first trimester of pregnancy, or at term.

Human chorionic stem cells are relatively accessible and easy to isolate in large numbers, can be sourced from surplus tissues at routine prenatal diagnostic procedures or at term delivery and thus used without ethical restrictions for allogeneic and autologous applications (Abdulrazzak, Moschidou et al. 2010). I have shown that the chorionic stem cells have an intermediate phenotype between MSC and hESC, showing features of both, and can be safely transplanted intra-peritoneal in neonatal *oim* mice and subcutaneously in a murine skin wound model without forming tumours. Others have shown these cells have potential for clinical use as both e-CSC (Poloni, Maurizi et al. 2011) and l-CSC (Fariha, Chua et al. 2011) can maintain a normal karyotype after long term expansion. The lack of telomerase activity and hTERT expression in e-CSC (Poloni, Maurizi et al. 2011) and l-CSC (Fariha, Chua et al. 2011; Koo, Park et al. 2012) means undifferentiated cells are unlikely to transform into cancer cells when transplanted, although they do not self-renew and will senesce in culture. In addition e-CSC and l-CSC, like fetal liver MSC (Gotherstrom, Ringden et al. 2004), have low immunogenicity as they do not express HLA class II and their expression of HLA class I is low, which means an immune reaction is unlikely following allogeneic use (Le Blanc, Tammik et al. 2003).

In addition to testing the repair capacity of chorionic stem cells, steps towards establishing the safety of placenta-derived cells are underway with preclinical testing. No adverse clinical signs were seen after administration of allogeneic term placenta derived stem cells in NOD/SCID mice (Ramot, Meiron et al. 2009) or when delivered by intramuscular injection to mice with critical limb ischemia (Prather, Toren et al. 2009). Likewise, transplantation of e-CSC into nude mice did not cause tumour formation and cells repopulated depleted hematopoietic tissues (Spitalieri, Cortese et al. 2009). Although I did not test tumorigenicity

in immune-deficient mice, I found no tumour formation in any of the mice transplanted with either e-CSC or l-CSC.

Finally, placenta derived stem cells isolated at term have already been used safely in clinical trials, although fetal origin of the cells was not tested. For example, these cells were safely infused in a patient with acute myeloid leukemia (Brooke, Rossetti et al. 2009). Placental cell therapy has also been used safely in Phase I clinical trials by Celgene Cellular Therapeutics of 12 patients with Crohn's disease, utilising the immuno-modulatory properties of l-CSC, no major toxicities were reported (Health. 2013). Finally term placenta derived stem cells are currently in Phase III clinical trials by Israel's Pluristem Therapeutics for patients with critical limb ischemia and peripheral artery disease (Trounson, Thakar et al. 2011). Clinical trials using e-CSC as well as rigorous quality control standards are now needed to generate GMP-grade cells and test their long-term safety for use in allogeneic or autologous cell therapy, as well as for cell or tissue banking (Portmann-Lanz, Schoeberlein et al. 2006; Si, Zhao et al. 2011; Poloni, Maurizi et al. 2012).

5.2. The more primitive characteristics of e-CSC are advantageous for cell therapy

I showed *in vitro* that the phenotype of CSC changes during development, with e-CSC offering advantages relevant for cell therapy when compared with their term counterparts such as smaller size, faster growth kinetics, later senescence and greater osteogenic and adipogenic differentiation potency. These differences translate into greater repair potential of e-CSC over l-CSC in two different mouse models; skin wound repair and osteogenesis imperfecta.

I hypothesised that the ability of transplanted cells to cross the trans-endothelial barrier of blood vessels and home to the target tissue sites may be aided by a smaller cell, which has less resistance to pass through the endothelium. However, engraftment of donor cells in *oim* bone was not significantly different 8 weeks after transplantation of e-CSC or l-CSC therefore the larger size of l-CSC did not appear to be the limiting factor to its therapeutic effects. Furthermore others have shown l-CSC can successfully migrate through the blood-

brain barrier of rats (Kholodenko, Yarygin et al. 2012) and engraft in bone immune-deficient model of medullary myeloma bone disease (Li, Ling et al. 2011). It is also unlikely that the faster intrinsic growth kinetics of e-CSC over l-CSC were responsible for the differences in bone mechanical properties measured in the bone of e-CSC and l-CSC transplanted *oim*, since engraftment of donor cells was similar. Although, the increased expansion potential of e-CSC may be an advantage for its long term survival and functionality in host tissues.

Instead my results suggest the greater inherent osteogenic differentiation potency of e-CSC compared to l-CSC translated to better results when these cells were transplanted in *oim* mice. *In vitro* e-CSC showed enhanced osteogenic differentiation with higher levels of osteogenic gene expression and more collagen type I protein produced than l-CSC. Similarly *in vivo* transplanted e-CSC showed higher expression of human specific osteogenic genes than l-CSC indicating more osteogenic differentiation, which corresponded with greater bone plasticity. Analysis of e-CSC transplanted *oim* also showed the production of COL1A2 and late osteoblast markers by donor cells indicating their differentiation to functional osteoblasts. Therefore the differentiation potency of donor cells seems to be important for the effectiveness of cell therapy and may in part explain why e-CSC were better than l-CSC in improving the mechanical properties of *oim* bones.

Strategies to increase exogenous cell osteogenesis and increase production of COL1A2 could further improve the ability of e-CSC to treat OI, and could also be used to enhance the treatment success of l-CSC. In the literature various protocols have been tested to enhance osteogenic differentiation of MSC, which could be applicable. It has been found that spermine, found in platelet-rich plasma, increased *Runx2* expression in MSC and subsequently enhanced osteoblastogenesis, resulting in more maturation of osteoblasts (Guidotti, Facchini et al. 2013). Histone deacetylase (HDAC) inhibitors, which are modulators of cell fate, also promote osteogenesis capacity of adult MSC and could be used to pre-treat cells before transplantation in orthopaedic applications (Ye, Fan et al. 2012; Hu, Zhang et al. 2013). Whilst Vitamin MK-7 co-supplementation with vitamin D3 has been shown to increase osteogenesis and angiogenesis genes in adult MSC and has potential for use with cell therapy (Gigante, Bruge et al. 2012). Alternatively bone healing has been shown to be promoted when irradiated with low-intensity laser therapy (Ebrahimi, Moslemi et al.

2012), or electromagnetic field stimulation, which enhanced chondrogenesis and osteogenesis of adipose derived stem cells (Chen, Lin et al. 2012). Further research with adipose derived stromal cells showed that the CD90+ cells have greater osteogenic capacity, and could be a potential candidate for selection prior to transplantation (Chung, Liu et al. 2012).

5.3. Investigation of the trophic mechanisms of tissue repair

My findings provide some insight into the mechanisms of action of e-CSC showing their osteoblast differentiation in *oim* bone. I also show that transplanted e-CSC may have an effect on endogenous chondrogenesis, which was associated with increased bone plasticity. This reflects the findings of Horwitz *et al.* in *oim* mice, that showed bone marrow MSC indirectly increased endogenous chondrocyte proliferation at the growth plate (Otsuru, Gordon et al. 2012). The concept that transplanted cells can influence endogenous progenitor cells is not new and has been reported in a wide range of other models. For example, transplanted bone marrow stem cells increased host pancreatic β cells in a diabetic mouse model (Urban, Kiss et al. 2008) and increased angiogenesis via endogenous endothelial differentiation in a primate model of myocardial infarction (Yoshioka, Ageyama et al. 2005), whilst transplanted neural progenitors increased endogenous neurogenesis in a rat model of stroke (Jin, Xie et al. 2011). These effects have been shown to be mediated by direct exchange of cytoplasmic components across cellular contacts (Plotnikov, Khryapenkova et al. 2008; Cselenyak, Pankotai et al. 2010), but also release of soluble factors (Ankrum and Karp 2010).

Paracrine action by transplanted cells, such as release of trophic factors, has also been shown to aid tissue repair by reducing tissue inflammation, apoptosis and fibrosis and increasing tissue angiogenesis (Togel, Weiss et al. 2007; Zhang, Mal et al. 2007). My findings demonstrated tissue repair 7 days after transplantation of e-CSC into a skin wound mouse model, which was likely the result of beneficial paracrine factors affecting the endogenous differentiation and tissue repair. To test if soluble factors released by the transplanted cells were responsible for tissue repair, conditioned media from e-CSC could be applied instead to the cells.

Critically, more analysis of the paracrine effects of e-CSC in tissue regeneration in the skin wound model and *oim* is needed to understand how the cell phenotype translates to therapeutic benefits *in vivo* and to understand the fundamental mechanisms of action. In order to address this it would be essential to measure local and systemic growth factors produced by the transplanted cells (e.g. those that may affect bone and cartilage growth such as HIF-1, IGF-1, β -FGF, CTGF and PDGF), as well as to measure levels of inflammatory cytokines to determine if the exogenous cells had an anti-inflammatory response. Investigation into levels of proangiogenic molecules and quantification of blood vessels would indicate the effect of transplanted cells on angiogenesis. Whilst measurements of anti-apoptotic factors would show if transplanted cells promoted endogenous cell survival and proliferation, which would be beneficial to tissue repair (Phinney 2007; Dominici, Rasini et al. 2009). Comparing the number and activity of immune cells at sites of tissue repair (e.g. fractures) in transplanted and non-transplanted mice would also give an indication of the effect of transplanted cells on the immune response of the mice.

Adult BM-MSC are known to be immune-modulatory with suppression of T-cell activation and proliferation, natural killer cell cytotoxicity, B-cell maturation and induction of regulatory T-cells (Francois and Galipeau 2012). This property is an advantage for application in autoimmune diseases such as type I diabetes (Fiorina, Jurewicz et al. 2009), multiple sclerosis (Martino, Franklin et al. 2010; Tyndall 2010), rheumatoid arthritis (Bouffi, Djouad et al. 2009) and graft versus host disease (Trento and Dazzi 2010; Francois and Galipeau 2012), although obtaining sufficient numbers of cells is difficult. Immune-suppressive properties have been demonstrated in both term and first trimester placenta derived stem cells (Cargnoni, Gibelli et al. 2009; Karlsson, Erkers et al. 2012; Lee, Jung et al. 2012; Poloni, Maurizi et al. 2012). However, a comparative study of the two CSC populations with adult BM-MSC is now needed to assess if a more primitive phenotype is an advantage or disadvantage in terms of immunosuppressive capacity.

5.4. Enhanced effectiveness of cell therapy by altering stem cell properties

I showed that the effects of cell therapy in *oim* could be improved by priming blood hfMSC with SDF1 prior to transplantation, which upregulated the expression of external CXCR4 and subsequently led to increased homing of donor cells to bone. Preliminary tests have shown e-CSC does not respond in the same way to SDF-1 priming, with external CXCR4 levels remaining the same after incubation with SDF-1. Subsequent ongoing work within the group is investigating if increasing the number of cells transplanted results in increased engraftment within the *oim* bone.

The CXCR7 receptor also plays an important role in chemokine SDF-1 mediated migration and is required for cell growth, survival, endothelial adhesion and transendothelial migration (Burns, Summers et al. 2006; Mazzinghi, Ronconi et al. 2008). CXCR7 may therefore be an additional target, alongside CXCR4, to increase stem cell homing to the site of tissue repair. For example, hypoxic pre-conditioning has been shown to upregulate CXCR4 and CXCR7 in bone marrow MSC, which led to improved migration, adhesion and survival of MSC (Liu, Xue et al. 2010) and improved therapeutic potential of MSC (Hu, Yu et al. 2008; Rosova, Dao et al. 2008). This can also be achieved using chemicals or acidosis that induce hypoxia-inducible factor-1, desferrioxamine and cobalt chloride (Liu, Xue et al. 2010; Cencioni, Melchionna et al. 2011). Alternatively a combination of gene and cell therapy could be used to upregulate expression of the *Cxcr4* and *Cxcr7* genes in the donor cells. The *Cox2* gene could also be targeted, due to its role in MSC recruitment (Lau, Kothari et al. 2013). The successful transfer of genes into CSC using vectors has already been carried out in l-CSC (Han, Li et al. 2012), but has yet to be determined for e-CSC.

5.5. Reprogramming e-CSC to pluripotency for wider cell therapy and tissue engineering applications

My findings showed e-CSC endogenously expressed basal levels of the key pluripotency genes required for reprogramming; *Klf4*, *Sox2* and *c-Myc* and the *variant 1* isoform of *Oct4A*, critical for self renewal of pluripotent cells (Atlasi, Mowla et al. 2008; Ryan, Pettit et al. 2011), albeit at a considerably lower level of expression than hESC.

Induced pluripotent stem cells (iPSC) have raised the possibility to generate unlimited patient specific pluripotent stem cells for use in a wide range of regenerative medicine settings, as well as for investigation of disease pathologies and drug screening. They were first generated using retroviral vectors to ectopically express four key pluripotency genes (*Oct4A*, *Klf4*, *Sox2* and *c-Myc*) from fibroblasts (Takahashi and Yamanaka 2006). However, viral integration methods have low efficiency, risk viral induced tumorigenicity from random integration and lack stability of the phenotype, whilst non-integration methods show promise but remain both inefficient and technically challenging (Mukherjee and Thrasher 2011).

Recent work from Dr Pascale Guillot's group has shown that first and second trimester human amniotic fluid stem cells (AFSC) can be reprogrammed to functional pluripotency without ectopic expression of transcription factors (Moschidou, Mukherjee et al. 2012; Moschidou, Mukherjee et al. 2012), due to the endogenous expression of *Oct4A*, *Sox2*, *Klf4* and *c-Myc* in the parental population. This means AFSC are ideal to generate patient specific iPSC, which can be rapidly grown to sufficient numbers for application in cell therapy or tissue engineering, whilst maintaining an identity that can differentiate to all the lineages of the three germ layers (Li, Zhou et al. 2009; Ye, Chang et al. 2009; Galende, Karakikes et al. 2010). Induced pluripotent AFSC were generated by expansion of cells on Matrigel in low growth factor media supplemented with β -FGF and HDAC inhibitor valproic acid (VPA) (Moschidou, Drews et al. 2012; Moschidou and Guillot 2012; Moschidou, Mukherjee et al. 2012; Moschidou, Mukherjee et al. 2012). In combination with environmental cues HDAC inhibitors upregulate gene transcription by increasing acetylation of histones and causing chromatin relaxation (Han, Lu et al. 2007). Identifying other clinically available sources that can be reprogrammed using only small molecules will further advance the movement of this field to the clinic and maximise the tissue types that can be banked for later therapeutic use.

As a small side project with a BSc student [Hyein Chun] we investigated if the human chorionic stem cells could also be grown under hESC conditions and if, like the AFSC, had potential to be reprogrammed using small molecule supplementation. Our findings so far show that e-CSC, but not l-CSC, can survive and be expanded on Matrigel in low growth factor media supplemented with β -FGF (Nutristem, Sigma). The e-CSC formed compact

colonies on top of large fibroblastic flat colonies (Appendix 3A), as was the case for AFSC (Moschidou, Mukherjee et al. 2012; Moschidou, Mukherjee et al. 2012). Various epigenetic modifying chemical compounds were supplemented into the hESC media and e-CSC were assessed for cell survival and pluripotency gene expression. The HDAC inhibitor VPA (1mM) or the DNA methyltransferase inhibitor RG108 (100µM) compounds upregulated basal expression levels of *Oct4A*, *Sox2* and *Nanog* in the e-CSC with minimal cell death (Appendix 3B) [Supervised work of Hyein Chun, December 2012]. Further analysis, including teratoma formation, is now required to determine if e-CSC have been reprogrammed to full pluripotency using this method.

If successful it will be important to test if the reprogrammed cells can be fully differentiated to c-MYC negative cells that can be safely applied in regenerative medicine and to assess their differentiation potency. For applications in autoimmune diseases determining if the reprogrammed cells could be reverted to an MSC phenotype with immune-suppressive properties would provide a practical alternative to adult BM-MSC. These reprogrammed cells could also have wide applications in gene targeted therapy, once the feasibility and safety of vector gene transfer in the reprogrammed cells has been assessed, since they do not contain any previous viral integration. This greatly broadens the opportunities to use e-CSC safely in cell based therapies to treat a wide variety of disease pathologies as well as in tissue engineering. In combination with the fact e-CSC can be harvested during pregnancy, without causing harm to the fetus, these cells offer huge benefits over using adult bone marrow for use in prenatal and neonatal autologous or allogeneic therapy.

5.6. Conclusion

My work investigated the evolution of the stemness characteristics of chorionic stem cells during fetal development and demonstrates the therapeutic benefits of e-CSC to treat OI in a mouse model. Strategies to improve the therapeutic effects of these cells in OI are required before human trials if cell therapy using first trimester chorionic stem cells can become a standardized treatment for OI.

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Appendix 1 – Controls for characterisation of MSC phenotype in CSC

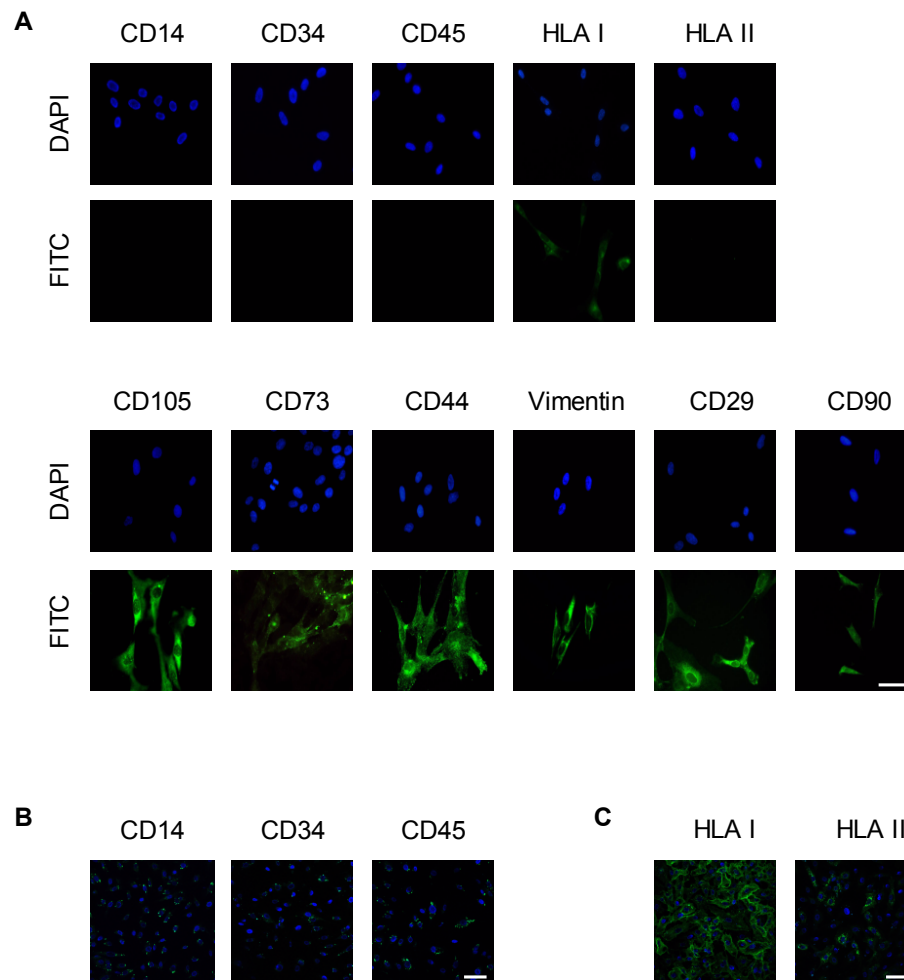


Figure 48 – Controls used for MSC characterisation.

(a) Human first trimester fetal bone marrow mesenchymal stem cells are negative for CD14, CD34, CD45 and HLA II, with some expression of HLA I and were used as a positive control for expression of CD105, CD73, CD44, Vimentin, CD29 and CD90. (b) Human umbilical vein endothelial cells were used as a positive control for expression of CD14, CD34 and CD45. (c) Interferon gamma stimulated human umbilical vein endothelial cells were used as a positive control for expression of HLA-ABC (HLA I) and HLA-DP, DQ, DR (HLA II). All genes stained with FITC (green). Nuclei stained with DAPI (blue). All scale bars are 100 μ m.

Appendix 2 – Controls for investigation of PGC origin in CSC

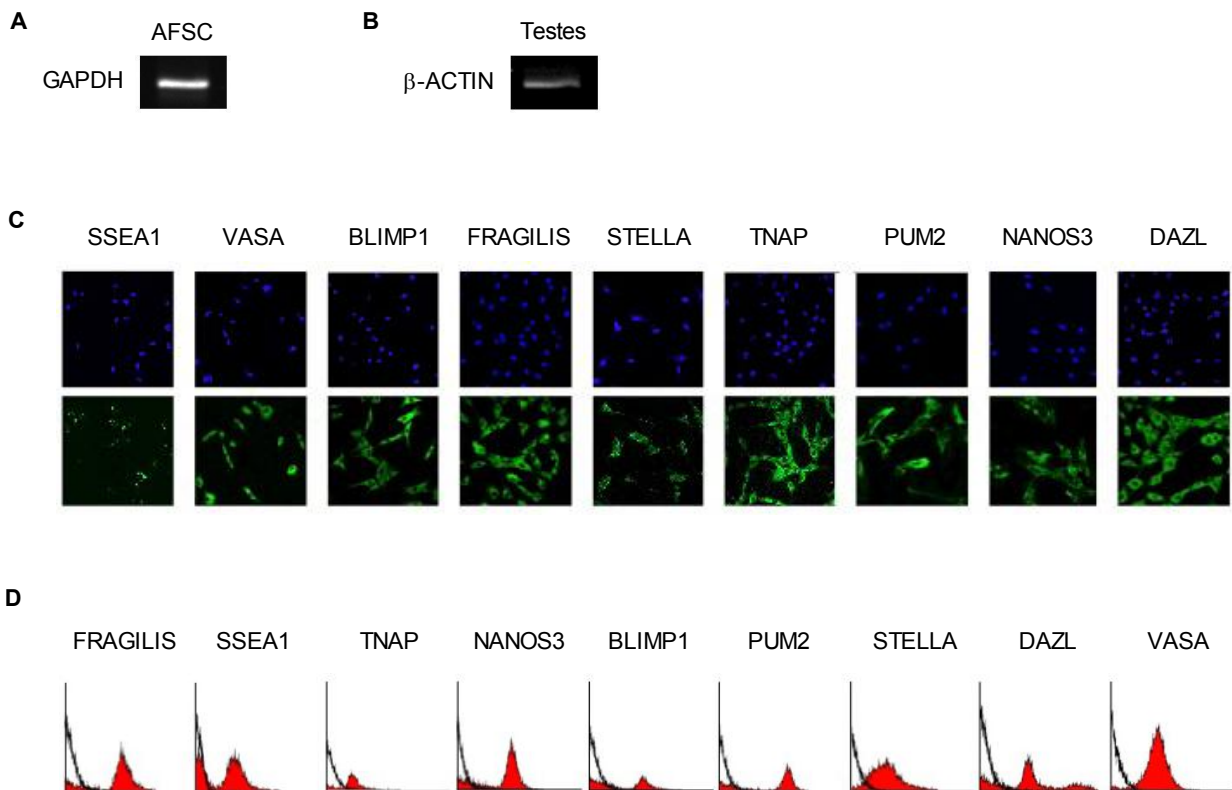


Figure 49 – Positive controls for PGC markers.

(a) Gene expression of loading control *Gapdh* for human second trimester amniotic fluid stem cells (AFSC), which were used as a positive control of *c-kit* expression in Figure 20. (b) Gene expression of loading control β -actin for mouse testes, which were used as a positive control of *Vasa* expression in Figure 20. (c) Representative images of confocal immuno-fluorescence for protein expression of PGC markers; *SSEA1*, *VASA*, *BLIMP1*, *FRAGILIS*, *STELLA*, *TNAP*, *PUM2*, *NANOS3* and *DAZL* using positive control first trimester AFSC, which exhibits characteristics of PGC (Moschidou, Mukherjee et al. 2012) (d) Representative flow cytometry for PGC markers in positive control first trimester AFSC (isotype control in black).

Appendix 3 – Reprogramming e-CSC using small molecules

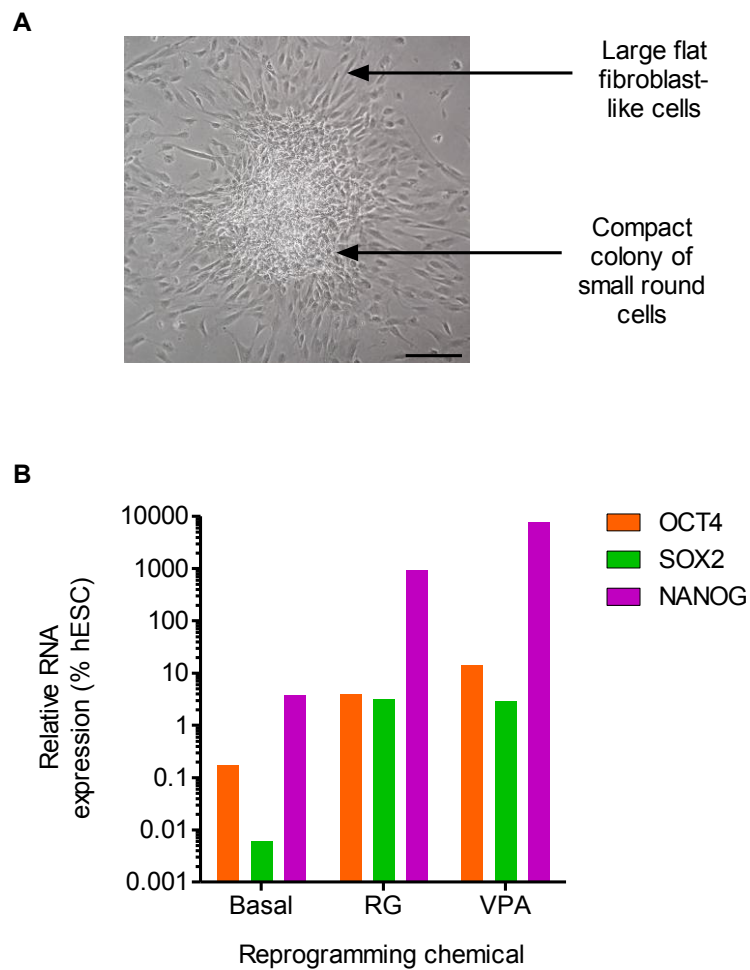


Figure 50 – Small molecule supplementation upregulates pluripotency genes

(a) Photograph of morphology of e-CSC after 1 month of growth and passaging in low growth factor medium supplemented with β -FGF. Scale bar is 200 μ m. (b) Quantitative real time PCR results showing increase in expression of Oct4A (orange), Sox2 (green) and Nanog (pink) following 7 days growth of e-CSC in low growth factor media supplemented with β -FGF and small molecules; RG108 (RG) or valproic acid (VPA), or without media supplementation (Basal). Expression is normalised to Gapdh and given as a percentage relative to human embryonic stem cell levels (hESC).