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Ectopic Bone Formation by Mesenchymal Stem Cells Derived from Human Term Placenta and the Decidua

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Abstract

Mesenchymal stem cells (MSCs) are one of the most attractive cell types for cell-based bone tissue repair a derived MSCs and maternal-derived MSCs have been isolated from chorionic villi of human term placenta a attached to the placenta following delivery, respectively. Chorionic-derived MSCs (CMSCs) and decidua-deri generated in this study met the MSCs criteria set by International Society of Cellular Therapy. These criteria to plastic; (ii) >90% expression of CD73, CD105, CD90, CD146, CD44 and CD166 combined with <5% expr and HLA-DR; and (iii) ability to differentiate into osteogenic, adipogenic, and chondrogenic lineages. *In vivo* implantation into SCID mice showed that both bromo-deoxyuridine (BrdU)-labelled CMSCs and DMSCs whe with hydroxyapatite/tricalcium phosphate particles were capable of forming ectopic bone at 8-weeks post-tra Histological assessment showed expression of bone markers, osteopontin (OPN), osteocalcin (OCN), biglyc sialoprotein (BSP), and also a marker of vasculature, alpha-smooth muscle actin (α-SMA). This study provid CMSCs and DMSCs as cellular candidates with potent bone forming capacity.

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Introduction

Mesenchymal stem cells (MSCs), which are also referred to as multipotent stromal cells, are found in many t capable of multipotent differentiation, allowing them to contribute to bone regeneration and repair since MSC

differentiate into osteocytic lineages [1]. Moreover, MSCs are readily isolated, their numbers can be greatly e cryopreserved for later use, and importantly, they display low immunogenicity, are immunomodulatory and ha profile. According to the International Society for Cellular Therapy (ISCT), MSCs must; (i) adhere to untreate express CD105, CD73, and CD90 but not CD34, CD14, CD19, CD11b, CD79α or HLA-DR, and (iii) differenti adipogenic and chondrogenic lineages *in vitro* [2].

The human term placenta is an abundant, readily accessible and non-controversial source of MSCs. MSCs I from fetal derived placental tissues including the amnion, chorion and chorionic villi, and from maternal deriv attached to placental tissue following delivery i.e. the *decidua parietalis* and *decidua basalis* [3–7]. The perip placenta on the maternal side that is in contact with the uterine wall (called the basal plate) comprises the ch side, and maternal *decidua basalis* on the other. Following delivery of the placenta, the *decidua basalis* rema maternal side of the placenta. Thus, careful preparation and characterization needs to be carried out to confi of *decidua basalis* MSCs (DMSCs) and the fetal origins of the chorionic villous MSCs (CMSCs). As such, in stipulated by the ISCT, Parolini et al. proposed that CMSCs should have <1% maternal cells in the populatio issue of the presence of maternal cells in human placental MSCs cultures was reviewed recently [10]. There study was to isolate and characterize CMSCs and DMSCs according to the criteria described above and to c fetal and maternal origins of these cells. This characterization was an essential prerequisite to the use of CM *vivo* assays.

While studies have reported osteogenesis by CMSCs and DMSCs *in vitro*, bone formation *in vivo* has not be studies are essential for evaluating the functional capacity of CMSCs and DMSCs and their potential for clini Therefore, we initiated the study using a mouse model of ectopic bone formation to explore the possibility tha CMSCs and DMSCs were capable of regenerating ectopic bone-like structure *in vivo*.

The orthotopic bone formation assay is commonly used to study osteogenesis *in vivo*. Compared to the orth ectopic bone forming assay has unique advantages since there is no requirement for bone cytokine stimulati interaction with endogenous bone-forming cells [11]. In addition, a variety of ectopic locations can be used fo including subcutaneous and intramuscular sites and the kidney capsule [11]. Subcutaneous implantation is th experimental model of ectopic bone formation. Mouse models are preferable and most widely used due to th folds that can accommodate large implants, and the availability of immunodeficient mice that will accept impl Another important consideration is the lack of naturally occurring bone-forming stem cells within the intraderm therefore newly-formed bone can be confidently attributed to the exogenous stem cells. The most pertinent c subcutaneous implantation is the lack of robust bone growth which may be due to poor blood flow. However, formation can be stimulated by incorporating hydroxyapatite and tricalcium phosphate (HA/TCP) together wi are currently used as bone graft substitutes, are biocompatible and form bonds between bone and ceramic i second and principal aim of this study was to evaluate *in vivo* bone formation capacity of CMSCs and DMSC subcutaneous implantation together with HA/TCP.

Materials and Methods

Tissue collection

Placental samples were collected from healthy women with normal pregnancies following elective Caesarea delivery at term (n = 6). The placental tissue had no obvious signs of calcification, infarcts or meconium stair were women who smoked or had a twin or triplet pregnancy, drug dependency, intrauterine infection, prolong membranes or placental abruption. Informed written consent was obtained from all participants before delive approved by the Royal Women's Hospital Human Research Ethics Committee.

Isolation of CMSCs

CMSCs were isolated using the explant method as described previously [7] with the following modifications. made through the fetal membranes near the umbilical cord insertion site and 1 g of chorionic villous tissue w approximately 1–2 cm below the chorionic plate. Pieces of chorionic tissue with typical villous morphology w gauge needle under a dissecting microscope to remove non-villous tissue. Cleaned villi were finely diced an trypsin for 40 min at 37°C. The trypsin was inactivated with FBS and tissues were washed in PBS. The diges in Amniomax C100 complete medium (Life Technologies) in 25 cm^2 tissue culture flasks maintained at 37°C CO_2 incubator. After 7 days, villous tissues were removed from the flask and the adherent cells arising from were grown until at least 80% confluent before expanding to reach P5.

Isolation of DMSCs

We have previously reported the isolation of DMSCs from the *decidua basalis* adhering onto the maternal si
About eight grams of placental tissue was dissected from the basal plate, washed four times in PBS, finely m
trypsin (0.25%; Life Technologies, CA, USA) and DNAse 1 (50 μg/mL; Worthington, NJ, USA) at 4°C overnig
(FBS; Thermo Scientific, MA, USA) was added to inactivate the trypsin and the digest was centrifuged at 20(
pelleted tissue was digested in type 1 collagenase (10 mg/mL; Worthington) and DNAse 1 (50 μg/mL, Worth
37°C and strained through a 100 μm stainless steel sieve. The filtrate was layered over Histopaque (Sigma-A
separated by density gradient centrifugation at 400*g* for 30 min. Mononuclear cell layers containing the DMS
centrifuged at 200*g* for 5 min. DMSCs were maintained in α-MEM medium (Sigma-Aldrich) with 10% FBS, p
(100 U/mL and 100 mg/mL, respectively; Life Technologies) and 2 mM L-glutamine (Sigma-Aldrich). P0 DMS
after reaching 80% confluence and cells were expanded up to P5.

Fluorescence in situ hybridisation (FISH)

FISH was used to determine whether the DMSCs were maternal and the CMSCs fetal in origin as described
Briefly, term placentae delivered from pregnancies carrying male babies (n = 3) were used to prepare DMSC
were lifted with TrypLE Express, washed in Hank's buffered saline solution (HBSS; Life Technologies) and pl
coated glass slides (Thermo Scientific). After fixing in 3:1 methanol to acetic acid solution at room temperatu
hybridized with labelled chromosome X (Spectrum Green) and chromosome Y (Spectrum Orange) probes (A
USA). Approximately 200 cells per slide were examined.

Flow cytometry

To determine whether expanded cells expressed positive and negative markers characteristic of MSCs, cells
cytometry for CD73, CD105, CD90, CD146, CD44 and CD166 and the absence of CD45, CD19 and HLA-DR
with each of the primary antibodies or equivalent concentrations of matched isotype controls (Table 1) as des
Cells were then washed in HBSS containing 2% FBS and centrifuged at 1000 rpm for 5 min. Cell pellets wer
μL of HBSS with 2% FBS and 1 μg/mL DAPI (Sigma-Aldrich). The cells were analyzed on a LSRII flow cytor
software (BD Biosciences, CA, USA).

Antibody*	Conjugate	Clone	Volume / 100μl	Manufactur
CD45	APC-Cy7	2D1	1μl	BD Biosciences
CD73	PE	AD2	1μl	BD Biosciences
CD105	APC	SN6	0.5μl	eBioscience
CD90	PE	5E10	0.25μl	BD Biosciences
CD146	PE	P1H12	1μl	BD Biosciences
CD44	PE	G44-26	1μl	BD Biosciences
CD166	PE	3A6	1μl	BD Biosciences
HLA-DR	APC	G46-6	1μl	BD Biosciences
CD19	APC-Cy7	SJ25C1	0.25μl	BD Biosciences
IgG1 isotype control	PE	MOPC-21	1μl	BD Biosciences
IgG2a isotype control	APC	G155-178	1μl	BD Biosciences
IgG1 isotype control	APC-Cy7	MOPC-21	0.25μl	BD Biosciences

* anti-human antibodies raised in mice.

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Table 1. Antibodies used for characterizing CMSCs and DMSCs by flow cytometry.
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In vitro differentiation into mesenchymal lineages

Differentiation of DMSCs and CMSCs into adipogenic, osteogenic, and chondrogenic lineages was assessed
differentiation was carried out with bullet kits as described [5, 6, 13]. Adipogenic and osteogenic differentiatio
Mesencult basal medium together with the respective differentiation supplements according to manufacturer
Cell Technologies, BC, Canada). Chondrogenic differentiation was carried in DMEM/F12 medium (Life Techn
and chondrogenic supplements (both from R&D Systems, MN, USA) according to the manufacturer's instruc
stained with Oil Red O solution, Alizarin Red solution and Safranin O (Sigma-Aldrich) to visualize adipogene
chondrogenesis respectively.

In vivo ectopic bone formation assay

CMSCs and DMSCs (n = 3 each) were expanded to reach approximately $1x10^7$ cells per sample (P2-P3). A
MSCs from each donor were mixed with 40 mg hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic parti
USA) and then subcutaneously transplanted into the dorsal surface of eight-week-old SCID mice (National In
bg-nu-xid; Harlan Sprague-Dawley, IN, USA) as described previously [14, 15]. Each mouse received two imp
procedures were performed in accordance with guidelines of an approved small-animal protocol (South Aust
Ethics Committee #139/09). After 8 weeks, the implants were removed, fixed in 10% formalin overnight at 4°

for 2 weeks in 0.5 M EDTA, prior to paraffin embedding. For histological analysis, 5 μm sections of the impla stained with haematoxylin and eosin (H&E). Expression of the specific osteogenesis markers osteocalcin (O (OPN), biglycan (BGN), and bone sialoprotein (BSP), were analyzed by immunohistochemistry using previou [16]. The implanted cells were labelled with BrdU at 24 and 48 hrs prior to implantation to evaluate the locali transplanted cells. Immunohistochemical staining using an anti-BrdU antibody was carried out as previously

Results

Isolation and expansion of CMSCs and DMSCs

CMSCs were isolated using the explant method [5, 7]. CMSCs migrated from the explants approximately 7 c and expanded CMSCs exhibited the characteristic the fibroblast-like morphology of MSCs (Fig 1Ai). DMSCs *decidua basalis* attached to chorionic villi of term placentae adhered onto tissue culture flasks within 24 h of previous findings [18–20], P0 DMSCs were initially heterogeneous and became more homogeneous followir the fibroblastic morphology characteristic of MSCs (Fig 2Ai). Given that CMSCs and DMSCs were morpholo after passaging, it was crucial that CMSCs and DMSCs used in these experiments were well-characterized v surface markers expression, origin, and differentiation potential.

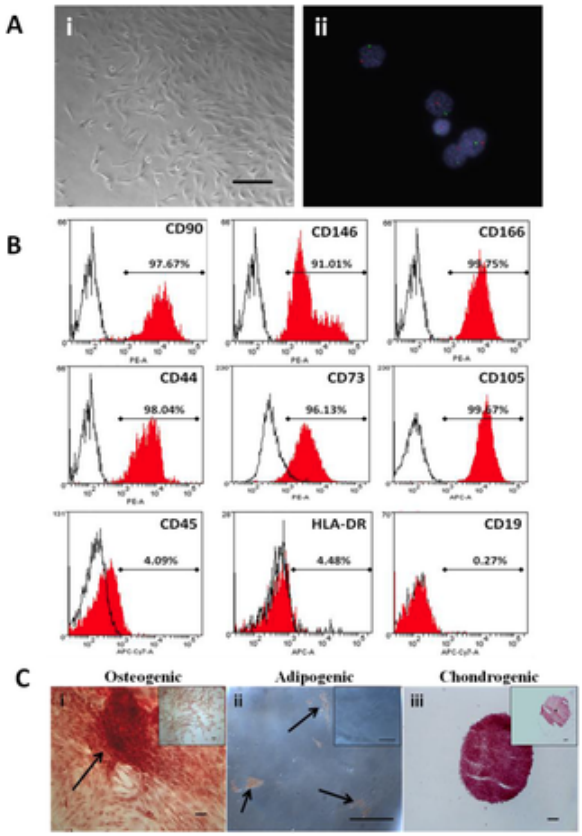

Fig 1. CMSC phenotypic characterization.

A. (i) Bright field microscopy image of CMSCs at P0. Magnification is 100X and scalebar is 100 μm. (ii) CI placentae of male newborns were analyzed using interphase FISH on MSC nuclei. CMSCs showed one c (Spectrum Green) and one chromosome Y (Spectrum Orange) signals. Cell nuclei were stained blue with is 630X. B. Primary CMSCs cell surface markers expression. Histograms of representative primary CMSC expression of CD90, CD146, CD166, CD44, CD73, CD105, CD45, HLA-DR, and CD19. The red histogra marker antibody staining while the white histogram shows the corresponding isotype control antibody stai phycoerythrin dye, APC: allophycocyanin dye, APC-Cy7: allophycocyanin-Cy7 dye. C. Representative ph showing CMSCs differentiation into mesenchymal lineages. (i) Osteogenic differentiation, Alizarin Red sta 5weeks growth in osteogenic induction medium. Arrows show calcium depositions. (ii) Adipogenic differer staining in cells after 14 days growth in adipogenic induction medium. Arrows show fat droplets. (iii) Chon

differentiation, Safranin O staining for proteoglycans depositions in cells after 21 days growth in chondrog
medium. Inset shows control uninduced CMSCs. Scalebar is 100 μm.
doi:10.1371/journal.pone.0141246.g001

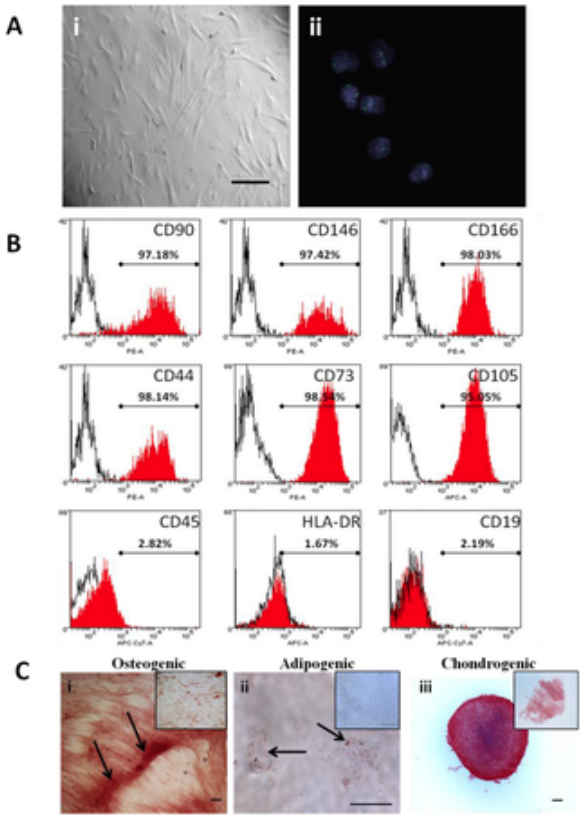

Fig 2. DMSC phenotypic characterization.
A. (i) Bright field microscopy image of DMSCs at P0. Magnification is 100X and scalebar is 100 μm. (ii) DI
placentae of male newborns were analyzed using interphase FISH on MSC nuclei. DMSCs showed two X
(Spectrum Green) signals. Cell nuclei were stained blue with DAPI. Magnification is 630X. B. Primary DM
markers expression. Histograms of representative primary DMSC at P3 depicting the expression of CD90
CD44, CD73, CD105, CD45, HLA-DR, and CD19. The red histogram shows the MSC marker antibody sta
white histogram shows the corresponding isotype control antibody staining. PE: phycoerythrin dye, APC:
dye, APC-Cy7: allophycocyanin-Cy7 dye. C. Representative photomicrographs showing DMSCs different
mesenchymal lineages. (i) Osteogenic differentiation, Alizarin Red staining in cells after 5weeks growth in
induction medium. Arrows show calcium depositions. (ii) Adipogenic differentiation, Oil Red O staining in
growth in adipogenic induction medium. Arrows show fat droplets. (iii) Chondrogenic differentiation, Safra
proteoglycans depositions in cells after 21 days growth in chondrogenic induction medium. Inset shows c
DMSCs. Scalebar is 100 μm.
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Phenotypic characterization of CMSCs and DMSCs

CMSCs and DMSCs at P3-P5 were analyzed by flow cytometry for cell surface markers present on expande
than 90% of CMSCs (Fig 1B and DMSCs (Fig 2B) expressed the MSC markers CD90, CD146, CD166, CD4
while <5% were CD45, CD19 and HLA-DR positive. These findings were consistent with the expression prof
Cells beyond P5 were not analyzed since studies have reported that MSCs undergo cell death or senescenc
[4, 21–23].

Given the risk of cross contamination between fetal and maternal cells, firstly we analyzed CMSCs and DMS

that pure cell populations had been isolated. CMSCs and DMSCs were isolated from placentae of women de
and approximately 200 cells of each type were analyzed for signals in interphase nuclei using X/Y chromoso
showed the XY phenotype (Spectrum Green and Orange labelled chromosomes, respectively) and were the
Evaluation of 200 interphase nuclei revealed that CMSCs used in this study had 94% XY (6% XXYY), 100%
(0.5% XXYY). Two Spectrum Green labelled X chromosomes were visible in DMSCs and therefore DMSCs
Evaluation of 200 interphase nuclei showed that DMSCs used in this study had 100% XX, 92.5% XX (7.5% X
(1% XXXX). Cases of tetraploidy were always XXYY from fetal CMSCs and XXXX from maternal DMSCs pr
is a common artefact of cell culturing and does not preclude the use of cell preparations for further analysis [

Differentiation of expanded CMSCs and DMSCs into osteogenic, adipogenic and chondrogenic lineages was
further verify their *in vitro* MSC properties. Alizarin Red stained calcium deposits indicative of bone formation
maintained in osteogenic induction medium (Fig 1Ci). Oil Red O stained lipid droplets were observed arounc
stimulated in adipogenic differentiation medium (Fig 1Cii). CMSCs aggregated into a three-dimensional sphe
approximately 24 h stimulation in chondrogenic differentiation medium. Safranin O staining in sections taken
of the cell pellet after 3 weeks stimulation showed the presence of proteoglycans, which are normally secrete
matrix by cartilage cells (Fig 1Ciii). Control CMSCs cultures maintained in the appropriate basal medium did
differentiation into these lineages (Fig 1Ci–1Ciii insets). DMSCs also differentiated into the osteogenic, adipo
chondrogenic mesenchymal lineages (Fig 2Ci, 2Cii and 2Ciii respectively) [13].

In vivo ectopic bone formation assay

CMSCs and DMSCs (n = 3 per group) were assayed for their capacity to develop bone-like tissue following e
into SCID mice with HA/TCP particles as a vehicle. All implants showed new bone formation throughout the
3E and 3F show representative sections of CMSC and DMSC implants, respectively stained with H&E. The
performed with bright pink H&E staining is indicative of mineralized tissue [17]. New bone was formed (area
HA/TCP (indicated by dashed lines) and directly interfaces the ceramic surface. The new bone contains oste
within the matrix indicating that bone formation was active and progressive. Further histological examination
formation was surrounded by the presence of adipocytes (honeycomb-like structures), fibrous tissue, and sm
Anti-BrdU staining demonstrated the presence of implanted cells associated with areas of mineral formation
fibrous tissue formation (Fig 3C, 3D, 3G and 3H). The transplanted CMSCs and DMSCs exhibited the capac
and fibrous tissues *in vivo*.

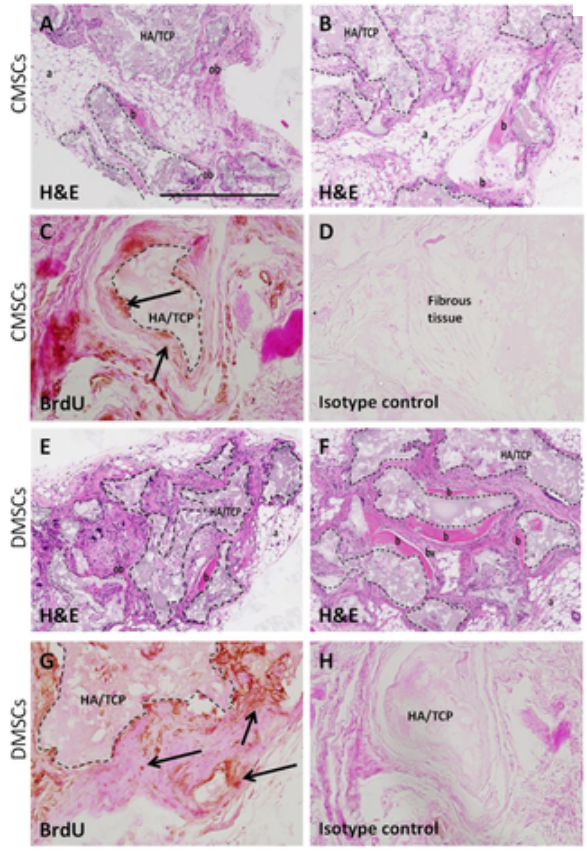

Fig 3. Histology of CMSCs and DMSCs transplants.

Cross sections are representative of CMSCs transplants (A-B) and DMSCs transplants (E-F) after 8 week Haematoxylin and Eosin (H&E). In the transplant, the HA/TCP carrier surfaces (dashed lines) are lined wi formation (b), areas of immature bone (ob) together with the surrounding fibrous and hematopoietic tissue vessel (bv). Representative BrdU staining for localization of implanted CMSCs (C-D) and DMSCs (G-H). I implanted cells were found lining the mineralized matrix (black arrows) and surrounding fibrous tissue. Br is indicative of DAB reactivity. There was no immunoreactivity present in sections stained with isotype-ma HA/TCP: hydroxyapatite/tricalcium phosphate particles. Magnification is 100X and scalebar is 500 μm.

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Immunohistochemical staining with several osteogenesis markers was performed to confirm that cells with o were formed in the transplants. Implanted CMSCs and DMSCs lining or embedded within the mineralized su bone-related markers: BSP, OCN, OPN, and BGN (Fig 4A–4H). The reactivity of these markers showed that and DMSCs contributed to the generation of osteogenic cells. In addition, the presence of newly formed ves was also indicated by immunostaining with α-SMA (Fig 4I and 4J). Overall, the data demonstrated the prese formation with no obvious qualitative differences between CMSCs and DMSCs.

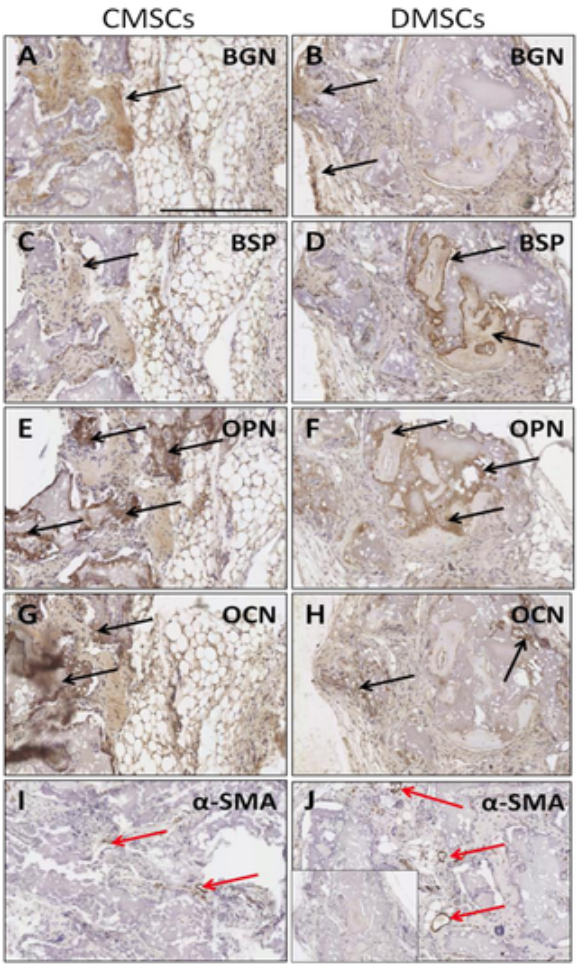

Fig 4. Immunoreactivity of osteogenesis markers after *in vivo* transplantation of primary CMSCs and DMSCs into immun
(A-B) BGN: biglycan expression. (C-D) BSP: bone sialoprotein expression. (E-F) OPN: osteopontin expre
osteocalcin expression. (I-J) α-SMA: alpha-smooth muscle actin as negative control. Inset shows represe
sections stained with isotype-matched antibodies. Colour detection was performed using DAB reaction. M
and scalebar is 300 μm. Black arrow show bone-forming surfaces and red arrows show blood vessels.
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Discussion

We demonstrated the isolation and expansion of two different MSC populations obtained from human term p
CMSCs and maternal-derived DMSCs. Both CMSCs and DMSCs are shown to have typical MSC features: (
(b) ability to differentiate *in vitro* into adipocytes, osteocytes, and chondrocytes; and (c) expression of MSC s
CD146, CD166, CD44, CD73, and CD105). FISH analysis confirmed that pure populations of fetal CMSCs a
could be obtained using the isolation methods employed. Thus, the CMSCs and DMSCs adhered to the crite
placental MSCs [2, 9].

MSCs from different sources such as adipose tissue, bone marrow, and dental pulp have been subjected to
ectopic bone formation assay [14, 15, 25, 26]. This study investigated CMSCs and DMSCs for the first time,
implanted with these cells into the subcutaneous space of immunodeficient mice could differentiate into new
characteristics of bone. This indicates that donor MSCs has the capacity for long-term survival and could co
generation of different tissue types *in vivo*. The efficacy of this *in vivo* assay was demonstrated in another st
were implanted with human foreskin fibroblasts and HA/TCP only controls were tested and showed only fibr
no indication of osteogenesis or haematopoiesis [26].

In this study, we set out to confirm that the implanted cells had survived and contributed to tissue formation v principle, BrdU stain incorporates into the DNA of dividing cells and diminishes as those cells further divide. post-implantation, a proportion of MSCs may have lost their BrdU expression due to their initial proliferation physical location within tissue (i.e. osteocytes in lacunae), antigen retrieval and DNA denaturing protocol ma exposed the antigen for its detection by immunohistochemistry. Whilst we acknowledge the limitation of usin implanted cells, this protocol has been widely used and accepted [27–29].

It is also of interest to note that development potential of BMMSCs *in vivo* was similar to CMSCs and DMSC contributed to new bone formation together with surrounding fibrous and adipocytes accumulation [14, 15, 3 support other studies demonstrating placenta-derived MSCs *in vivo* osteogenesis capacity. Intrabone, but nc injection of placenta adherent cells into a mouse model of myeloma-associated bone loss promoted bone fo differentiation of the host's osteoblasts [31]. Furthermore, placenta-derived MSCs grown in a silk fibroin/HA s transplanted in a rabbit radius defect model and improved bone repair as evidenced by formation of new lam bone and a number of osteoblasts [32]. In addition, there was evidence of angiogenesis with evidence of ne formation. The presence of newly formed vasculature is in agreement with previous studies that have reporte cells when implanted using the similar *in vivo* mouse model [14, 16]. Another study showed amnion-derived neovascularisation in an *in vivo* mouse model [33].

Porous HA/TCP showed good tissue tolerance with no immunological or toxic reaction, and that bone tissue upon their surfaces. This property is important for bone graft substitutes because without it, fibrous tissue ca interface between bone tissue and the graft, and cause loosening of the graft [12, 26]. The choice of osteoin (HA/TCP) combined with the appropriate choice of animal model are potentially crucial to determine *in vivo* CMSCs and DMSCs. To conclude, this is the first evidence of *in vivo* differentiation potential of DMSCs and transplantation in the mouse model of ectopic bone formation.

Conclusions

In this study, we have isolated human CMSCs and DMSCs and both cell types demonstrated the characteris Subcutaneous transplantation of CMSCs and DMSCs embedded in a HA/TCP biomatrix, into a mouse mode formation led to the formation of a bone-like structure. BrdU labelling indicated that transplanted cells were r and contributed to tissue formation. Bone-specific markers such as OPN, OCN, BGN, and BSP were presen without any qualitative difference. These data suggest that human CMSCs and DMSCs have potent *in vivo* b and may be worthwhile candidates for *in vivo* bone tissue repair.

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Author Contributions

Conceived and designed the experiments: GDK BK. Performed the experiments: GDK DM MDP. Analyzed t BK. Contributed reagents/materials/analysis tools: GDK DM SG MHA BK MDP. Wrote the paper: GDK UM B MDP.

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