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Comparison of Human Placenta- and Bone Marrow–Derived Multipotent Mesenchymal Stem Cells

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Bone marrow is the traditional source of human multipotent mesenchymal stem cells (MSCs), but placenta appears to be an alternative and more readily available source. This study comprehensively compared human placenta–derived MSC (hpMSC) and human bone marrow–derived MSC (hbmMSC) in terms of cell characteristics, optimal growth conditions and in vivo safety specifically to determine if hpMSC could represent a source of human MSC for clinical trial. MSC were isolated from human placenta (hpMSC) and human bone marrow (hbmMSC) and expanded ex vivo using good manufacturing practice–compliant reagents. hpMSC and hbmMSC showed similar proliferation characteristics in different basal culture media types, fetal calf serum (FCS) concentrations, FCS heat-inactivation experiments, flask types and media replacement responsiveness. However, hpMSC and hbmMSC differed with respect to their proliferation capabilities at different seeding densities, with hbmMSC proliferating more slowly than hpMSC in every experiment. hpMSC had greater long-term growth ability than hbmMSC. MSC from both sources exhibited similar light microscopy morphology, size, cell surface phenotype, and mesodermal differentiation ability with the exception that hpMSC consistently appeared less able to differentiate to the adipogenic lineage. A comparison of both hbmMSC and hpMSC from early and medium passage cultures using single-nucleotide polymorphism (SNP) GeneChip analysis confirmed GTG-banding data that no copy number changes had been acquired during sequential passaging. In three of three informative cases (in which the gender of the delivered baby was male), hpMSC were of maternal origin. Neither hpMSC nor hbmMSC caused any acute toxicity in normal mice when injected intravenously at the same, or higher, doses than those currently used in clinical trials of hbmMSC. This study suggests that human placenta is an acceptable alternative source for human MSC and their use is currently being evaluated in clinical trials.

Introduction

The mesenchymal stem cell (MSC) is a stem cell located within the stroma of the bone marrow and other organs including placenta. They have been phenotypically characterized using a variety of markers [1–3]. When isolated by plastic adherence and expanded ex vivo, these cells have been shown to differentiate into cell types of mesenchymal origin including chondrocytes, adipocytes, and osteocytes [1]. In the bone marrow they provide support for hematopoiesis [4]. In addition, they are able to differentiate into endothelial cells, form capillaries in vitro and secrete growth factors important in angiogenesis including vascular endothelial growth factor [1]. It has also been shown that MSC demonstrate plasticity beyond their traditional mesodermal lineage, in that they have been induced to generate tissues of both ectodermal (neurons) and endodermal (hepatocytes) nature [5,6]. In support of these observations, undifferentiated MSC express many lineage-specific genes other than those of mesenchymal lineage [7]. Their ability to differentiate into a wide variety of cell types, together with their reproducibility of isolation, high expansion potential and capacity for useful modification using molecular biological engineering techniques, make them good candidates for the repair and regeneration of a large variety of tissues. They have been shown

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in preclinical studies to improve myocardial function (after acute myocardial infarction), cerebral function (after cerebral infarction), liver and joint damage [8–11].

Importantly, MSC appear to have a major advantage over many other cell types for cellular therapy, in that they are immunologically privileged and even in large outbred animals can generally be transplanted across major histocompatibility complex (MHC) barriers without the need for immune suppression [12]. The mechanism for this is not fully understood at present, but appears to be an active process that leads to suppression of T-cell function [13–15]. This has important implications for the therapeutic application of MSC, because MSC derived from healthy unrelated volunteer donors can be cryopreserved, thus making them available in a timely manner for patients in a variety of acute and chronic clinical settings. MHC-identical, MHC-haploidentical, and MHC-unmatched MSC have been used successfully in the clinic [16–18].

Bone marrow is the traditional source of human MSC. However, human MSC have been generated from a wide variety of tissues and organs including placenta [19–21], cord blood [22], amnion [23], amniotic fluid [24], fat [25], lung [26], and liver [27]. Most of these sources, including large volumes of normal bone marrow, are relatively difficult to access as a tissue source for the isolation of MSC. In contrast, placenta is readily and widely available. To determine whether human placenta–derived MSCs (hpMSCs) represent an appropriate alternative source of human MSC for use in clinical trials, we optimized ex vivo expansion conditions for both types of MSC. The literature describes a number of differences in the culture conditions described to generate human MSC, including various types of basal media, concentrations of fetal calf serum (FCS), with and without heat inactivation of FCS, seeding densities, flask types, and medium replacement schedule. We explored all these variables for generating hpMSC and bone marrow–derived MSC (hbmMSC). We also compared the following biological characteristics of the two types of MSC: morphology, size, cell surface phenotype, mesodermal differentiation ability, karyotype, single-nucleotide polymorphism (SNP), and in vivo safety in murine recipients. Wherever possible we used good manufacturing practice–compliant reagents, so that the protocols were able to be directly translated to the clinical trial setting.

Materials and Methods

Cell harvest

Bone marrow. Human bone marrow (3–5 mL) obtained from the iliac crest of healthy adult donors was diluted 1:5 with Hank's Balanced Salt Solution (HBSS) (Invitrogen). The cell suspension was underlayed with 12 mL 1.073 g/mL Percoll™ (GE Healthcare) and centrifuged (540*g*, 20°C, 20 min) without a brake. Cells from the interface layer were washed twice with HBSS (first at 540*g*, 20°C, 10 min, then 250*g*, 20°C, 5 min). The mononuclear cells were plated at a density of between 0.5 and 1×10^5 cells/cm^2 in tissue culture flasks for ex vivo expansion.

Placenta. Human placentas were obtained from healthy mothers during routine Caesarian section births. In a biohazard cabinet the cord blood was drained and the umbilical cord and external membranes were removed. Placental tissue (including amnion, chorion and desidua basalis) was then dissected into pieces ~5 g in size (300 g in total). This tissue was placed in beakers and washed with 500 mL HBSS/100 g tissue. The pieces of placenta were then divided equally between 50 mL tubes at ~10 g/tube. Dulbecco's modified Eagle's medium–Low Glucose (DMEM-LG; JRH Biosciences) with 100 U/mL Collagenase, type I (Worthington Biochemical Corporation) and 5 µg/mL DNase I (Roche) was added to each tube up to a total volume of 50 mL. Tubes were incubated on a shaker (220 rpm, 37°C, 2 h), then pulse centrifuged at 540*g* to remove large particulate matter and the cell suspensions were passed through 70 µm filters (Becton-Dickinson). The remaining tissue was washed with HBSS and the resulting cell suspension also filtered through 70 µm filters. The combined filtered cells were centrifuged (540*g*, 5 min, 20°C) and resuspended in 30 mL HBSS, and 12 mL 1.073 g/mL Percoll™ was underlayed. Samples were centrifuged (540*g*, 20°C, 20 min) and the interface removed and washed twice with HBSS (first at 540*g*, 20°C, 10 min and second at 300*g*, 20°C, 5 min). The mononuclear cells were then plated at 2–4 $\times$ 10^5 cells/cm^2 in tissue culture flasks for ex vivo expansion.

Cell culture

Depending upon the experiment bone marrow or placental cells were plated into either 25 or 75 cm^2 tissue culture flasks (Nunc). The standard tissue culture medium was basal media (e.g., DMEM-LG) with 20 % (v/v) Australian-sourced FCS and 50 µg/mL gentamicin (Pharmacia). Initial experiments compared five different lots of nonheat-inactivated FCS (Invitrogen; catalogue number: 10099-141; lots: 560339 and 1188431; Invitrogen; catalogue number: 10099-158; lot: 1180078; and JRH Biosciences; catalogue number: 12003-500M; lot: 4J0046). One of these (12003-500M) was selected for all subsequent experiments. Cultures were incubated in humidified 5 % CO_2 incubators and the media was replaced twice a week. Cells were isolated by plastic adherence in expansion cultures and nonadherent cells were washed off the cultures after 3 days.

Cell cultures were passaged when 90–95% confluent. For passaging, the flasks were washed with HBSS and incubated with TrypLE™ Select (Invitrogen) for 5–10 min at 37°C. Dissociated cells were removed, then pelleted by centrifugation (540*g*, 5 min, 4°C). The supernatant was discarded and cells were resuspended in tissue culture media. At passage 1, hbmMSC and hpMSC were seeded into three replicate 25 cm^2 flasks per experiment. From passage 2, cells were seeded from one 25 cm^2 flask to another 25 cm^2 flask.

For the experiments comparing basal media, FCS concentration (20 or 10% v/v) and heat inactivation of FCS (60 min at 56°C), hpMSC and hbmMSC were plated at 3–4 $\times$ 10^4 cells/flask (1,600 cells/cm^2) at every passage in each experiment from passage 1 onwards. MSC were cultured in one of five different basal culture media: DMEM-LG, Minimum Essential Medium, α-Modification (α-MEM, JRH Biosciences; catalogue number: 51451-500M), DMEM-High Glucose (DMEM-HG; Invitrogen; catalogue

number: 11960-044), Iscove's Modified Dulbecco's Medium (IMDM; Invitrogen; catalogue number: 31980-030) and RPMI 1640 Medium (RPMI; Invitrogen; catalogue number: 21870-076).

For the experiment on seeding density, hpMSC and hbmMSC were plated at either 2.5×10^3 cells/flask (100 cells/cm^2) or 2.5×10^4 cells/flask (1,000 cells/cm^2).

For the experiments on flask type and frequency of medium change (weekly vs. twice weekly), hbmMSC were plated at 5.6×10^3 cells/flask at every passage in each experiment, and hpMSC were plated 4×10^4 cells/flask. hpMSC and hbmMSC were cultured in one of three different 25 cm^2 flask types: Nunc (catalogue number: 156367), BD Falcon (BD Biosciences; catalogue number: 353108), and Corning (catalogue number: 3056).

Mesodermal lineage differentiation

Osteogenic lineage. Osteogenic differentiation was induced by culturing 90% confluent MSC for 3 weeks in DMEM-HG, 10% FCS, 0.1 μM dexamethasone, (Mayne Pharma; Australia Register Number: 16375; Melbourne, Victoria, Australia), 50 μM L-ascorbic acid-2-phosphate (Sigma; catalogue number: A8960-5G; Castle Hill, New South Wales, Australia), 10 mM β-glycerol phosphate disodium salt pentahydrate (Sigma; catalogue number: 50020) and 0.3 mM inorganic (sodium) phosphate (Sigma) [28,29]. Osteogenic differentiation was assessed by staining cells in wells with Alizarin Red S.

Chondrogenic lineage. Chondrogenic differentiation was induced by culturing pellets of 5×10^5 MSC for 3 weeks in DMEM-HG, 0.1 μM dexamethasone, 1 mM sodium pyruvate (Sigma; catalogue number: P5280-25G), 50 μM L-ascorbic acid-2-phosphate, 35 mM l-proline (Sigma; catalogue number: 81710), 10 ng/mL TGFβ_1 (R&D Systems; catalogue number: 243-B3) and 50 mg/mL ITS Premix (human recombinant insulin, human transferrin, and selenious acid; BD Biosciences; catalogue number: 354351) [5]. Chondrogenic differentiation was assessed by staining frozen sections of the cell pellets with periodic acid Schiff.

Adipogenic lineage. Adipogenic differentiation was induced by culturing 80% confluent MSC for 3 weeks in DMEM-HG, 1 μM dexamethasone, 5 μg/mL insulin (Sigma), 60 μM indomethacin (Sigma; catalogue number: 17378-5G), and 0.5 mM 3-Isobutyl-1-methylxanthine (IBMX; Sigma; catalogue number: I5879) [28]. Adipogenic differentiation was assessed by staining cells in wells with Oil Red O. It should be noted that these differentiation protocols were originally optimised for use with bone marrow as a source of MSC.

RNA extraction and reverse transcriptase polymerase chain reaction for mesodermal lineage markers. RNA was isolated from differentiated or undifferentiated MSC using an RNeasy kit (Qiagen) according to manufacturer's guidelines. Primers were designed for human messenger RNA (mRNA) and all RNA samples were DNase pretreated. The reverse transcriptase reaction was performed with oligo-dT and Superscript III (Invitrogen) according to manufacturer's instructions. PCR was performed on complementary DNA using Taq accuprime Supermix (Invitrogen) according to manufacturer's instructions for 32 cycles: 30 s denaturation at 94°C, 30 s hybridization at 56°C, 1 min elongation at 68°C. Product size and primer sequences used were, 5′–3′: Perilipin (PLIN adipogenic differentiation marker) 123 bp (5′)AAACAGCATCAGCGTTCCCATC (3′)AG TGTTGGCAGCAAATTCCG, Runt Related Transcription Factor 2 (RUNX2 osteogenic differentiation marker) 114 bp (5′)CGCAAAACCACAGAACCACAAGTGCG (3′)GTTGG TCTCGGTGGCTGGTAG, Aggrecan (ACAN chondrogenic differentiation marker) 150 bp (5′)CGGGTCTCACTG CCCAACTACCCG (3′)GCCTTTCACCACGACTTCCAG, and Actin 458 bp (5′)ATCCTCACCCTGAAGTACC (3′)CTCCTTAATGTCACGCACG. Samples were analysed on 1.5% agarose gels and stained with SYBR® Safe DNA gel stain (Invitrogen).

Flow cytometry

Cell surface phenotype and cell size estimation. Cells were detached from flasks using TrypLE select (Invitrogen), washed and added to wells of a 96-well plate. Cells were incubated for 10 min at room temperature with unconjugated mouse anti-human IgG_1, IgG_{2A}, IgG_{2B}, CD29, CD34, CD44, CD45, CD49d, CD73, CD90, CD105, CD166, MHC I, and MHC II antibodies (BD Biosciences). Excess antibody was removed by washing wells with phosphate-buffered saline. Donkey anti-mouse phycoerythrin secondary antibody (Jackson Laboratory, Bar Harbor, ME) was added to each of these wells and cells incubated for 10 min at room temperature. After further washing, flow cytometry analysis was performed on a FACS Calibur (Becton-Dickinson) using FCS Express Version 3 software. For cell size estimation, 29.6 μm Spheroblank size calibration beads were used (Spherotech, Lake Forest, IL).

Genetic profiling

Cytogenetic analysis of GTG-banded metaphases were performed on MSCs from five placental and four bone marrow samples. Genetic stability was further explored using GeneChip SNP-based human mapping analysis to evaluated genomic gains and losses.

Karyotype analysis. Flasks containing cells from five hpMSC and four hbmMSC cultures at passage 3 (all samples) and at passages 5 and 8 (two hbmMSC and all hpMSC samples) were processed using standard cytogenetic techniques. The flasks were harvested 1–3 days after receipt. Colchicine was added to the flasks for 3–18 h, after which the cells were released from the flask surface with trypsin/versene and treated with hypotonic KCl. The cells were fixed with methanol/glacial acetic acid 3:1 by volume and spread onto glass slides. Metaphase cells were GTG-banded using trypsin and Geimsa stain. As many metaphases as possible were karyotyped up to a maximum of 20 metaphases per sample.

SNP microarray analysis of genome copy number. DNA was extracted from frozen cell pellets using the QIAamp DNA Blood Mini Kit (Qiagen). Cells were protease digested for 10 min at 56°C before mixing with 0.5 volumes of ethanol and concentrated on the QIAamp Spin Column. The column was washed and genomic DNA eluted and 250 ng labeled using

the Affymetrix XbaI SNP GeneChip™ Labelling kit, as per manufacturer's instructions. In brief, DNA was digested with 1 U XbaI (NEB) for 2.5 h and ligated to Adaptor Xba oligomers using 250 U T4 DNA Ligase (NEB). Three PCR reactions were performed on a total of 75 ng of postligation DNA and purified using MiniElute 96 UF PCR Purification plates (Qiagen). Forty microgram of DNA was fragmented and end-labeled with dUTP-biotin before a 16-h hybridization on the 50K XbaI Human Mapping GeneChip. The GeneChip was washed according to Mapping 100 Kv1_450 washing protocol and scanned on a GS3000 scanner.

Human Mapping XbaI GeneChip data was extracted and analyzed using GCOS v1.3 and GDAS v3.0.1 from Affymetrix. Copy number estimates were produced using Chromosome Copy Number Tool v2.0.0.9 (CCNT) (Affymetrix) and graphed using Copy Number Analyser for GeneChip v2.0 (CNAG) [30].

In vivo toxicity studies. Passage 3 and 5 hpMSC were injected intravenously via the tail vein into Balb/c mice (Animal Resources Centre, Perth, Australia) at three doses: 2×10^4 cells/mouse (1×10^6 cells/kg), 2×10^5 cells/mouse (1×10^7 cells/kg), and 2×10^6 cells/mouse (1×10^8 cells/kg). At the request of the University of Queensland Animal Ethics Committee, each dose was administered to only two female Balb/c mice (for minimization of animal utilization) in 200 µl sodium chloride 0.9% (Baxter Australia) with 4% (v/v) FCS (not heat-inactivated) through 26-gauge needles. Cells were not filtered.

Passage 5 hbmMSCs were administered to mice in the same way and at the same doses. Mice were monitored closely for the first 5 h after cell administration, and then once each day for 3 days thereafter. A mouse health score sheet was used to assess animals during this experiment: One point was given if animals showed ≥15% weight loss, hunched posture at rest, decreased activity or ruffled fur. Mice were sacrificed at day 3, or beforehand if they reached a score of 4.

Data analysis

Every experiment used four bone marrow and four placenta samples with three replicates per experiment and sample. All line and bar graphs display median values with interquartile ranges. To analyze growth kinetics, cumulative cell number was determined at each passage in every experiment.

Cumulative cell number at passage X equals the sum of the three confluent cell cultures at passage X divided by the sum of the cells plated into the three passage X cultures multiplied by the cumulative cell number at passage X-1. Cumulative cell numbers were calculated from the number of cells seeded from passage 1.

The number of population doublings was determined in the long-term cultures at every passage. Two to the power of (population doublings at passage X) equals cumulative MSC number at passage X divided by MSC number at passage 0. Therefore, population doublings at passage X equals $\log_{10}$ (cumulative MSC number at passage X divided by MSC number at passage 0) divided by $\log_{10} 2$.

The rate of cell proliferation is illustrated in each expansion kinetics graph by the gradient of each line, which is the change in cell number/change in time. Differences in cell proliferation (using cumulative cell numbers and population doublings) and cell viability were determined using nonparametric statistical tests. For each time point of each experiment, and for each experiment overall, the Wilcoxon–Mann–Whitney test (two-tailed) was used to determine whether significant differences existed between two conditions. When more than two conditions were present, the Kruskal–Wallis test was used to determine whether statistically significant differences existed between at least two conditions. When significant differences were found, the Posthoc Kruskal–Wallis multiple comparisons test or Wilcoxon–Mann–Whitney test (one-tailed) was used to determine which conditions were significantly different from each other at each time point. Differences between variables were considered significant when $p \leq 0.05$.

Ethical approval

All experiments were approved by the Mater Health Services Human Research Ethics Committee and/or the Animal Ethics Committee of the University of Queensland.

Results

Growth optimization

Basal culture media. There was a significant difference in hpMSC proliferation at specific multiple time points and over the entire experiment ($p < 0.001$) when cultured in five different basal culture media (Fig. 1A) (*$p < 0.05$). By passage 2, hpMSC in RPMI had died, and so proliferation was significantly less in this medium than the other four media. By passage 3, hpMSC in DMEM-HG had also died, so cell proliferation was significantly less in this medium than DMEM-LG, α-MEM and IMDM. There was no significant difference in hpMSC proliferation between DMEM-LG, α-MEM, and IMDM. Viability was not different between passages (median 85%, range 75–100%).

There was no significant difference in hbmMSC proliferation (Fig. 1B) or viability (median 85%, range 75–95%) when cultured in DMEM-LG or α-MEM. hbmMSC proliferated more slowly than hpMSC and it thus took hbmMSC a longer time to reach a given number of cells than hpMSC (Fig. 1A and B).

FCS concentration. There was a significant difference in hpMSC proliferation over the entire experiment ($p < 0.001$), with hpMSC growing better in 20% FCS compared to 10% FCS. hbmMSC proliferated more slowly than hpMSC and numbers of both hpMSC and hbmMSC decreased in culture over time in 10% (v/v) FCS compared to 20% (Fig. 1 C and D). There was no significant difference in viability of hpMSC (median 90%) or hbmMSC (median 80%) between the two conditions from passage 1 through passage 3 (Fig. 2C and D).

FCS heat inactivation. There was no difference in hpMSC- or hbmMSC proliferation rate or viability when cells were cultured in medium with FCS that had been heat inactivated or not heat inactivated (data not shown).

Seeding densities. There was a significant difference in hpMSC proliferation over the entire experiment ($p = 0.042$),

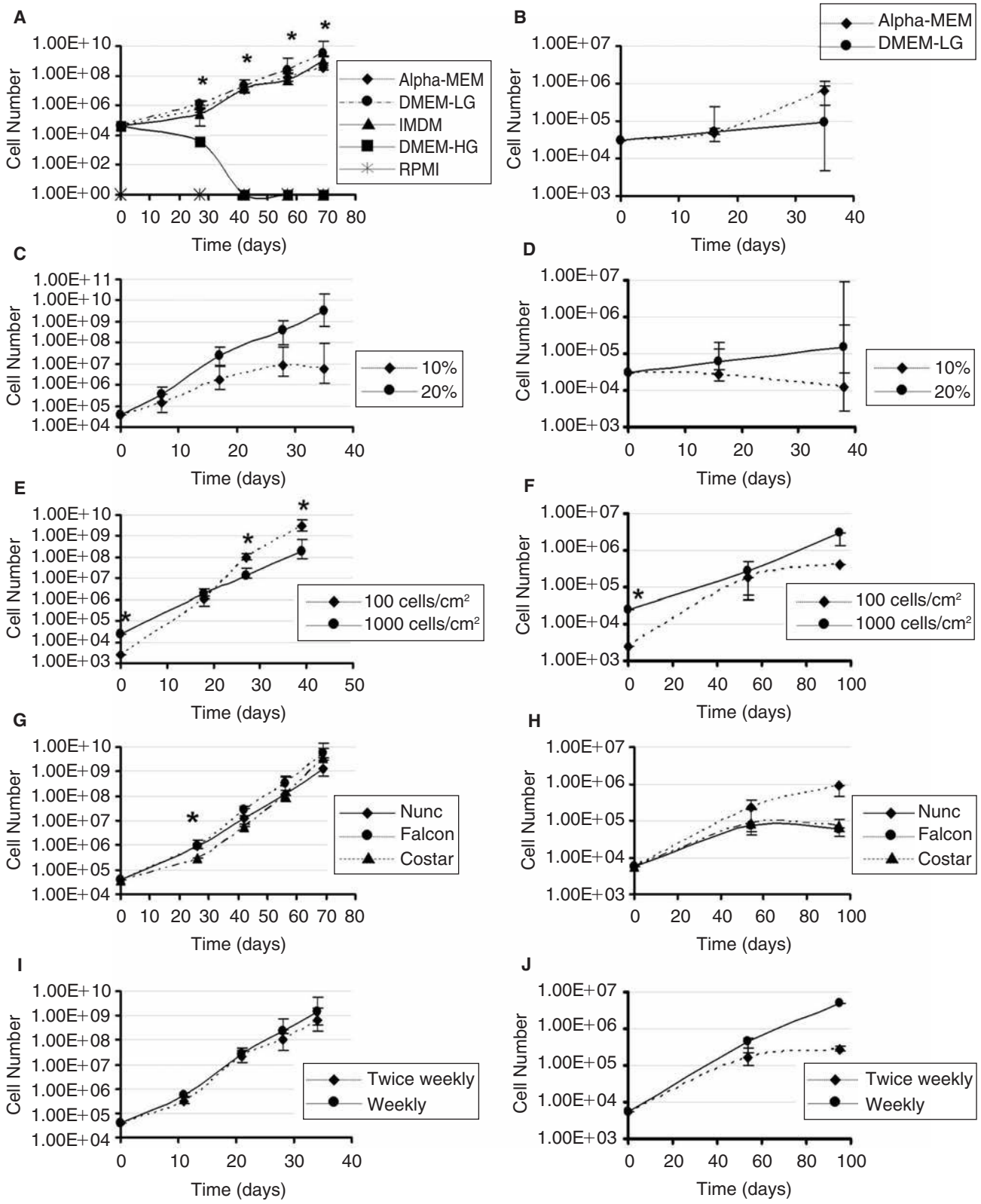


FIG. 1. Human MSC growth optimization. Effect of basal medium on ex vivo expansion ability of (**A**) hpMSC and (**B**) hbmMSC (*$p < 0.05$ at indicated time points). Effect of fetal calf serum concentration on ex vivo expansion ability of (**C**) hpMSC and (**D**) hbmMSC. Effect of seeding density at passage 1 on ex vivo expansion ability of (**E**) hpMSC and (**F**) hbmMSC (*$p < 0.05$ at indicated time points). Effect of flask type on ex vivo expansion ability of (**G**) hpMSC and (**H**) hbmMSC hpMSC (*$p < 0.05$ at indicated time points). Effect of medium replacement frequency on ex vivo expansion ability of (**I**) hpMSC and (**J**) hbmMSC.

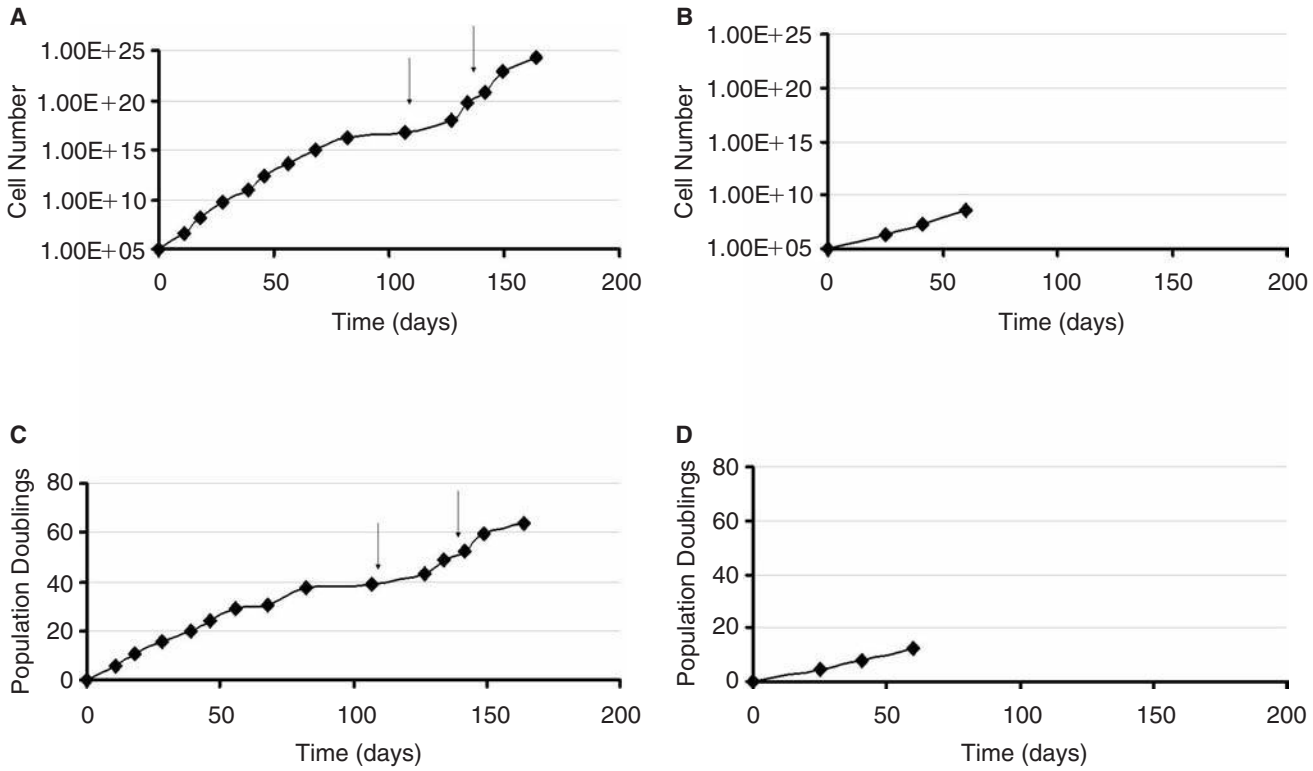


FIG. 2. Long-term growth ability of human MSC in vitro. Cell number (**A**) and population doublings (**C**) during long-term culture of hpMSC. Cell number (**B**) and population doublings (**D**) of hbmMSC during long-term culture. *Arrows* indicate where cells were thawed to continue cell culture.

with hpMSC growing better when seeded at 100 cells/cm^2 compared to 1,000 cells/cm^2. hpMSC showed significantly faster proliferation at days 28 and 38 (passages 3 and 4, respectively) when seeded at 100 cells/cm^2 compared to 1,000 cells/cm^2 (Fig. 1E) (*$p < 0.05$). At passage 2, hpMSC cultures seeded at 100 cells/cm^2 had better viability (85% vs. 76%) than cultures seeded at 1,000 cells/m^2 (*$p < 0.05$).

After passage 1, when hbmMSC were first divided into cultures seeded at 100 cells/cm^2 or 1,000 cells/cm^2, there was no significant difference in hbmMSC proliferation between the two conditions (Fig. 1F). Unlike hpMSC cultures, hbmMSC cultures seeded at 100 cells/cm^2 did not proliferate at a faster rate than cultures seeded at 1,000 cells/cm^2. There was no significant difference in hbmMSC viability between cultures which had been seeded at 100 or 1,000 cells/cm^2 (median 80%).

Flask type. There was a significant difference in hpMSC proliferation over the entire experiment ($p = 0.002$) with hpMSC growing better in Nunc and Falcon flasks compared with Costar flasks, including a significant difference at day 28 (*$p < 0.05$) (Fig. 1G and H).

Medium replacement. There was no difference in hpMSC proliferation rate or viability whether culture medium was replaced once or twice weekly (Fig. 1I and J). The growth of hbmMSC appeared to slow when medium was replaced twice weekly (Fig. 1J).

Long-term growth ability. Using culture conditions optimized as described above, we found that hpMSC proliferated for long periods of time. One long-term hpMSC culture underwent 14 passages for >160 days with 64 population doublings (Fig. 2A and C). Over 10^{24} cells would have been accumulated if every cell had been continued in culture from passage 0. In contrast, we were unable to maintain hbmMSC for such periods of time, the maximum being 3 passages for 60 days with 12 population doublings (Fig. 2B and D). Only 10^8 cells would have been accumulated if all cells from passage 0 had been continued in culture.

In summary, hpMSC consistently proliferated more robustly than hbmMSC. Optimal culture conditions for hpMSC and hbmMSC included the use of DMEM-LG or α-MEM basal medium, 20% nonheat-inactivated FCS, Nunc or Falcon flasks and weekly medium replacement. These conditions were adopted for subsequent experiments.

Characterization

Cell morphology. Both hpMSC and hbmMSC showed plastic adherence and a typical fibroblastic morphology by light microscopy (Fig. 3). hbmMSC (Fig. 3E–H) showed a more elongated morphology compared to the hpMSC (Fig. 3A–D). Seeding density at 100 versus 1,000 cell/cm^2 did not affect cell morphology in cultures at low or high confluence (Fig. 3).

Cell size. hpMSC (Fig. 4A) and hbmMSC (Fig. 4B) were large, granular cells with diameters ranging between 30 and 120 μm.

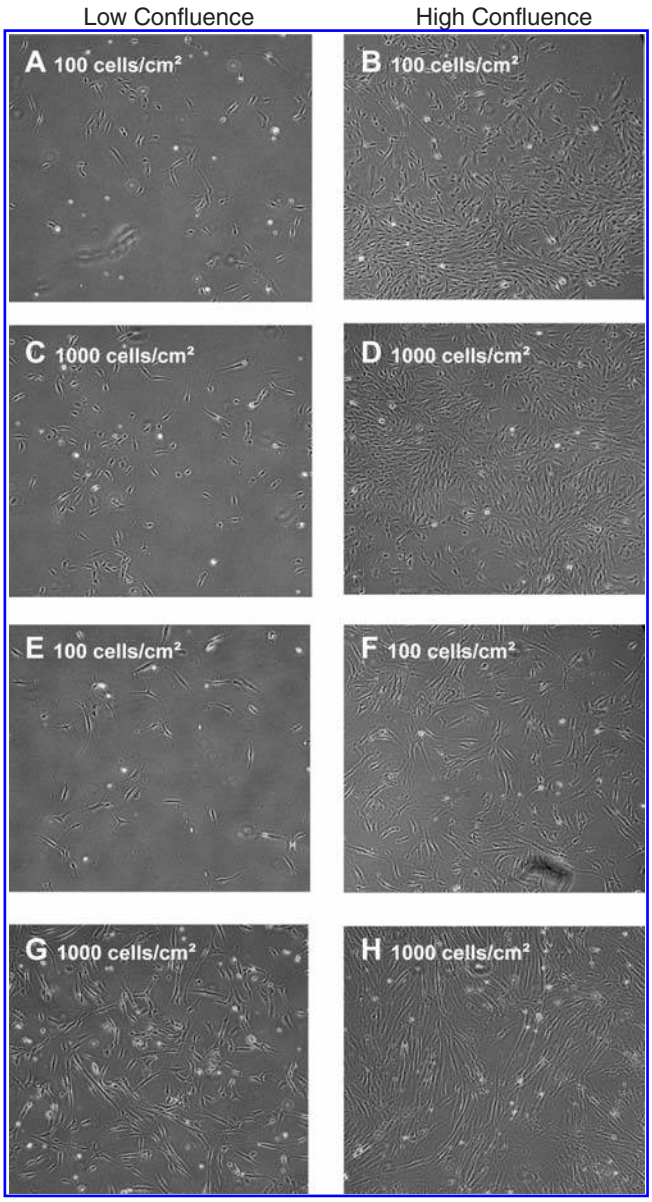


FIG. 3. Morphology and seeding density of human MSC. A typical fibroblastic morphology was observed for both hpMSC (**A–D**) and hbmMSC (**E–H**) by light microscopy, although hbmMSC showed a more elongated morphology. Seeding density at 100 versus 1000 cell/cm^2 did not affect cell morphology in cultures at low or high confluence.

Cell surface phenotype. Figure 4C illustrates the frequency of hpMSC isolated from placenta at passage 0 using flow cytometry. When gated for live cells, the CD73 and CD105 double positive population represents 1% of the CD45 negative cells and 0.3% of total live cell population. hpMSC and hbmMSC had a similar surface phenotype (Fig. 4D) with equivalent expression of CD29, CD44, CD73, CD105, CD166, and MHC I. However, hbmMSC expressed CD49d at lower levels than hpMSC and neither hpMSC nor hbmMSC expressed CD34, CD45, or MHC II.

Cell differentiation. Both hpMSC (Fig. 4E) and hbmMSC (Fig. 4F) differentiated into osteocytes, chondrocytes, and adipocytes under specific differentiation conditions. As assessed by this assay, hpMSC failed to differentiate into adipocytes as readily as hbmMSC. However, when cells were grown in the presence of differentiation-inducing factors, mRNA expression for specific markers of adipogenic (PLIN), osteogenic (RUNX2), and chondrogenic (ACAN) lineages was observed (Fig. 4G and H). Undifferentiated MSC did not express these mRNA for mesodermal lineages.

Cytogenetic and SNP analysis. Karyotype analysis was performed on GTG-banded metaphases from five hpMSC samples and four hbmMSC (Table 1). The hpMSC cultures performed well, with results obtained from passage 3 and 5 in all five samples, and results from passage 8 in four of the five samples. In contrast, the hbmMSC samples generally responded poorly to culture and harvest, with <20 metaphases available for analysis for most samples, and with results obtained beyond passage 1 in only one of four samples.

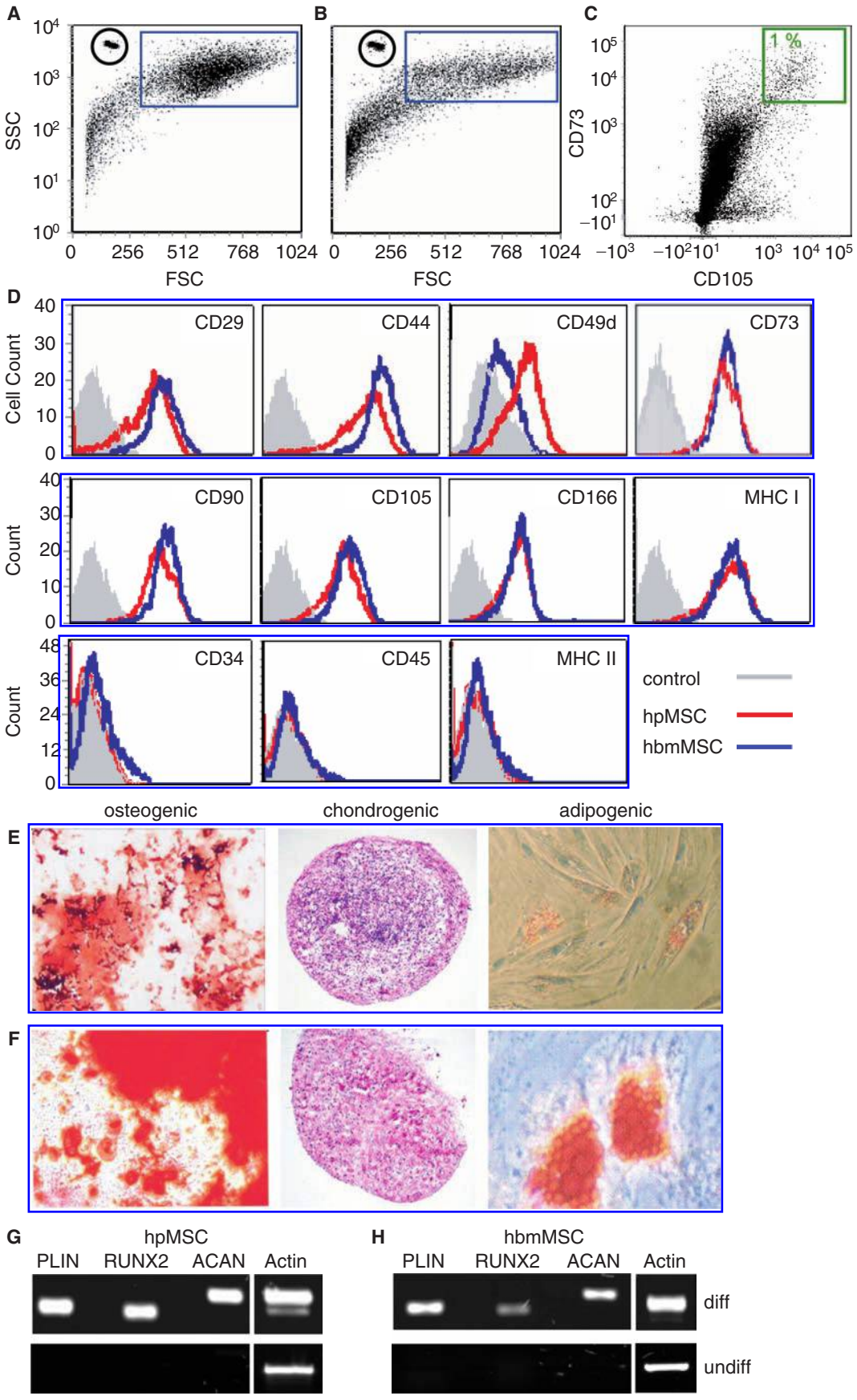
Eleven of 14 hpMSC metaphase spreads (from five different placentas) were normal. Three of the five hpMSC samples showed a cytogenetic change: a complex unbalanced rearrangement at passage 5 of sample 3, trisomy 18 at passage 8 of sample 4, and a balanced translocation at passage 3 of sample 6. Endoreduplication was present in samples 4 and 12. The remaining two hpMSC samples were normal and stable over time.

Four of five evaluable hbmMSC metaphase spreads (from four different bone marrow samples) were normal. At passage 3 hbmMSC samples 5, 9, and 30 returned a normal male (46,XY) or female (46,XX) karyotype. hbmMSC sample 15 was the only hbmMSC sample with cytogenetic anomalies; a single cell with trisomy 8 and another cell with an apparently balanced translocation between chromosomes 1 and 18 at passage 3. Neither of these anomalies were observed at passage 5. Cells from passage 8 grew poorly and failed to produce metaphases for analysis.

In no case where cytogenetically abnormal cells were observed was the abnormal cell line observed in a subsequent (higher) passage number. There appeared to be no selective advantage for the abnormal cells in the culture system.

To further explore the karyotype analysis and confirm the observed genomic stability GeneChip SNP and Copy Number estimation was performed. DNA from frozen pellets of early and late passages of hbmMSC 5.4 and hpMSC 13 were analyzed by Human Mapping GeneChips to confirm the relative genomic stability seen by karyotype analysis. The XbaI Mapping GeneChip provides SNP typing and gene-copy estimates at 58,494 positions spanning chromosomes 1–22 and X. MSC passages were selected that did not display any gross abnormalities by karyotyping and could thus provide further information on minor sequence changes (SNP typing) that would otherwise only be possible by extensive sequencing or microsatellite typing.

Passage 2 and passage 5 of hbmMSC 5 had a mean detection rate (MDR) of 99.52 and 99.89% and a SNP call rate of 94.48 and 97.25%, respectively. hpMSC passage 3 and passage 5 had a MDR of 99.52 and 99.89% and a SNP call rate of 94.48 and 97.25%, respectively. An estimation of gene


A
B
C
SSC
FSC
FSC
CD73
CD105
1 %
D
Cell Count
CD29
CD44
CD49d
CD73
Count
CD90
CD105
CD166
MHC I
Count
CD34
CD45
MHC II
control
hpMSC
hbmMSC
osteogenic
chondrogenic
adipogenic
E
F
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hpMSC
PLIN
RUNX2
ACAN
Actin
H
hbmMSC
PLIN
RUNX2
ACAN
Actin
diff
undiff

Table 1. Cytogenetic Analysis of Human Placental MSCs and Bone Marrow MSCs

Sample	*Passage number*	*Karyotype*	*Number of abnormal metaphases*
Placenta 3	3	46,XX	0/20
	5	46,XX/46,XX,add(11)(p11.2),der(15)t (11;15)(q13;p11.2)	10/20
	8	46,XX	0/15
Placenta 4	3	46,XX	0/20
	5	46,XX (endoreduplication present)	0/15
	8	46,XX/47,XX,+18	2/20
Placenta 6	3	46,XX/46,XX,t(1;11)(p34.2;p15)	1/13
	5	46,XX	0/20
	8	46,XX	0/6
Placenta 12	3	46,XX	0/20
	5	46,XX (endoreduplication present)	0/20
	8	46,XX	0/10
Placenta 13	3	46,XX	0/10
	5	46,XX	0/15
	8	No metaphases seen	Not applicable
Bone marrow 5	3	46,XX	0/20
Bone marrow 9	3	46,XX	0/20
Bone marrow 15	3	46,XY/47,XY,+8/46,XY,t(1;18) (q25;q21)	2/20
	5	46,XY	0/20
	8	No metaphase cells seen	Not applicable
Bone marrow 30	3	46,XY	0/9
	5	No metaphases seen	Not applicable

number copy using CCNT showed no gross copy number changes in either of the two hpMSC passages (Fig. 5A) or in either of the two hbmMSC passages (Fig. 5B). A distribution of minor gains was evident in the hpMSC. However, because these were present in both passage 3 and passage 5 of the cell population, it was not possible to discern if they were acquired during very early culturing or reflect copy number polymorphisms within the original donor cells. With the rise in use of whole-genome copy number estimations, it has been recognized that all individuals carry multiple small copy number polymorphisms (CNPs) of an apparent benign nature. To ascertain whether the hpMSC CNP was culture derived or inherited by the donor, the original donor would need to be resampled (fresh peripheral blood mononuclear cells), a situation precluded by the ethical constraints of deidentification at the time of sample collection.

In vivo toxicity testing

Balb/c mice survived when hpMSC were administered intravenously at 2×10^4 cells/mouse (1×10^6 cells/kg), or 2×10^5 cells/mouse (1×10^7 cells/kg) (data not shown). One mouse died and one survived when hpMSC were administered at 2×10^6 cells/mouse (1×10^8 cells/kg) intravenously. Mouse health was not affected when hpMSC were administered intravenously at 2×10^4 cells/mouse (1×10^6 cells/kg) or 2×10^5 cells/mouse (1×10^7 cells/kg). Mice did suffer some transient acute toxicity when hpMSC

FIG. 4. Characterization of ex vivo expanded human MSC. Size range of hpMSC (**A**) and hbmMSC (**B**). Forward and side scatter profiles are shown together with inclusion of 30 micron beads (*circled*) for size estimation of live MSC (*blue box*). (**C**) Frequency of hpMSC isolated from placenta at passage 0 using flow cytometry. When gated for live cells, the CD73 and CD105 double positive population (*green box*) represents 1% of the CD45 negative cells. (**D**) Cell surface phenotype analysis by flow cytometry of hpMSC (*red*) and hbmMSC (*blue*). Isotype control is shown as grey filled histogram. Mesodermal differentiation ability of (**E**) hpMSC and (**F**) hbmMSC. Left hand column shows osteogenic differentiation using Alizarin Red S. Middle column shows chondrogenic differentiation using periodic acid schiff (PAS) staining. Right hand column shows adipogenic differentiation using Oil Red O staining. RT-PCR analysis of mRNA for lineage-specific markers of adipogenic (PLIN), osteogenic (RUNX2), and chondrogenic (ACAN) differentiation from hpMSC (**G**) and hbmMSC (**H**). Cells were grown in differentiation medium (*upper panel*) or normal growth medium (*lower panel*), as described in the Materials and Methods section, and actin was used as a positive control.

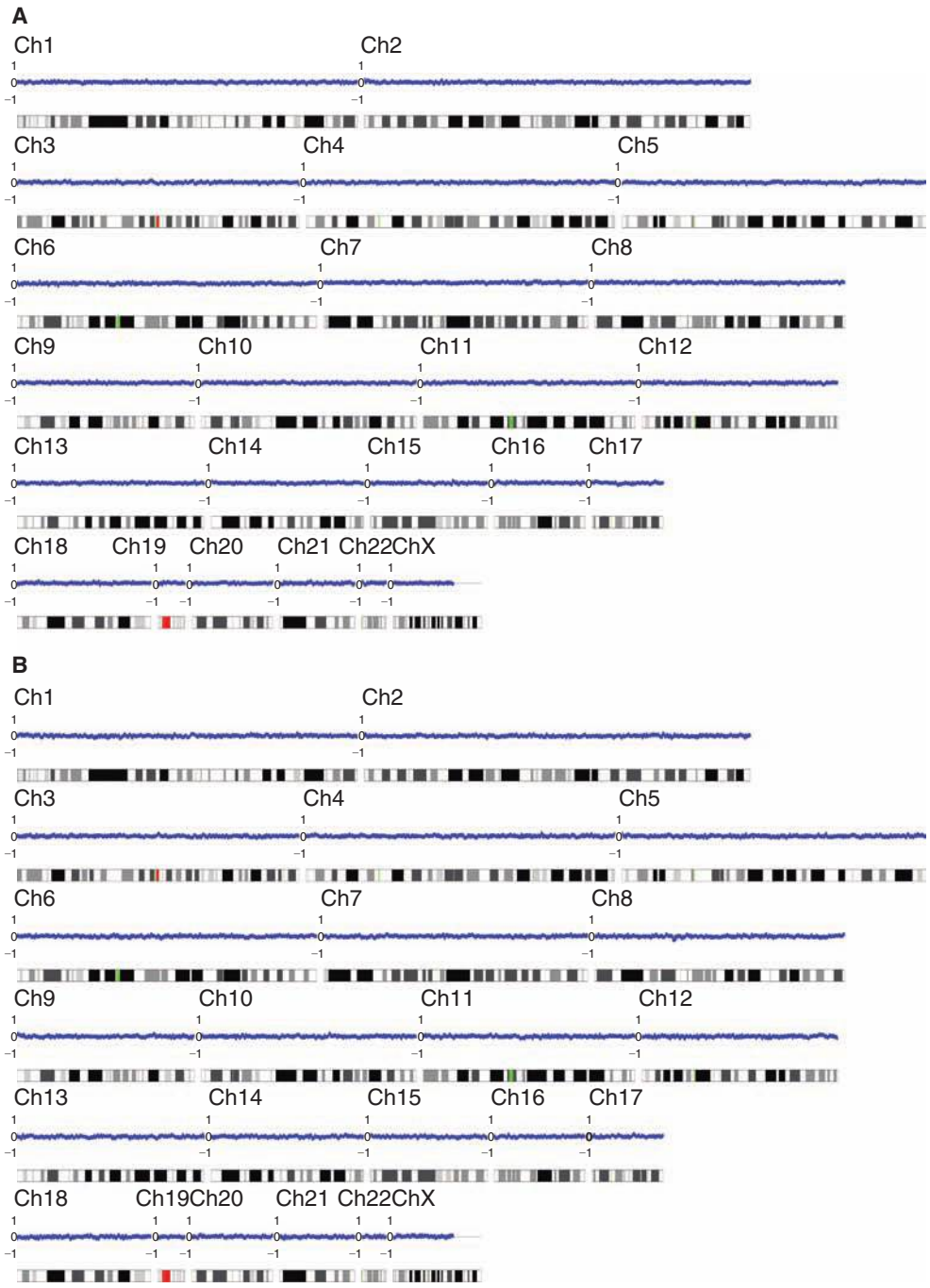

FIG. 5. Cytogenetic and SNP analysis of human MSC. (**A**) GeneChip SNP microarray analysis of hpMSC from placenta 13 (passage 5 graphed against passage 3). (**B**) GeneChip SNP microarray analysis of hbmMSC from sample 5 (passage 5 graphed against passage 2). Data analyzed using CNAT 2.0 software with copy number expressed on a $\log_2$ scale and SNP displayed by base pair distance.

were administered at 2×10^6 cells/mouse (1×10^8 cells/kg) intravenously.

Balb/c mice survived when hbmMSC were administered intravenously at 2×10^4 cells/mouse (1×10^6 cells/kg), 2×10^5 cells/mouse (1×10^7 cells/kg), or 2×10^6 cells/mouse (1×10^8 cells/kg) intravenously. Mouse health was not affected when hbmMSC were administered intravenously at 2×10^4 cells/mouse (1×10^6 cells/kg) or 2×10^5 cells/mouse (1×10^7 cells/kg). However, mice did suffer some transient acute toxicity when hbmMSC were administered at 2×10^6 cells/mouse (1×10^8 cells/kg) intravenously.

Discussion

In an attempt to increase the potential available sources of MSC for experimental and therapeutic use, we evaluated hpMSC and hbmMSC with respect to multiple aspects of their growth requirements and related biological properties. The growth requirements of hpMSC and hbmMSC were similar. Overall, however, we found that hpMSC consistently grew faster and more robustly than hbmMSC. hpMSC and hbmMSC exhibited a similar morphology, size, cell surface phenotype for characteristic MSC markers, and mesodermal

differentiation ability for osteogenic and chondrogenic lineages. In contrast, we consistently found hpMSC generally less readily able to demonstrate adipogenic differentiation using this culture assay. Chang et al. [31] found that cord blood–derived MSC had a reduced adipogenic differentiation ability as compared to bone marrow MSC. This study showed that leptin, which has been shown to be synthesized and secreted by the placenta, blocked the accumulation of lipid in adipocytes. The role of leptin in hpMSC differentiation is unknown. The size range of the cells was striking with diameters up to 120 μm, thus explaining the propensity of these cells to be held up in the pulmonary capillary bed after intravenous injection. This is a potential cause of acute toxicity. However, no toxicity was noted in mice intravenously injected with hpMSC or hbmMSC at doses equivalent to, or up to 1 log higher than currently used in clinical trials using hbmMSC. Mice did have toxicity when injected with the equivalent of 1×10^8 MSC/kg. However, this dose is 10–100 times higher than has been used in clinical trials to date and a dose of 1×10^7 MSC/kg was safe in these experiments.

We found in the experimental conditions assessed in this study that a lower seeding density was associated with a faster proliferation rate of hpMSC but not of hbmMSC. However, for clinical trial manufacturing purposes, it is probably preferable to obtain as many MSCs in as few passages as possible, in order to minimize the risk of genetic mutation. For clinical trial manufacturing we have found a seeding density of 2,000 cells/cm^3 to be optimal.

Other groups have used similar methodologies to derive MSC from placenta [19–21]. The method of preparing the placenta has utilized dissection, followed by enzymatic digestion, then gradient centrifugation to obtain a mononuclear interface prior to washing and plating on plastic flasks in basal medium with 10–20% FCS at 37°C in 5% CO_2. Interestingly, when amnion or amniotic fluid have been used as a source, the derived MSC were found to be of fetal rather than maternal origin [19], and amniotic tissue–derived MSCs may have significantly greater differentiation potential than those derived from placenta [24]. In our study, cytogenetic analysis in three of three informative cases (when the delivered baby was a male), hpMSCs were shown to be of maternal origin. Other reports on placenta-derived MSCs have shown variable results in this regard: Wulf et al. [20] used whole placenta after removal of the amniotic membranes and also found the resulting MSCs to be exclusively of maternal origin. In contrast, In 't Anker et al. [19] found MSCs derived from placenta to be of maternal origin, but those derived from amnion or amniotic fluid were predominantly of fetal origin. Clearly it is possible to derive MSCs of both maternal and fetal origin from these gestational products. It will be interesting to compare the multipotent differentiation potential of MSCs from each source. Of note, Yen et al. found that hpMSC express the pluripotent cell surface markers SSEA-4, TRA 1-60, and TRA 1-81, but these were absent on adult bmMSC [21]. Another study found these markers to be present on first-trimester fetal blood, liver, and bone marrow–derived MSC but absent on adult bmMSC [32]. As we have shown, the placenta-derived MSC isolated by our methods resulted in cells of maternal origin and not fetal origin. The expression of these pluripotent cell surface markers is likely reflective of the adult or fetal source of the stem cells in question.

The culturing of cells for the purpose of their subsequent administration to a patient has highlighted questions around the genomic stability of cells grown in culture long term. During both cultures of cells for routine cytogenetic analysis and cultures of stem cells, spontaneous chromosomal changes are relatively frequently observed. These changes do not obviously increase the rate of cell proliferation, and do not accumulate through successive passages, with most studies indicating that a normal karyotype is maintained for 30–40 passages in vitro [33]. However, a second category of cytogenetic anomaly in cultured cells has been reported [34]. This report described several cases of cell cultures with specific chromosomal changes. These abnormal cells proliferated within the culture to the detriment of the normal 46,N cells, rapidly leading to a population where all cells in the culture expressed the chromosomal abnormality. Although this second category has been described only in cells that have undergone over 30 passages, it highlights the dilemma that an optimal dosage of MSC must be weighed against the time in culture required to reach sufficient cell numbers for effective treatment. To address this issue and to establish a criterion for growing MSC in culture for clinical trial use, metaphase karyotyping was performed on hbmMSC and hpMSC samples. Chromosomal changes were detected in a minority of both hbmMSC and hpMSC cultures at passage number 3, 5, and 8. These changes included trisomies, balanced translocations, and unbalanced translocations leading to partial duplication/deletion. These cell lines did not appear in subsequent passages, and seemed to have no selective advantage over normal cells. This result is in concordance with the observation that cytogenetic changes at low passage numbers rarely survive. Thus, it appears from this study that cells grown up to passage 8 may be used in clinical trials. However, the incidence of cytogenetic abnormality observed strongly suggests that samples destined for use in patients be karyotyped before use, at least until a comprehensive inter-laboratory investigation defines the scope of the problem of karyotype instability and its relation to culture conditions and passage number. In our own MSC clinical trials, normal karyotype is currently a release criterion for the clinical use of the cells.

Cytogenetic analysis does not detect microdeletions/duplications or very subtle rearrangements. It is possible that these subtle anomalies are present and yet are not detectable by conventional G-banded analysis. Therefore a higher resolution methodology using GeneChip-based SNP analysis was undertaken. The aim of using GeneChip-based SNP analysis was to provide a detailed comparison between culture passages of two MSC lines for the potential acquisition by the cells of genomic alterations too small to be identified by conventional karyotyping. The GeneChip data were used to generate both an internal comparison of copy number and an internal comparison of SNP type.

Neither of the two samples had any change in copy number between early and later passages, using early passage as a baseline measurement representing a diploid state.

This supports the karyotype data, carried out to passage 8, that the cell culture environment is prompting growth but not simultaneously applying selective pressure that could cause an outgrowth of cytogenetically abnormal cells with acquired growth advantage through mutation.

Although the combination of karyotyping and GeneChip-based SNP and copy number estimation fall short of a complete genomic scan (only possible by complete genome sequencing), both techniques give a measure of genome integrity, providing information about potential rearrangements and copy number changes. An internal comparison of early and late passage samples showed no loss of heterozygosity, an alteration of which could reflect a cell culture–selected mutation with growth advantages. A small number of individual SNP sites (0.33% for hbmMSC and 0.21% for hpMSC) did vary between early and late passage number in both hbmMSC and hpMSC, suggesting that minor alterations had occurred, and that the significance of such changes will need to be evaluated in future studies.

While whole genome assays provide a result at the sample population level and may not be able to identify individual cells with a mutation-conferring growth advantage, it has been demonstrated here that utilizing hpMSC out to passage 5 will provide a population highly similar to early passage cells.

Our group and others have recently demonstrated the immunosuppressive capacity of hpMSC and it appears equivalent to that of hbmMSC, at least in terms of the ability to suppress an alloproliferative T-cell response in mixed leukocyte culture [35,36], thus indicating that hpMSC could be explored for such therapeutic applications as the treatment of corticosteroid-refractory acute graft-versus-host disease [18,25], in addition to their potential in tissue repair and regeneration.

In conclusion, this is the first study to compare the optimal growth conditions required to expand ex vivo hpMSC and hbmMSC. Both populations were similar in terms of growth condition requirements and in terms of subsequent biological characterization. hpMSC grew more robustly under controlled culture conditions than hbmMSC. While karyotypic changes were detected in a minority of metaphases in both hpMSC and hbmMSC, SNP analysis indicated genomic stability between passages. Both types of MSC were tolerated without short-term toxicity in mice at the dosages normally encompassed in clinical trials of MSC. This study provides a basis for the use of placenta-derived MSC in clinical trials, and we have recently completed our first manufacturing production run of placenta-derived MSC for this purpose.

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