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Preferential chondrogenic differentiation potential of human bone marrow-derived mesenchymal stem cells

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Abstract

Objective: Mesenchymal stem cells are multipotent cells capable of differentiation into several mesenchymal lineages. These cells have been isolated from various tissues such as adult bone marrow, placenta and fetal tissues. Since a specific phenotypical marker for MSCs is lacking, MSCs are currently characterized on the basis of phenotype and capacity to differentiate into multiple mesenchymal lineages. However, how the potential of these cells to differentiate into the chondrogenic lineage is influenced by the tissue of origin has not been examined. The aim of this study was to investigate whether MSCs isolated from different sources exhibit differential multilineage differentiation potential.
Design: MSCs from fetal and adult tissues were phenotypically characterized and examined for their differentiation capacity, based on morphological criteria and expression of extracellular matrix components.
Results: Our results show that both fetal and adult MSCs undergo chondrogenesis under appropriate conditions. Nevertheless, MSCs of bone marrow origin, either fetal or adult, exhibit a higher chondrogenic potential than fetal lung and placenta derived MSCs, as demonstrated by the appearance of typical morphological features of cartilage, the intensity of Toluidine Blue staining and the expression of collagen type II, IX and X after culture under chondrogenic conditions. In addition, the capacity of MSCs to differentiate into chondrocytes was reduced upon passaging of cells.
Conclusions: MSCs are an attractive source for cartilage tissue engineering strategies. Hence, exploring the chondrogenic potential of different sources is of great interest for such a purpose. Our study indicates that bone marrow is to be considered as the preferred MSC source for cartilage engineering.

Introduction

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Articular cartilage has a limited capacity of healing after injury. Traumatic damage and degenerative diseases of the cartilage such as osteoarthritis or rheumatoid arthiritis are common health problems worldwide. Therefore a lot of interest has recently emerged in techniques for cartilage tissue engineering. The current strategies of cartilage repair, based on the use of autologous chondrocytes, have some limitations including the small number of cells available with restricted proliferative capacity and the further damage at donor site of harvest. For these reasons, new techniques are now focusing on the use of mesenchymal progenitor cells to be delivered within an appropriate carrier system to repair and regenerate pathologically altered cartilage [1,2]. In fact, mesenchymal stem cells (MSCs) play a role in bone and cartilage homeostasis and it has been shown that the chondrogenic activity of these cells is reduced in patients with advanced osteoarthritis (OA) [3]. One of the mechanisms involved in the repair of damaged articular cartilage may be the *de novo* chondrogenesis from MSCs [4,5]. Therefore, based on the *in vitro* observation of the differentiation into chondrocytes, on their expandability and availability, MSCs can be considered as an attractive candidate for purposes of cartilage engineering [6,7].
MSCs are multipotent cells with the ability to differentiate into several mesenchymal lineages, including osteoblasts, adypocytes and chondrocytes [8-10]. Although MSCs were originally isolated from bone marrow [11], they have also been isolated from other tissue sources. MSCs have been identified in fetal tissues such as lung, bone marrow, liver and spleen in first- and second-trimester [12,13]. Placenta has been shown to be another rich source of MSCs of both fetal and maternal origin [14];

and MSCs have been also isolated from umbical cord blood, although in low frequency, and adipose tissue. At present no unique phenotype has been identified for MSCs; therefore the isolation and characterization of MSCs relies on the expression of a number of characteristic markers on culture expanded cells and on their ability to differentiate into the various mesenchymal differentiation lineages.
In the present study, we investigate the multilineage differentiation potential of MSCs derived from 4 different sources and whether this capacity is influenced by the tissue of origin.

Materials and methods

Isolation and culture of human MSCs

Fetal tissues

Fetal lung (fL) and fetal bone marrow (fBM) were obtained from the same fetus from women undergoing elective termination of pregnancy between 15 and 22 weeks of gestation. The study was approved by the hospital ethical committees and informed consent was obtained. Single cell suspensions of fetal lung were made by mincing and flushing the organ through a 100 mm nylon filter with IMDM medium (Cambrex, Verviers, Belgium) containing 1% penicillin/streptomycin (P/S; Cambrex, Verviers, Belgium) and 2% heat-inactivated fetal calf serum (FCS; Cambrex, Verviers, Belgium), i.e. washing medium. Single cell suspensions of fetal bone marrow were obtained by penetrating the long bones with a needle (23 gauge) and flushing the bones with washing medium. After washing, the cell suspension was depleted of red cells by incubation for 10 minutes in NH_4Cl (8,4 g/L)/$KHCO_3$ (1/g) buffer at 4 °C. The cells were subsequently plated at 160.000/cm^2 in culture medium consisting of M199 (Gibco, Paisley, Scotland) supplemented with 10% FCS, P/S, Endothelial Cell Growth Factor (ECGF) 20 mg/ml (Roche Diagnostics GmbH, Mannheim, Germany) and heparin 8 U/ml in tissue culture flasks (Greiner Bio-One GmbH, Mannheim, Germany) previously coated with 1% gelatin for 30 minutes at room temperature.

Placenta

Placentas (PL) were derived from second- and third-trimester pregnancies after informed consent and approval by the hospital ethical committees. Tissue specimens of placenta (maternal origin) were first washed with PBS; single cell suspensions were made by mincing and flushing the tissue through a 100 mm nylon filter with washing medium. The cells were subsequently cultured as described above. To confirm the maternal origin of these cells, molecular HLA-typing was performed on the PL-MSCs cultures.

Adult bone marrow

Adult bone marrow (aBM) samples were obtained from healthy donors for allogeneic stem cell transplantation under a protocol approved by the Ethical Review Board. Mononuclear cells were isolated by density gradient (Ficoll, 1.077 g/ml) and plated at 160.000/cm^2 in culture medium consisting of DMEM-Low Glucose (Gibco, Paisley, Scotland) supplemented with 10% FCS and P/S.
All cells were kept in a humidified atmosphere at 37 °C with 5% CO_2. Three to five days after plating, non-adherent cells were removed and the medium was refreshed. When grown to confluency, adherent cells were detached with trypsin/EDTA (Cambrex, Verviers, Belgium) for 5 minutes at 37 °C and reseeded for expansion or differentiation.

Flow cytometric analysis

Culture expanded fL-, fBM-, aBM- and PL-MSCs were phenotypically characterized by flow cytometry (FACSScan, Becton Dickinson). Fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated antibodies against CD166 (CLB, Amsterdam The Netherlands), CD105 (Ancell Corporation, Bayport, MN, USA), CD90 (Pharmingen, San Diego, CA), CD34, CD45 and CD80 (Beckton Dickinson, San Jose, CA), CD31 (DAKO, Glostrup, Denmark), HLA-ABC (Instruchemie, Hilversum, The Netherlands), HLA-DR (Beckton Dickinson, San Jose, CA) were used, as well as isotype controls.

Osteogenic and adipogenic differentiation

The adipogenic and osteogenic differentiation capacity of MSCs from the 4 different sources was determined as previously described [13]. In short, to induce osteogenic differentiation, cells were cultured in a-MEM supplemented with 10% FCS, P/S, dexametasone (10^{-7} M) and ascorbic acid (50 mg/ml). b-glycerophosphate (5 mM) was added from day 7 onwards. For adipogenesis, insulin (10 mg/ml), indomethacin (0,25 M) and 1-methyl-3-isobutylxantine (IBMX, 50 mM) were added to this medium. Cells were incubated in differentiation medium for 3 weeks, with medium replacement twice a week, at 37°C with 5% CO_2. To detect the osteogenic differentiation cells were stained for alkaline phosphatase (AP) activity using Fast Blue and for calcium depositions with Alzarin Red. The adipogenic differentiation was evaluated through the morphological appearance of fat droplets.

Chondrogenic differentiation

For chondrocyte differentiation, 200,000 MSCs were placed per well in 96-well suspension culture plates, U-shape, (Greiner Bio-One GmbH, Mannheim, Germany) and centrifuged at 1200 rpm for 4 minutes to a pellet. Pellets were cultured at 37 °C with 5% CO_2 in 200 ml of serum-free chondrogenic medium consisting of DMEM-High Glucose (Gibco, Paisley, Scotland), 40 mg/ml proline (Sigma, USA), 100 mg/ml sodium pyruvate (Sigma, USA), 50 mg/ml ITS+Premix (BD Biosciences, Bedford, MA), 1% Glutamax (Gibco, Paisley, Scotland), P/S, 50 mg/ml ascorbate-2-phosphate (Sigma, USA), 10-7 dexametasone (Sigma, USA), 10 ng/ml Transforming growth Factor-b3 (TGF-b3, R&D Systems, Minneapolis) and 500 ng/ml Bone Morphogenetic Protein-6 (BMP-6, kindly provided by dr. S. Vucikevic) The medium was refreshed every 3 days for 21 days. We investigated at least three samples per source and 3 different passages per sample.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from 1 million undifferentiated MSCs and from pellets at day 21 of the differentiation period, from each source, using RNAeasy kit (Quiagen Gmbh, Hilden). RNA was reverse transcribed into cDNA using First Strand cDNA Synthesis kit for RT-PCR (Roche Diagnostics Gmbh, Mannheim, Germany) according to the manifacturer's instructions. cDNA was amplified using a GeneAmp PCR System 2400 (Applied Biosystems, Foster City, CA). Expression of collagen type II, IX and X mRNA was quantified by real-time quantitative PCR using the Bio-Rad iCycler with SYBR Green. Data were corrected for β_2-microglobulin expression. The following oligonucleotide primers were used: collagen type II: forward 5'-CCCTCTCCCACACCTTCCTC-3' and reverse 5'-GGGTGAGGGATTCCAGGGAAA-3'; collagen type

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IX: forward 5’-AGGACACAAGGGTGAAGAAGGT-3’ and reverse 5’-TTTTCCCCTTTGTCCCCAACTATG-3’; collagen type X: forward 5’-TTTTGCTGCTAGTATCCTTGAACTT-3’ and reverse 5’-AGGAGTACCTTGCTCT CCTCTTACT-3’; β_2-microglobulin: forward 5’-TGCTGTCTCCATGTTTGATGTAT CT-3’ and reverse 5’-TCTCTGCTCCCCACCTCTAAGT-3’. All PCR reactions were performed with 5 ng cDNA and according to the manufacturer’s protocol of the qPCR Core Kit (Eurogentec, Southampton, UK) in a final volume of 25 µl.
The cDNA was amplified using the following thermal cycling conditions: one cycle at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each sample was assayed in triplicate and water was used as a negative control. Fluorescence spectra were recorded and the threshold cycle number (Ct) was read. For each source mean Ct was calculated and from this value the fold difference from expression in the human growth plate according to the equation $2^{-\Delta\Delta Ct}$. For visualization, this value was log-transformed and expressed in figure 2C.

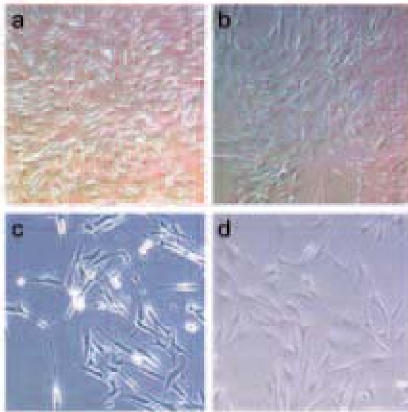
Histological analysis

After 21 days of culture in chondrogenic medium, pellets were fixed in 10% formalin, dehydrated by treatment with graded ethanols and incubated in butanol overnight. Thereafter pellets were embedded in paraffin and cut into 5 mm sections using a Reichert Jung 2055 microtome (Leica, Rijswijk, The Netherlands). The sections were then mounted on glass slides and stained with Toluidine Blue.
The immunohistochemical staining for collagen type X was performed on deparaffinized sections. To block non-specific activity, sections were pretreated with hydrogen peroxidase. Sections were stained with mouse monoclonal antibody against collagen type X (Quartett, Berlin, Germany). The binding of mouse IgG was detected by biotinylated rabbit-anti-mouse IgG (DAKO, Glostrup, Denmark), followed by incubation with horseradish-peroxidase-conjugated-streptavidine (Amersham Biosciences, UK). The peroxidase activity was revealed using 3-amino-9-ethylcarbazole (AEC) substrate. After sections were washed, they were counterstained with hematoxylin.

Results

Morphology and immunophenotypic characterization of MSCs

MSCs isolated from all four different sources displayed the characteristic MSC-like spindle-shape, however subtle differences in morphology were present (Figure 1A). fL- and PL-MSCs showed a more elongated and thin shape compared to the rounder and thicker shape of BM-MSCs.
MSCs from all samples were immunophenotypically analyzed at passage 2 or 3. The phenotypes of fL-, fBM-, aBM- and PL-MSCs were similar and in agreement with previous publication [9,13,14], i.e. CD90, CD105, CD166, HLA-ABC positive and CD34, CD45, CD31, CD80, HLA-DR negative (Figure 1B).



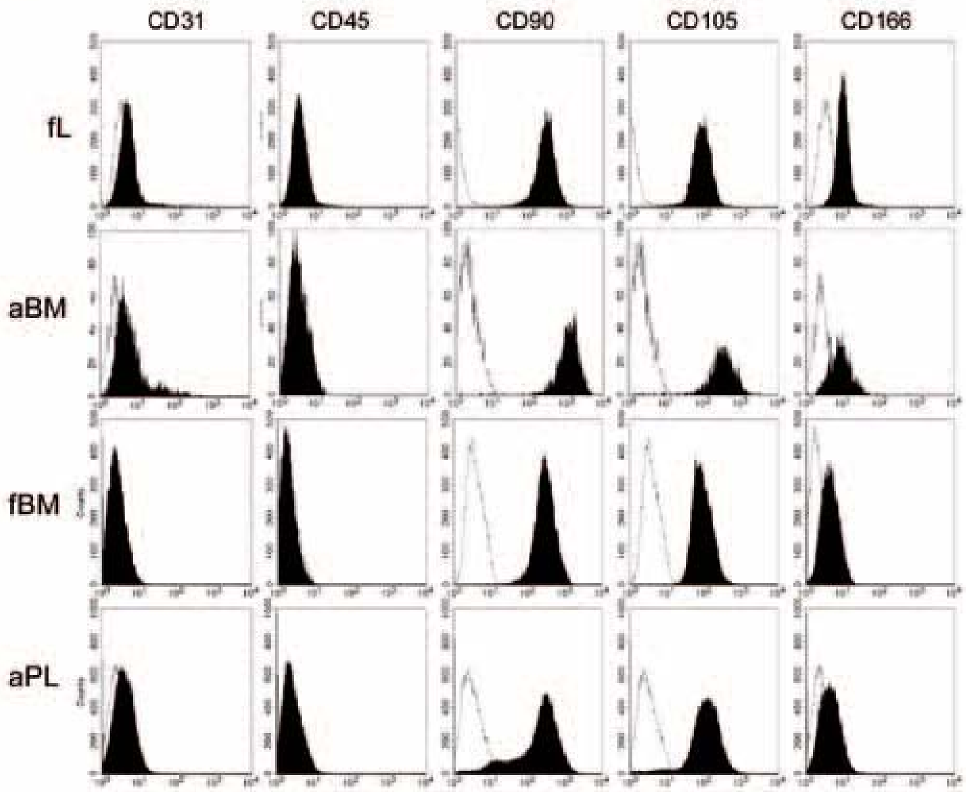


Figure 1. Morphology and immunophenotype of culture-expanded MSCs.

(A) MSCs from the four different sources display the characteristic spindle-shaped morphology, however subtle differences are present. **a, b:** Morphology of fBM- and aBM-MSCs respectively, showing a splindle-round shape. Magnification x5. **c, d:** Morphology of fL- and PL-MSCs respectively, displaying a spindle-thin shape. Magnification x10. **(B)** Immunophenotypic characterization of MSCs from a representative sample.

Osteogenic and adipogenic differentiation

To examine the differentiation capacity of MSCs from the different sources, cells were induced into osteoblasts and adipocytes and examined by histological stainings. fL-, fBM-, aBM-MSCs and PL-MSCs were all able to differentiate into osteoblasts as demonstrated by the histologic detection of alkaline phosphatase activity and calcium depositions, and into adipocytes as revealed by the formation of lipid droplets (data not shown). However, PL-MSCs showed a lower ability to form both osteoblasts and adipocytes, while fL-MSCs were less capable to differentiate into the adipogenic lineage, as compared to bone marrow sources (Table 1).
The differentiation capacity into osteoblasts and adipocytes was maintained, unmodified, untill passage 7 (P7).

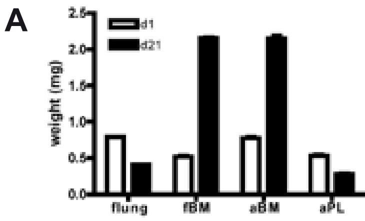
Table 1. Differentiation potential of MSCs derived from different tissue sources

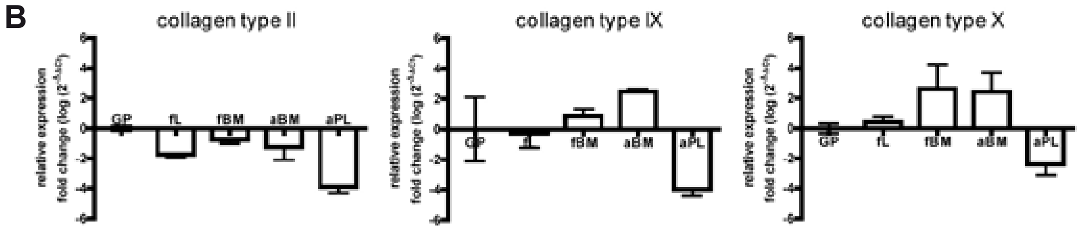
	Adipocyte-like morphology	Alzarin Red staining Intensity	Toluidine Blue Intensity	Chondrocyte-like morphology	N. of positive pellets/total N. of pellets evaluated
fL-MSC	+	++	+/-	+/-	0/4
fBM-MSC	++	++	++	++	3/3
aBM-MSC	++	++	+	++	4/4
PL-MSC	+/-	+/-	-	-	0/4

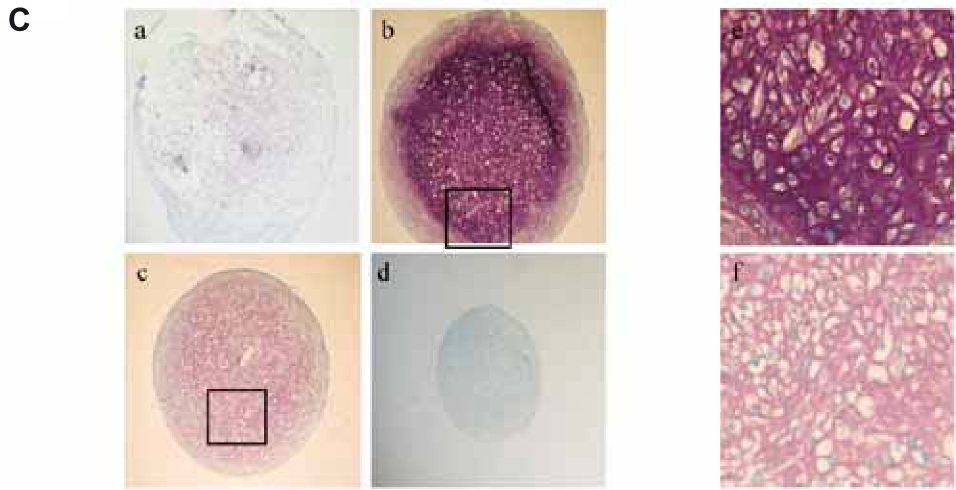
The presence of adipocyte-like morphology, the intensity of Alzarin Red and Toluidine Blue staining and the chondrocyte-like morphology are scored as: -, +/-, +, ++. The adipocyte-like morphology is defined by the appearance of fat droplets. The chondrocyte-like morphology is defined by the following features: decrease in cell density with distance between cells, rounded morphology of cells and nuclei, deposition of extracellular matrix, presence of chondrocytic lacunae. Only pellets that are scored + or ++ for both the Toluidine Blue staining and the chondrocyte-like morphology are considered positive for cartilage formation (last column on the right).

The chondrogenic differentiation capacity of MSCs is influenced by the tissue of origin

Next, the chondrogenic differentiation capacity of MSCs of different origin was examined and compared. FL- (n= 4 samples), fBM- (n= 3 samples, derived from the same fetus), aBM- (n= 4 samples) and PL-MSCs (n= 4 samples, maternal origin) were centrifuged into micromasses, cultured as pellets and differentiated in serum-free medium containing ascorbate-2-phosphate, dexamethasone, TGF-b3 and BMP-6.
MSCs from all sources formed a pellet after centrifugation, although pellets consisting of fL- and PL-MSCs were frequently less stable and showed a more irregular shape compared to the pellets from fBM- and aBM-MSCs that were firm and spherical. Pellets derived from fBM- and aBM-MSCs were larger in size after 21 days of culture compared to those derived from fL- and PL-MSCs. In particular, fBM- and aBM-MSCs showed an increase in both the diameter and the area of the pellets after 3 weeks of culture in chondrogenic medium (data not shown). Furthermore, a 4.2- and a 2.9-fold increase in weight was observed in pellets formed by fBM and aBM-MSCs respectively at day 21 of culture, whereas fL- and PL-MSCs exhibited a decrease in weight of pellets over the culture period (Figure 2A). These observations suggest that cartilage extracellular matrix has been syntesized and deposited in pellets from bone marrow cells, leading to the increase in size and weight.
To more specifically analyze the process of chondrogenesis, the expression of collagen type II, IX and X was measured by Q-PCR on undifferentiated cells and on pellets 3 weeks after induction (Figure 2B). Pellets formed by fBM- and aBM-MSCs showed a marked increase in the expression of collagen type II, IX and X after the induction period, compared to fL- and PL-MSCs pellets. These data were confirmed by the histological analysis: fBM- and aBM-MSCs produced more proteoglycans, hence more extracellular matrix, and expressed more chondrocyte-like *lacunae*, as indicated by the intensity of the Toluidine Blue staining (Figure 2C). Moreover, the immunohistochemical staining for collagen type X was positive only in sections derived from fBM and aBM samples, suggesting that only these cells started to terminally differentiate into hypertrofic chondrocytes (Figure 2D). A scoring system was used to compare the different degrees of chondrogenic differentiation between the 4 sources; evaluating the intensity of the Toluidine Blue staining and the appearance of the typical chondrocyte-like morphology, both scored from – to ++. The stained sections from both fBM- and aBM-MSCs obtained the highest score with 100% of the samples evaluated demonstrating differentiatiation into chondrocytes; in fact, fBM- and aBM- cells showed the most intense Toluidine Blue staining and the typical morphological features of differentiated cartilage such as the decrease in cell density with distance between cells, the rounded morphology of cells, the deposition of extracellular matrix stained in purple and the presence of chondrocytic lacunae, whereas samples from fL- and PL-MSCs didn't (Table 1).


A
d1
d21
weight (mg)
flung
fBM
aBM
aPL


B
collagen type II
collagen type IX
collagen type X
relative expression
fold change (log (2-ΔΔCt))
GP
fL
fBM
aBM
aPL


C
a
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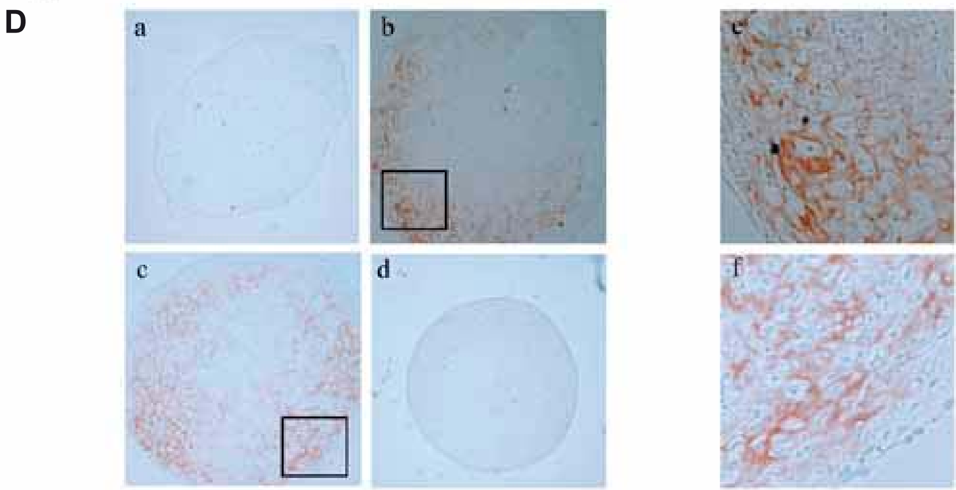
D
a
b
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e
f

Figure 2. Chondrogenic differentiation of MSCs from different sources. (page 156)
(A) Wet weights of the pellets from the four different sources at day 1 and 21 of the induction period (n = 3). **(B)** Real-time RT-PCR analysis of collagen type II, IX and X during chondrogenic differentiation corrected for the housekeeping gene b2 microglobulin. Values are fold difference compared to expression in human growth plate (GP) and expressed as log ($2^{-\Delta\Delta Ct}$) **(C)** Toluidine Blue staining on paraffin embedded sections after 3 weeks of differentiation. Representative pictures from each source at passage 3 are shown: a) fL-MSCs; b) fBM-MSCs; c) aBM-MSCs; d) PL-MSCs; e) and f) are enlargements of figure 2C b) and c) respectively, for detailed morphology. **(D)** Immunohistochemical staining for collagen type X on deparaffinezed sections after the induction period. Representative pictures at passage 3 are depicted: a) fL-MSCs; b) fBM-MSCs; c) aBM-MSCs; d) PL-MSCs; e) and f) are enlargements of figure 2D b) and c) respectively, for detailed morphology.

The chondrogenic differentiation capacity of MSCs decreases with passage number

In order to examine whether the number of cell passages has an influence on the chondrogenic differentiation capacity of MSCs, different passages of fL-, fBM-, aBM- and PL-MSCs were evaluated. When considering fBM- and aBM-MSCs, a passage-dependent decrease in cartilage formation was observed (Table 2). In particular, the early passages (P2-3) displayed the most intense Toluidine Blue staining and all the typical features of differentiated cartilage, whereas the later passages (P4-5 and P6-7) showed only a moderate or mild staining with less evidence of cartilage differentiation (data not shown). Moreover, the capacity of BM-MSCs to differentiate into chondrocytes declined between passage 6 and 8. For fL- and PL-MSCs, the influence of cell passage was not evident since little or no differentiation towards the chondrogenic lineage was observed overall.

Table 2. Influence of cell passage on the chondrogenic capacity of MSCs from different tissue sources.

	P2-3	P4-5	P6-7
fL-MSC	0/4	0/4	0/4
fBM-MSC	3/3	3/3	1/3
aBM-MSC	4/4	4/4	2/4
PL-MSC	0/3	0/3	0/4

The differentiation capacity is expressed as number of positive samples on the total number of samples evaluated per passage-group. A sample is scored positive when satisfying the criteria described in Table 1.

Discussion

Skeletal defects resulting from disease, malformation or injury are an interesting area of application for stem cell therapy. The therapeutic effect of the transplantation of MSCs in children with *Osteogenesis Imperfecta*, a genetic disorder resulting in the abnormal production of collagen type I [15-17], suggests the potential of MSCs to ameliorate bone disorders. MSCs are an ideal candidate for strategies of tissue engineering since they are easily isolated and can be rapidly expanded to numbers that are required for clinical application [10,18,19].

In this study we compared the chondrogenic differentiation potential of culture expanded MSCs derived from fetal BM and lung, placenta and adult BM. In comparison with fetal lung and placenta, fetal BM derived MSCs exhibited a significant enhanced capacity to differentiate into chondrocytes, as evidenced by the increase in weight, diameter and area of pellets formed by bone marrow cells over the differentiation period, the increased levels in the expression of mRNA of extracellular matrix components, such as collagen type II, IX and X, the positivity of the immunohistochemical staining for collagen type X and the marked intensity of the Toluidine Blue staining. A similar prefered chondrogenic potential was observed for adult BM-derived MSCs, showing that BM as a source of MSCs was responsible for this differentiation potential, rather that the fetal developmental stage of the tissue. The chondrogenic differentiation was reduced and ultimately lost, after prolonged passages of the cultures. Although in vivo experiments are required to further substantiate the biological significance of these findings, our results suggests BM as the preferred source for cartilage tissue engineering.

MSCs have been shown to require specific culture conditions to induce differentiation towards the chondrogenic lineage. These requirements include a high cell density facilitating cell-cell contact and the use of serum-free medium with the addition of bioactive factors [20]. In particular BMP-6, a member of the TGF-b superfamily of growth factors, has been demonstrated to enhance the chondrogenic differentiation of human MSCs in a pellet culture system [21,22]. Our results confirm these findings and add that not only aBM-MSCs but also MSCs isolated from fetal tissues, namely lung and bone marrow, can undergo chondrogenesis under the same conditions.

In a study from *in't Anker* and colleagues [13] it was shown that MSCs isolated from second-trimester fetal bone marrow, lung, liver and spleen exhibit a different potential to differentiate into osteoblast and adipocytes, despite a similar immunophenotype. Moreover, a recent study from *Im et al* [23] demonstrated that adipose tissue-derived MSCs possess a lower osteogenic and chondrogenic potential than BM-derived MSCs. Also in our experiments, despite MSCs derived from the four different sources showed a comparable phenotypic characterization and morphology, the capacity to form cartilage was more expressed in fBM- and aBM-MSCs. Moreover, we found that PL-MSCs were less capable to form both osteoblasts and adipocytes, while fL-MSCs showed to have a lower ability to differentiate into the adipogenic lineage compared to bone marrow sources. These differences in the differentiation potential might reflect some intrinsic diversities of MSCs residing in the various tissues, suggesting that the relation between immunophenotype and function of MSCs needs to be further investigated. Alternatively, the frequency of cells with lineage-specific differentiation capacity may differ between tissue sources. Indeed, the identification of specific markers enabling to distinguish between different populations of MSCs would be an important tool in the understanding and employment of these cells in the clinical setting.

We found that the chondrogenic potential of MSCs decreases with the increase of the cell passage, as shown by the inferior growth in size and the mild intensity of the Toluidine Blue staining in pellets from the later passages (P6-7). These findings are in agreement with the study from *Sekiya*

et al [22] in which a decrease in the chondrogenic potential of adult bone marrow-derived MSCs was seen after each consecutive passage under very similar culture conditions. In *Sekiya* experience, only a selected population of MSCs, the so called small and rapidly self-renewing cells (RS cells), retained the ability to form cartilage at P5. This loss of chondrogenic potential may be due to the graduate elimination of MSCs with chondrogenic potential and overgrowth by MSCs that lack differentiation potential, or may be due to functional alterations in chondrogenic MSCs.
In conclusion, our results show that: I) MSCs of both fetal and adult origin undergo chondrogenesis under appropriate culture conditions; II) fBM- and aBM-MSCs express a higher chondrogenic ability than fL- and PL-MSCs, based on morphological, molecular, histochemical and immunohistochemical criteria; III) an inverse correlation between passage number and chondrogenic differentiation capacity of MSCs is present. Based on these observations and considering the attractive role that MSCs could play in strategies of cartilage tissue engineering, our data suggest that bone marrow cells are to be considered as the preferred MSC source to be employed for cartilage repair.

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