Mesenchymal stem cells in human second-trimester bone marrow, liver, lung, and spleen exhibit a similar immunophenotype but a heterogeneous multilineage differentiation potential

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Background and Objectives. We previously found that human fetal lung is a rich source of mesenchymal stem cells (MSC). Here we characterize and analyze the frequency and function of MSC in other second-trimester fetal tissues.

Design and Methods. Single cell suspensions of fetal bone marrow (BM), liver, lung, and spleen were made and analyzed by flow cytometry for the expression of CD90, CD105, CD166, SH3, SH4, HLA-ABC, HLA-DR, CD34 and CD45. We assessed the frequency of MSC by limiting dilution assay.

Results. The frequency of MSC in BM was significantly higher than in liver, lung, and spleen ($p<0.05$). On primary non-expanded cells from fetal liver, lung and spleen the number of cells positive for *mesenchymal* markers was significantly higher within the CD34 positive population than within the CD34 negative population. The phenotype of the culture-expanded MSC was similar for all fetal tissues, i.e. CD90, CD105, CD166, SH3, SH4 and HLA-ABC positive and CD34, CD45 and HLA-DR negative. Culture-expanded cells from all tissues were able to differentiate along adipogenic and osteogenic pathways. However, adipogenic differentiation was less in MSC derived from spleen, and osteogenic differentiation was reduced in liver-derived MSC ($p<0.05$).

Interpretation and Conclusions. Our results indicate that culture-expanded MSC derived from second-trimester fetal tissues, although phenotypically similar, exhibit heterogeneity in differentiating potential. We speculate that these differences may be relevant for the clinical application of MSC.

Key words: mesenchymal stem cells, fetal tissues, limiting dilution assay, multilineage differentiation potential

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Human mesenchymal stem cells (MSC) are multipotent cells that are able to differentiate along different pathways including the osteogenic, adipogenic and chondrogenic lineages.[1-5] MSC can be isolated from various sources. Their presence was first identified in human adult bone marrow, and later also in adult peripheral blood, periosteum and muscle connective tissue.[6-8] MSC play an important role in early tissue formation; therefore their frequency in fetal tissues is likely to be higher than in adult tissues.[9] In agreement with this, MSC have also been isolated from first-trimester fetal blood, liver, bone marrow[10] and second-trimester fetal lung.[11] However, the frequency of the MSC in these fetal tissues was not assessed.

Preliminary studies have indicated that MSC may be applied therapeutically for the correction of disorders of mesenchymal origin. Allogenic bone marrow transplantation in children with *osteogenesis imperfecta* resulted in development of donor-derived osteoblasts, along with an increased bone density.[12,13] Another possible therapeutic application of MSC is based on their ability to enhance the engraftment of hematopoietic stem cells (HSC) after transplantation. We previously showed that fetal lung-derived MSC enhance the engraftment of umbilical cord blood (UCB)-derived CD34+ hematopoietic cells in NOD/SCID mice.[14] Others have demonstrated that co-transplantation of bone marrow stromal cells promote the engraftment of HSC after *in utero* transplantation in pre-immune fetal sheep.[15,16]

As a consequence of their low frequency in adult tissues, the phenotype of primary MSC precursor cells has not been established[17] and phenotypic characterization has been confined to culture-expanded cells (CD90, CD105, CD166, SH3 and SH4 positive).[2,4] The isolation of MSC therefore still relies on the ability of these cells to adhere to plastic and their *ex vivo* expansion potential. In the present study, we analyzed single cell suspensions from fetal bone marrow, liver, lung and spleen for the expression of markers known to be expressed on culture-expanded mesenchymal stem cells. In particular, we studied whether these primary non-cultured cells displayed a *MSC immunophenotype*. Furthermore, we determined the frequency of MSC in fetal bone marrow, liver, lung and spleen by limiting dilution assay, and we tested their multilineage differentiation capacity. Our results indicate, that culture-expanded MSC derived from fetal tissues, although phenotypically similar, exhibit heterogeneity in differentiation potential.

Design and Methods

Collection of tissues

Fetal tissues were obtained after informed consent from women undergoing elective termination of pregnancy (TOP) between 15 and 22 weeks of gestation. The Medical Ethics Review Board of the Leiden University Medical Center approved the protocol for collecting fetal tissues for research purposes (01/116-E). The gestational age was estimated by ultrasonic biparietal diameter and femur length measurements before and foot length measurements of the fetus after TOP. The mean gestational age of these fetuses was 20^{+2} (SD$\pm1^{+4}$) weeks.

Single cell suspensions of fetal lung, liver and spleen were made by mincing and flushing the organ through a 100 μm nylon filter (Falcon, Becton Dickinson) with IMDM medium (Bio-Whittaker, Verviers, Belgium) containing, 20 U/mL penicillin and 20 μg streptomycin (P/S) and 2% heat-inactivated fetal calf serum (FCS, Gibco Laboratories, Grand Islands, NY, USA), i.e. washing-medium. Single cell suspensions of fetal bone marrow were obtained by punching the long bones with a needle (23 gauge) and flushing the bones with washing-medium. After washing, the cell suspensions of bone marrow, lung, liver and spleen were depleted of red cells (incubation for 10 minutes in NH_4Cl (8.4 g/L)/KHO_3 (1 g/L) buffer at 4°C). The total number of nucleated cells in each cell suspension was counted using a Sysmex F-800 (Toa Medical Electronics Co, LTD, Kobe, Japan).

Expansion of mesenchymal cells

Single cell suspensions of fetal bone marrow, liver, lung and spleen were cultured in M199 (Gibco Laboratories, Grand Islands, NY, USA) supplemented with 10% heat-inactivated FCS, P/S, endothelial cell growth factor (ECGF) (20 μg/mL, Roche Diagnostics GmbH, Mannheim, Germany) and heparin (8 U/mL). For expansion of the cells, 3000 cells were plated per cm^2 in a culture flask (T25, Greiner Bio-One GmbH, Frickenhausen, Germany). Tissue culture plates and flasks were coated with 1% gelatin (30 minutes at room temperature). Plates and flasks were kept in a humidified atmosphere at 37°C (5% vol/vol CO_2). When grown to confluency, adherent cells were detached with trypsin/EDTA (5 minutes at 37°C).

Immunophenotyping

Freshly obtained and culture-expanded fetal bone marrow, liver, lung and spleen cells were phenotypically characterized by flow cytometry (FACSCaliber, Becton Dickinson). We used fluorescein isothiocyanate (FITC), phycoerythrin (PE) or allophycocyanin (APC) conjugated antibodies against CD34 (Beckton Dickinson, San José, CA, USA), CD105 (Ancell Corporation Bayport, MN, USA), CD90 (PharMingen San Diego, CA, USA), CD166 (CLB, Amsterdam, The Netherlands), SH3, SH4 (a kind gift from Dr. A. Moseley, Osiris Therapies, Baltimore), and CD45 (PharMingen San Diego, CA, USA).

Culture-expanded MSC were analyzed more extensively by flow cytometry (FACScan, Becton Dickinson) by staining with FITC- or PE-conjugated antibodies against CD31 (DAKO, Glostrup, Denmark), CD11a, CD14, CD25, CD45, HLA-DR (Beckton Dickinson, San José, CA, USA), CD49d, CD49e, CD80, CD127 (Immunotech Coulter Company, Marseille, France), CD106, CD120a, CD120b (R&D Systems, Abingdon, UK), CD40 (Serotec, Oxford, UK), CD44, CD50, CD86, CD123 (PharMingen San Diego, CA, USA), CD62E, CD62P, CD62L (Bio-Whittaker, Verviers, Belgium), CD58 (Southern Biotechnology Associates, Birmingham, USA), CD54, CD71, (CLB, Amsterdam The Netherlands) and HLA ABC (Instruchemie, Hilversum, The Netherlands).

The percentages of lymphocytes, granulocytes, and monocytes in the different fetal tissues were measured according to the SIHON procedure.[18] Staining was achieved with antibodies against CD66e (CLB, Amsterdam, The Netherlands), CD14, and CD45 (Beckton Dickinson, San José, CA, USA). LDS-751 (4 ng/mL, Exciton, Daton, OH, USA) was used to adjust a live gate and to exclude dead cells and debris. Positive cells were identified by comparison with isotype controls (APC-, FITC- and PE-conjugated mouse IgG1, IgG2a or IgG2b).

Functional characteristics

The adipogenic and osteogenic differentiation capacity of cultured MSC was determined as previously reported.[2,19] Briefly, culture-expanded cells (passage 1 or 2) from fetal bone marrow, liver, lung and spleen were detached and re-seeded at a density of 2.5×10^4 cells/cm^2 in a 24-well plate and these cells were cultured in 500 μL α-MEM supplemented with 10% heat-inactivated FCS, P/S, ascorbic acid (50 μL/mL), and dexamethasone (10^{-7}M). β-glycerophosphate (5 mM) was added to the medium from day 7 onwards. For induction of adipogenesis 50 μM indomethacin, 1.6 μM bovine insulin and 0.5 mM 1-methyl-3-isobutylxanthine (IBMX) were added to this medium. Cells were cultured at 37°C (5% vol/vol CO_2) and the medium was replaced twice a week. After three weeks of culture, cells were washed with phosphate-buffered 0.9% NaCl (PBS) and fixed with 10% formalin in PBS for 10 minutes and analyzed for adipogenic and osteogenic differentiation and DNA content.

Quantification of adipogenic and osteogenic differentiation

Adipogenic differentiation was assessed by staining with 0.3% Oil red O in 60% isopropanol for 10 minutes at room temperature. Thereafter, cells were washed with 60% isopropanol and staining was stopped with distilled water. Three hundred microliters of ethanol (100%) were added to each well to

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extract the Oil red O from the cells in order to quantify adipogenic differentiation. The amount of Oil red O released was determined spectrophotometrically at 550nm with a reference of 650nm and compared to an Oil red O standard titration curve.

To visualize osteogenic differentiation, cells were stained for alkaline phosphatase (AP) and calcium deposition. To evaluate AP expression, cells were washed with PBS and subsequently incubated for 15 minutes with substrate solution (0.2 mg/mL α-naphthyl-1-phosphate, 0.1 M Tris buffer pH 8.9, 0.01% magnesium sulphate and 0.6 mg/mL fast blue RR acid) resulting in the formation of a purple reaction product. After washing with PBS, 400 μL 0.05N NaOH in ethanol were added to each well. AP extraction was measured spectrophotometrically at 550 nm.

To detect calcium deposition, cells were washed with PBS, and incubated with 2% alizarin red S solution adjusted to pH 5.5 with 0.5% NH_4OH for 2-5 minutes. Mineralization was indicated by the presence of red deposition. Cells were washed extensively with distilled water. Three hundred microliters of 10% cetylpyridimium chloride in phosphate buffer (8 mM Na_2HPO_4 + 1.5 mM KH_2PO_4) were added and incubated overnight.[20] Alizarin red S release was measured spectrophotometrically at 550 nm and compared to a standard titration curve. The Oil red O, alkaline phosphatase and calcium values were related to DNA content from each well.

DNA content

Cells were washed with PBS and kept at 4°C until use. After extensive washing with H_2O, 300 μL 0.067% pronase in lysis buffer (100 mM NaCl, 10 mM Tris pH 8.0, 25 mM EDTA, 0.05% SDS) were added to each well and incubated overnight at 37°C. To 10 μL aliquots, 100 μL 1*SSC was added and the DNA content was measured by the method of fluorescence enhancement using Hoechst 33258 (ICN Biomedical BV, Zoetermeer, The Netherlands) binding to DNA[21] and calibration against a DNA standard titration curve (0.5-10 μg/mL). The amount of DNA was expressed in μg/well. To quantify the differentiation potential in the various lineages, we divided the amount of released Oil red O and alizarin red S by the amount of DNA. These values were expressed in μg/μg. The value obtained after dividing the amount of the AP extraction by the amount of DNA was expressed in mOD/min/μg.

Limiting dilution assay (LDA)

To assess the MSC precursor frequencies in fetal bone marrow, liver, lung and spleen we cultured 10,000, 7,000, 5,000, 3,000, 1,500, 1,000, 700, 500, 300, 100, 70, 50, 20, 10, 5, 2 cells and 1 cell from the same cell suspension per well in 1% gelatin-coated wells of 96-well plates. Sixteen replicates from the same cell concentration were cultured. After each week, over six weeks, each single well was individually scored. A well in which more than ten adherent spindle-shaped cells were seen was considered positive. The frequency of mesenchymal precursor cells in the different tissues was determined by means of Poisson statistics after six weeks.[22] Cultured cells were phenotypically analyzed for the expression of *mesenchymal markers*. The multilineage differentiation capacity of the culture-expanded cells was tested.

Table 1. Composition of fetal bone marrow, liver, lung, and spleen. Values are percentage (±SD).

Cell types	Bone marrow (n=11)	Liver (n=11)	Lung (n=11)	Spleen (n=7)
CD34+ cells	30.8±11	11.4±5	47.7±14	25±9
Lymphocytes	25.25±15.17	5.96 ±4.25	3.27±1.85	34.33±20.97
Granulocytes	11.62±9.17	1.62±1.44	0.69±0.27	3.93±2.10
Monocytes	5.40±5.77	1.14±0.76	0.57±0.46	1.87±0.89

Statistical analysis

Differences were calculated using Student's T-test and ANOVA testing. A p value of <0.05 was considered as significantly different.

To estimate MSC frequencies, we used the generalized linear model (GLM) for limiting dilution assays as described before.[22] We used the Matlab Statistics Toolbox (version 3, on Matlab 6), which handles GLM with complementary log-log link.

Results

Percentages of $CD34^+$ cells, lymphocytes, granulocytes and monocytes in fetal tissues

The percentages of $CD34^+$ cells in fetal organs are presented in Table 1. The highest proportion of $CD34^+$ cells was found in fetal lung.

These cells were $CD45^-$, indicating their non-hematopoietic origin (Table 2). This $CD34^+CD45^-$ cell fraction was significantly greater in fetal lung than in fetal bone marrow, in which only a small population of these cells was observed (43.33±13.84% versus 4.81±3.09%, p<0.001: Student's T-test). The percentage of $CD45^+$ cells in fetal lung was very low, indicating that contamination by fetal blood was rather low. Most of the $CD34^+$ cells in bone marrow were also $CD45^+$ (26.02±12.17%). Fetal spleen contained both a $CD34^+$ $CD45^-$ (12.56±5.09%) and a $CD34^+$ $CD45^+$ (13.43±11.53%) population. In liver a small $CD34^+CD45^-$ population (7.49±3.97%) was found. The percentages of lymphocytes, granulocytes, and monocytes in fetal bone marrow, liver, lung and spleen are shown in Table 1. The proportions

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Table 2. Immunophenotype of cells derived from human fetal bone marrow, liver, lung, and spleen.

Cell subset	Bone marrow (n=6)	Liver (n=6)	Lung (n=6)	Spleen (n=6)
$CD34^+CD45^-$	4.81 (3.09)	7.49 (4.79)	43.33 (13.84)	12.56 (5.09)
$CD34^+CD45^+$	26.02 (12.17)	3.97 (2.07)	2.19 (1.41)	13.43 (11.53)
$CD34^-CD45^+$	38.79 (16.27)	4.64 (2.35)	2.10 (0.82)	36.58 (8.66)

Data are expressed as mean (SD).

of lymphocytes and granulocytes were much higher in fetal bone marrow and spleen than in fetal lung and liver ($p<0.001$: ANOVA).

Immunophenotyping of freshly obtained cells from fetal bone marrow, liver, lung and spleen

Markers known to be expressed on culture-expanded MSC, i.e. CD90, CD105, CD166, and CD73 (SH3 and SH4), were tested on single cell suspensions derived from freshly obtained fetal bone marrow, liver, lung and spleen. The number of cells positive for these markers was higher within the $CD34^+$ population of fetal liver, lung and spleen than within the $CD34^-$ population (Figure 1). Within the non-expanded $CD45^-$ population from both fetal lung and spleen a significantly higher number of cells expressed the *mesenchymal* markers than within the $CD45^+$ population in these tissues (Figure 2). In fetal bone marrow and liver, the number of cells expressing *mesenchymal* markers was almost equal within the $CD45^+$ and the $CD45^-$ population, only the number of cells positive for SH3 was significantly higher within the $CD45^+$ population in fetal bone marrow and within the $CD45^-$ population of fetal liver ($p<0.05$).

Frequency of MSC and phenotype of cultured MSC in fetal tissues

The number of positive wells increased during the first three weeks of culture and then stabilized during the subsequent three weeks. Since by six weeks a steady-state had been reached in the growth of adherent cells from the different tissues we assessed the frequency of MSC in the different tissues by LDA after a culture period of six weeks. The percentage of adherent cells in fetal bone marrow was significantly higher than the percentages in liver, lung and spleen ($p<0.05$, ANOVA). The estimated MSC frequency in fetal bone marrow was 2.5 (95%CI 2.15-2.91) per 10^3 cells (Figure 3). The frequency of MSC precursors in fetal liver was 0.28 (95% CI 0.23-0.34) in 10^3 cells, in fetal lung 1.39 (95% CI 1.19-1.62) and in fetal spleen 1.72 (95% CI 1.47-2.03) per 10^3 cells. Culture-expanded adherent cells expressed *mesenchymal markers* and were able to differentiate into osteoblasts and adipocytes.

The phenotype of culture-expanded mesenchymal

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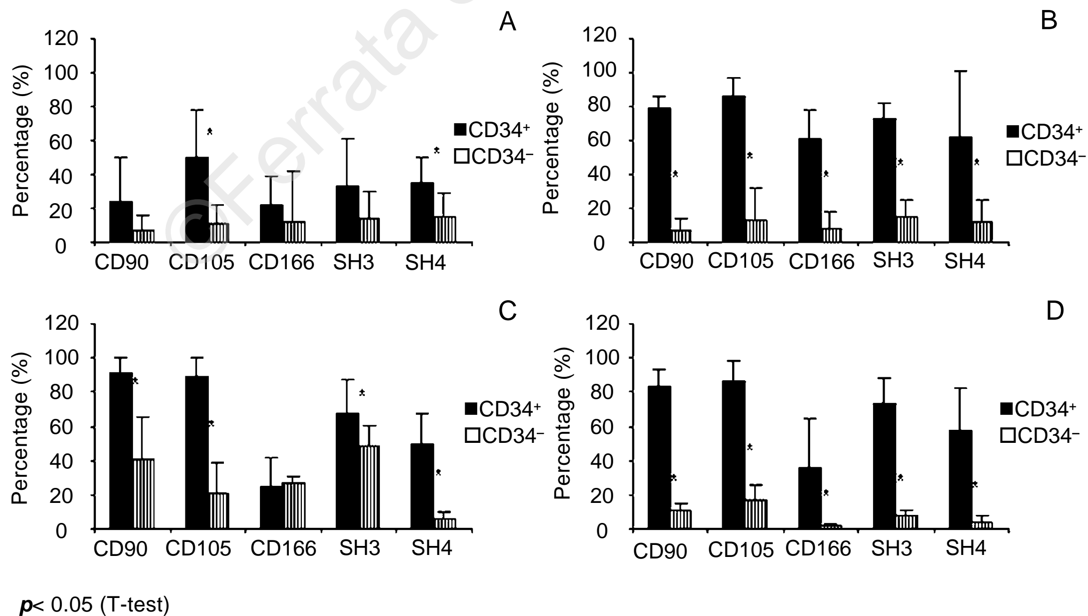

Figure 1. Percentages of cells positive for CD90, CD105, CD166, SH3 and SH4 within the CD34 positive (black bars) and CD34 negative cell population (hatched bars) of freshly obtained second-trimester fetal bone marrow (A), liver (B), lung (C), and spleen (D).

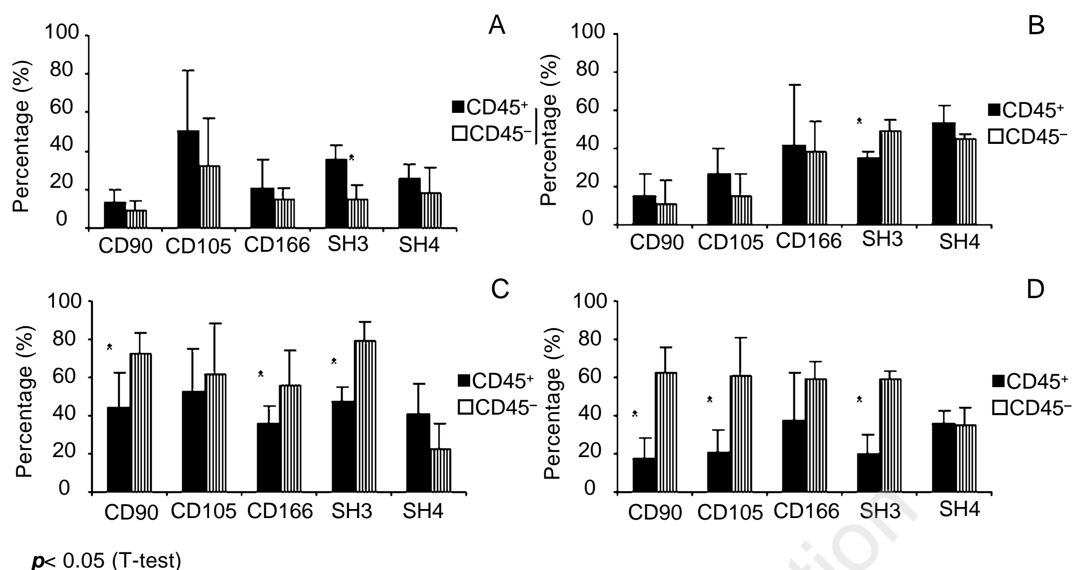

Figure 2. Percentages of cells positive for CD90, CD105, CD166, SH3 and SH4 within the CD45 positive (black bars) and CD45 negative cell population (hatched bars) of freshly obtained second-trimester fetal bone marrow (A), liver (B), lung (C), and spleen (D).

cells was comparable for fetal bone marrow, liver, lung and spleen. Culture-expanded cells exhibited a high expression of the *mesenchymal markers*, i.e. CD90, CD105, CD166, SH3, and SH4 and no expression of hematopoietic and endothelial markers, i.e. CD34, CD45, and CD31 (Table 3).

Adipogenic and osteogenic differentiation potential

Culture-expanded MSC derived from fetal bone marrow, liver, lung and spleen were able to differentiate into both the adipogenic and osteogenic lineage (Figure 4 A and B).

Cells cultured from fetal spleen had a significantly lower adipogenic differentiating capacity than did cells cultured from fetal bone marrow, liver and lung ($p<0.05$: ANOVA).

The osteogenic differentiating capacity of cells cultured from fetal liver was lower than that of cells derived from fetal bone marrow, lung and spleen ($p<0.05$: ANOVA).

Discussion

In this study, we report the characterization, frequency and multipotency of MSC derived from second-trimester fetal bone marrow, liver, lung and spleen. We analyzed the fetal tissues for expression of markers known to be expressed on culture-expanded MSC. We performed LDA to determine the frequency of the MSC, and we assessed the multilineage potential for adipogenic and osteogenic differentiation.

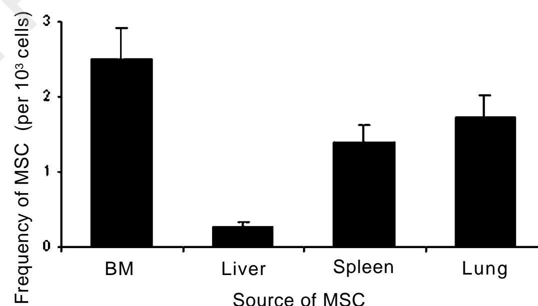

Figure 3. Frequencies of MSC in second-trimester fetal bone marrow, liver, lung, and spleen expressed in amount of MSC per 10^3 cells.

We found a significantly higher percentage of $CD34^+$ cells in fetal lung, bone marrow, and spleen than in fetal liver. In fetal lung the large majority of these cells were non-hematopoietic and did not express CD45, whereas in fetal spleen about half of the cells were $CD34^+ CD45^-$. In contrast, only a small population of $CD34^+CD45^-$ cells was found in fetal bone marrow. Since it has been proposed that the $CD34^+$ $CD45^-$ population contains MSC precursors,[14,23,24] we expected to find the highest frequency within the lung and spleen. However, the frequency of MSC, as assessed by limiting dilution analysis, was significantly higher in second-trimester bone marrow than in fetal lung, liver and spleen.

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Table 3. Immunophenotype of culture-expanded MSC from second-trimester fetal tissues.

Antigen	Bone marrow, Liver, Lung and Spleen	Antigen	Bone marrow, Liver, Lung and Spleen
CD90	+	**Ig superfamily**	
CD105	+	CD31	–
SH3	+	CD58	±
SH4	+	CD54	+
CD45	–	CD50	–
CD34	–	CD106	–
CD14	–	CD80 CD86	– –
HLA		CD166	+
HLA ABC	+	**Cytokine receptors**	
HLA DR	–	CD25	–
Integrins		CD123	±
CD49d	–	CD127	–
CD49e	+	CD120a	–
CD11a	–	CD120b	–
Selectins		**Other antigens**	
CD62E	–	CD71	±
CD62L	–	CD40	–
CD62P	–	CD44	+

+: positive; ±: weakly positive; –: negative.

There are two possible explanations for this unexpected result. Firstly, mesenchymal precursor cells may not be CD34+;[4,25,26] and secondly mesenchymal precursor cells may not exhibit the same phenotype in different tissues.[27]

Campagnoli *et al.* determined the frequency of MSC in first trimester fetal liver, bone marrow and blood by counting adherent fibroblast-like colonies cultured from unselected nucleated cells. They found frequencies of $11.3\pm2/10^6$, $12.6\pm3.6/10^6$ and $8.2\pm0.6/10^6$ for liver, bone marrow, and blood, respectively.[10] Caplan *et al.* found a frequency of 1/10,000 nucleated marrow cells in newborn bone marrow, although the method of measurement was not clearly described. In our study, the frequencies of MSC were evaluated using LDA. Analysis was performed after six weeks, at which time a steady-state had been reached in the growth of adherent cells. Analysis at earlier time points did not result in significantly higher or lower frequencies of MSC in the different tissues (*data not shown*). The MSC frequencies that we found in this study in fetal second-trimester bone marrow, liver, lung and spleen are much higher than those reported by Campagnoli and co-workers. This might be related to the LDA we used for the frequency analysis, which results in a more accurate determination of frequency than one-point analysis of counting colonies. Moreover, it is possible that fibroblast colony-forming units, leads counting to an underestimation in frequency because colonies may grow from more than one cell. The difference in culture medium we used could be another explanation for the higher frequency we found. Another possibility, however, is that there is a physiological explanation for the higher frequency, i.e. the frequency of MSC increases during gestational development.

The number of cells expressing markers considered to be characteristic of culture-expanded MSC, i.e. CD90, CD105, CD166, and CD73 (SH3 and SH4) was higher within the CD34 positive population than within the CD34 negative non-expanded cell population from fetal liver, lung and spleen. This suggests that cells with a phenotype similar to expanded mesenchymal stem cells are present in the CD34 positive population. In single cell suspensions derived from freshly obtained fetal lung and spleen, cells expressing markers known to be expressed on culture-expanded MSC were not only found within the CD45 negative population, but also within the hematopoietic CD45 positive population, most likely on B-lymphocytes and monocytes contained in this population. In the bone marrow and liver, the number of cells expressing *mesenchymal* markers was equal within the CD45 positive and CD45 negative cell populations. In bone marrow this conclusion must be viewed cautiously, since the CD45 negative cell population is very small. The number of cells expressing CD105 was relatively high within the CD45 positive population of bone marrow and liver, perhaps due to the presence of monocytes and B-lymphocytes that express this marker (*unpublished data*).

After expansion, MSC derived from the various fetal tissues were phenotypically similar on the basis of the studied markers and expressed CD90, CD105, CD166, SH3, and SH4 and did not express hematopoietic and endothelial markers, i.e. CD31, CD34, CD45 and CD14. Although, the MSC population of different tissues is similar, additional or unknown markers might exist that would reveal heterogeneity of the cultured MSC population. This phenotype was comparable to that reported for expanded fetal lung and adult bone marrow MSC.[4,14] We previously found that some antigens reported to be expressed on adult bone marrow-derived MSC were not expressed on fetal lung-derived MSC, i.e. CD50, CD102, CD106, CD62L, CD127, CD120a and CD120b. This difference was not related to the source of origin, since fetal bone marrow-derived MSC did not express these markers either. This difference in expression might, therefore, be ontogeny related.

The multilineage differentiation capacity of the

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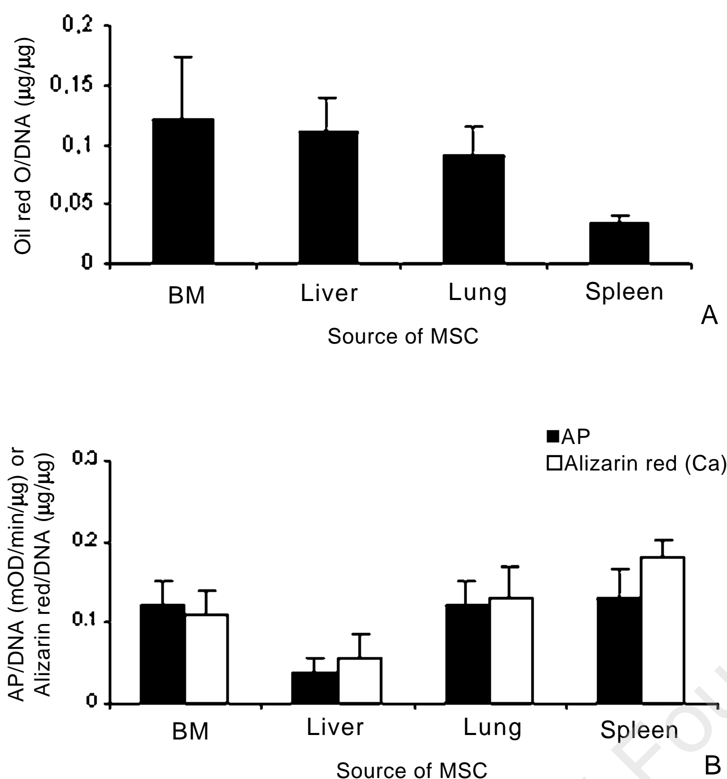

Figure 4. A: adipogenic differentiation potential of bone marrow, liver, lung, and spleen-derived cells was determined. The amount of Oil red O released from the cells was related to the DNA content of the same well. Each value is the mean ± SD of the results from three different wells and is the result of six different experiments. B: osteogenic differentiation capacity was measured by both alkaline phosphatase (AP) and alizarin red (Ca). The amount of AP and alizarin red was related to the DNA content. The amount of AP related to the amount of DNA is expressed in mOD/min/µg (black bars) and the amount of Alizarin red related to the amount of DNA is expressed in µg/µg (white bars). Each value is the mean ± SD of the results from three different wells and is derived from six different experiments.

cultured mesenchymal cells from fetal bone marrow, liver, lung and spleen was tested by culturing these cells under specific osteogenic and adipogenic culture conditions. Mesenchymal cells from all different fetal sources were able to differentiate into adipocytes and osteocytes and could therefore be considered as true MSC. However, there were differences in adipogenic and osteogenic differentiation capacity. MSC derived from bone marrow, liver and lung had a greater adipogenic differentiation capacity than did MSC from fetal spleen. The osteogenic differentiation potential of fetal bone marrow, lung and spleen cells was significantly higher than that of fetal liver cells. Thus, cells were phenotypically similar on the basis of the studied markers but differed in their multilineage potential. This difference in multilineage differentiation potential might have consequences for clinical applications i.e. the higher osteogenic differentiation potency of cells from fetal bone marrow, lung, and spleen as compared to cells from fetal liver suggest the former tissues would be preferential sources for bone repair. Since we demonstrated this increased potency *in vitro*, these findings need to be confirmed in animal models *in vivo*, before any conclusions can be drawn concerning their clinical use. Another possible future clinical application of MSC is related to their engraftment enhancing properties. Previously we demonstrated that co-transplantation of MSC promotes the engraftment of umbilical cord blood-derived CD34+ cells in NOD/SCID mice.[14] Since the mechanism of the engraftment promoting effect is not known, no prediction can be made about the source of MSC preferable for co-transplantation.

In summary, our results indicate that culture-expanded MSC derived from second-trimester fetal bone marrow, liver, lung and spleen exhibit similar immunophenotypes but differ in multilineage differentiation potential and frequency. We hypothesize that these differences may be relevant for the application of MSC in the clinical setting.

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Contributions

PS in 't A and WEF had the original idea and designed the study, interpreted the data and wrote the report. WAN, SAS, and HHHK supervised the analyses and wrote the report. RW wrote the report. CKvdK, ABK, RLvB and WB participated in the collection, analysis, and interpretation of data. All authors have seen and approved the final version. PS in 't A and WEF are primarily responsible for the manuscript. PS in 't A is primarily responsible for Tables 1, 2 and 3 and Figures 1, 2, 3, and 4.

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Disclosures

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In the following paragraphs, Professor Cazzola summarizes the peer-review process and its outcomes.

What is already known on this topic

Human fetal lung is a rich source of mesenchymal stem cells.

What this study adds

Mesenchymal stem cell derived from second-trimester fetal tissues have heterogeneous differentiating potential, and this may be relevant for clinical applications.

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