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Engraftment potential into NOD/SCID mice of CD34+ cells derived from human fetal liver as compared to fetal bone marrow

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Background and Objectives. We hypothesized that qualitative or quantitative differences in hematopoietic stem cells from fetal liver (FL) and fetal bone marrow (FBM) may be the cause of their organ specificity.

Design and Methods. To analyze possible differences *in vivo*, we compared the engraftment potential of equal numbers of CD34+ cells isolated from human FL or FBM into immunodeficient NOD/SCID mice.

Results. Mice showing engraftment following transplantation of CD34+ cells from FL demonstrated 14% (range 2-76%) CD45+ cells of human origin in the bone marrow compared to significantly lower levels of engraftment (4%, range 2-20%, $p < 0.04$) of FBM CD34+ cells. Likewise, the percentage of CD34+ CD38- cells in FBM was 4 times lower than the percentage in FL (1.4±0.9% and 5.6±0.7%, respectively). Similar organ distribution of engrafted human cells was found. Subset analysis of human cells in bone marrow of engrafted mice revealed identical distribution of the lymphoid, myeloid and erythroid lineages after transplantation of CD34+ cells from FL or FBM.

Interpretation and Conclusions. The FL CD34+ cells showed a four-fold higher content of the CD34+ CD38- subset coinciding with a four-fold higher engraftment of CD34+ cells into NOD/SCID mice. Since the organ distribution and differentiation potential of the cells engrafted were similar, we concluded that CD34+ hematopoietic cells derived from FL and FBM have quantitatively different, but qualitatively the same potential for engraftment into NOD/SCID mice.

Key words: fetal hematopoiesis, stem cells, NOD/SCID mice, fetal liver, fetal bone marrow.

In utero stem cell transplantation and gene therapy are being developed as new therapeutic strategies for the treatment of congenital diseases. Extensive knowledge about the hematopoietic stem cell compartment present in the fetus is a prerequisite for successful application of these procedures. Fetal hematopoiesis occurs in various organs, depending on the gestational age.[1-3] Initially, hematopoiesis is mainly situated in the fetal liver (FL).[4] Later during ontogeny the fetal bone marrow (FBM) becomes the major source of hematopoietic cells and this continues into adult life.[5] Although during the second trimester of pregnancy the FL contains more white blood cells (WBC) than the total compartment of FBM, the absolute numbers of CD34+ cells in the two tissues are very similar.[6] The differential development of lymphohematopoiesis in FL and FBM may be due to qualitative differences in the stem cell compartments or may be due to differences in the microenvironment. It could be hypothesized that if the fetal organs hosted different types of hematopoietic stem cells, and if FBM contained only lymphoid committed precursor cells during the second trimester of pregnancy, after *in utero* stem cell transplantation during the second trimester of pregnancy hematopoietic progenitor cells (HPC) capable of multilineage engraftment might home only to the FL. Engraftment to the FBM or to a circulating pool would be necessary for sustained engraftment since the bone marrow is the only hematopoietic site that remains after birth. We hypothesized that if the functional properties of the CD34+ hematopoietic progenitor cell content of the various fetal hematopoietic organs were similar, the difference in phenotype would probably be due to differences in microenvironment, and that the HPC from the organs may be interchangeable.

The engraftment potential of human hematopoietic progenitor cells following intravenous injection in immune-deficient mice provides an *in vivo* assay for measuring the repopulating capacity of human stem cells.[7-9] The primitive human cells initiating engraftment in these mice are defined as SCID-repopulating cells (SRCs).[10;11] In this *in vivo* system, engraftment of human SRCs after transplantation is predominantly in the murine bone marrow where proliferation and differentiation occurs.[8,12,13]

To examine whether quantitative or qualitative differences in FL and FBM $CD34^+$ HPC capable of repopulating NOD/SCID mice would explain their organ specificity, we analyzed possible differences in engraftment and *in vivo* differentiation of $CD34^+$ cells isolated from FL or BM after transplantation into immunodeficient NOD/LtSz-*scid/scid* (NOD/SCID) mice. In this study we demonstrated that FL $CD34^+$ cells resulted in a four-fold higher engraftment than FBM $CD34^+$ cells but both had similar proliferation and differentiation patterns into the various lymphoid, myeloid and erythroid lineages. Taking into account the four-fold higher percentage of $CD34^+$ $CD38^-$ in FL, it can be concluded that the engrafting potential of SRC from FL and FBM is equal.

Design and Methods

Collection and purification of fetal liver and fetal bone marrow $CD34^+$ cells

Fetal liver (FL) and fetal bone marrow (FBM) were obtained after informed consent from women undergoing elective termination of pregnancy for social reasons between 14 to 22 weeks of gestational age. The medical Ethics Review Board of the Leiden University Medical Center approved the protocol of this study. FL and FBM were collected from 9 fetuses. Cell suspensions were made within 1-2 hours by mincing the liver thoroughly and flushing the long bones with medium consisting of RPMI 1640 supplemented with penicillin (20 U/mL) and streptomycin (20 µg/mL) (BioWhittaker, Verviers, Belgium) and 2% heat-inactivated fetal calf serum (FCS) (Gibco Laboratories, Grand Island, NY, USA). Cells were washed twice in medium and then used freshly (n=3) or first cryopreserved in 10% dimethylsulphoxide, 25% FCS and 65% medium and used after thawing (n=6). In individual experiments both FL and FBM cells from a single fetus were always used either freshly or after cryopreservation. Thawing was performed by stepwise dilution in medium containing 37% FCS, after which the cells were washed twice. Subsequently, $CD34^+$ cells were isolated from the cell suspensions using magnetic separation columns (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and antibodies recognizing the CD34 epitope QBEND/10 according to the manufacturer's procedure. A phycoerythrin (PE)-conjugated monoclonal antibody recognizing a different CD34 epitope (clone: 8G12, Becton Dickinson Immunocytometry Systems (BDIS), San José, CA, USA) was used to check the purity of the selected $CD34^+$ fraction. Purified $CD34^+$ cells were stained with CD38 PE (clone: HB 11, BDIS) and CD34 FITC (clone: 8G12, BDIS) to check the percentage of $CD34^+/CD38^-$ cells within the purified cells used for transplantation. The labeled cells were washed in phosphate buffered saline (PBS) supplemented with 1% pasteurized protein solution (PPS, CLB, Amsterdam) and subsequently labeled for another 30 minutes with LDS-751 (Exciton, Daton, OH, USA) to determine the purity of $CD34^+$ cells within a life gate. The samples were assessed by flow-cytometric analysis on a FACScan flow cytometer (BDIS).

Transplantation of $CD34^+$ FL or FBM cells into NOD/SCID mice

Female NOD/SCID mice, obtained from Bomholtgard Breeding and Research Center A/S Denmark, were housed in filtertop cages under pathogen-free conditions and received autoclaved food (Hope Farms, Woerden, The Netherlands) and acidified water containing ciprofloxacin (85 mg/L, Bayer AG, Leverkusen, Germany) and polymyxin B (70 mg/L) *ad libitum*. Mice were sublethally irradiated (3.5 Gy), and 4 to 12 hours later 240,000 to 600,000 $CD34^+$ purified cells from FL or BM were transplanted intravenously (iv.) into a lateral tail vein. Control mice were injected with 300,000 $CD34^-$ cells. After 8 weeks the mice were sacrificed using CO_2 and blood was obtained by heart puncture, and collected in tubes containing heparin. Red cells were depleted from the blood by a 10-minute incubation in NH_4Cl (8.4 g/L)/$KHCO_3$ (1 g/L) buffer at 4°C. Bone marrow was collected by flushing both femora with medium, and liver, spleen and thymus were also harvested. Single cell suspensions were made (cell strainer 100 µm, Falcon, BDIS), washed twice, and resuspended in IMDM with 10% FCS. Total numbers of nucleated cells in the tissues were counted using a sysmex F-800 (Toa Medical Electronics Co., LTD, Kobe, Japan). The percentage of engrafted human cells was detected by flow cytometry. Staining was performed with monoclonal antibodies specific for human hematopoietic progenitor cells (CD34, CD45), B-cells (CD19, CD20), T-cells (CD2, CD4, CD8), myeloid cells (CD33) and erythroid cells [Glycopherin A (GPA)].

LDS-751 was used to adjust a live gate and exclude red cells and dead cells and debris from the measurements. Samples were analyzed on a FACScan. Positive cells were identified by comparison with isotypic controls (mouse IgG1, IgG2a or IgG2b, FITC-conjugated and PE-conjugated) and with cells harvested from control (not transplanted) NOD/SCID mice stained with the same antibodies. Engraftment was determined as ≥ 1% human CD45+ cells. Absolute human CD45+ cell numbers were calculated by measuring the cell numbers harvested from two femora, liver, spleen and blood and determining the percentage of human cells. Cells harvested were stained with huCD45, huCD34 and human lymphoid and myeloid monoclonal antibodies. Lymphoid, myeloid and CD34+ cells were expressed as percentage of the total number of human CD45+ cells.

Human progenitor cell assay

To compare the nature of committed progenitor cells within the CD34+ fraction derived from FL and FBM, equal numbers of CD34+ cells were assessed in semi-solid medium progenitor cell assays. Of each cell suspension, 500 CD34+ cells were assayed in duplicate in plastic 35-mm tissue culture dishes. The culture medium consisted of IMDM (BioWhittaker) supplemented with 10 ng/mL (granulocyte colony-stimulating factor G-CSF; Amgen, Thousand Oaks, CA, USA), 10 ng/mL (granulocyte-macrophage colony-stimulating factor GM-CSF; Sandoz AG, Basel, Switzerland), 10 ng/mL interleukin-3 (IL-3) (Sandoz AG, Basel, Switzerland), 3 U/mL erythropoietin (EPO; Organon Technica, Turnhout, Belgium), 50 ng/mL stem cell factor (SCF; Amgen), 1% deionized bovine serum albumin (BSA), 0.47 g/L human transferrin (Behringwerke, Marburg, Germany) saturated with $FeCl_3$. $6H_2O$, 5×10^{-5} M β-mercaptoethanol (ME, Sigma Chemicals, St. Louis, MO, USA), 30% FCS and 1.1% methylcellulose (Methocel 4000 cps; Fluka, Freiburg, Germany). Cultures were incubated at 37°C in 100% humidified atmosphere containing 5% CO_2. Hematopoietic colonies (BFU-E, burst-forming unit-erythroid; CFU-GM, granulocyte-macrophage colony-forming unit and CFU-GEMM, colony-forming unit granulocyte-erythroid-macrophage-megakaryocyte), were scored after 2 weeks using an inverted microscope. Progenitor assays were also performed in four control experiments to test the bone marrow of recipient mice for the presence of HPC by plating 2000 CD34+CD45+ cells derived from the bone marrow of recipient mice into methylcellulose cultures as described above.

Statistical analysis

Student's t-test was used to calculate differences between percentages. A p value of < 0.05 is indicated as statistically significantly different. Means are given ± SEM (standard error of mean).

Results

Collection and purification of fetal liver and fetal bone marrow CD34+ cells

The engraftment capacity of CD34+ cells derived from FL and FBM from 14 to 22 weeks of gestation were compared (n=9). Flow cytometric analysis of the purified CD34+ cells showed that the percentage of viable cells, as determined by the LDS-751 gate, was not different between FL and FBM. The purity of the viable CD34+ cells isolated from FL and FBM was 80.4±22.4% and 94.9±5.4%, respectively. The lower purity of CD34+ cells isolated from the FL cell suspension compared to FBM cells was due to a higher number of contaminating nucleated red blood cells present in the FL samples. Injected cell suspensions were corrected according to the purity, resulting in injection of equal numbers of CD34+ cells. The CD34+ population of FL cells contained four times more CD38- cells than the CD34+ FBM population (5.6±0.7% and 1.4±0.9% CD34+/CD38- cells, respectively, $p<0.05$).

Transplantation of CD34+ FL or FBM cells into NOD/SCID mice

To determine whether stem cells from FL and BM exhibited differences in engraftment potential, a total of 43 NOD/SCID mice were transplanted with 240,000 to 600,000 CD34+ FL or FBM cells. Forty mice were transplanted in paired experiments with equal numbers of FL or FBM CD34+ cells from the same fetus. Since from selected fetuses the number of CD34+ cells harvested from the FBM exceeded the number of FL cells, three additional mice were transplanted with 3×10^5, 4×10^5 or 6×10^5 FBM CD34+ cells. Figure 1 shows the numbers of CD45+ cells present in murine bone marrow, liver, spleen and blood after transplantation of CD34+ cells from FL or FBM. In 58% of all transplanted mice > 1% human cells (CD45+) could be measured in bone marrow of the mice after transplantation (Table 1). Mice showing engraftment following transplantation with CD34+ cells from FL demonstrated a median of 14% (range 2-76%) cells of human origin (CD45+) in the bone marrow (Table 2). In 45% of these mice with bone marrow engraftment, human cells could also be detected in the blood (median 1.8%, range 0-22%). Mice showing engraftment after transplantation with CD34+ cells

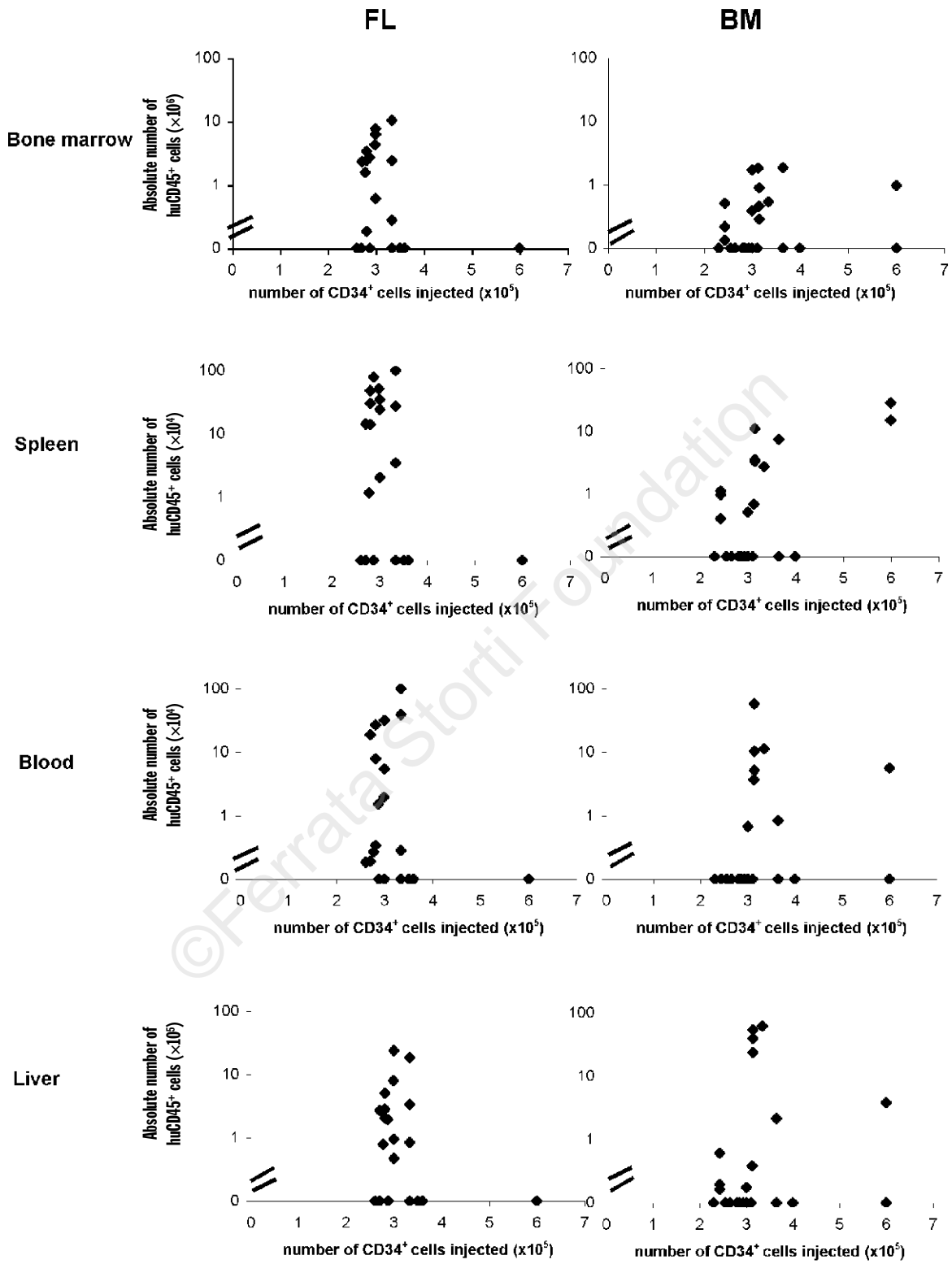

Figure 1. Relationship between number of injected FL CD34+ cells or FBM CD34+ cells and engraftment levels of human cells in NOD/SCID mice. Chimerism was defined as the absolute number of human CD45+ cells detected in bone marrow of two femora, spleen, peripheral blood (in 1 mL) or liver in recipient mice 8 weeks post-transplantation.

Table 1. Number of NOD/SCID mice with engraftment of huCD45+ cells.

CD34+ cell transplant	*Number of mice*	*Engraftment*
Fetal liver	20	13 (65%)
Fetal bone marrow	23	12 (52%)
Total	43	25 (58%)

Number of NOD/SCID mice with engraftment of huCD45+ cells after transplantation of equal numbers of CD34+ cells derived from human fetal liver or bone marrow.

from FBM demonstrated significantly lower levels of engraftment in the bone marrow (median 4%, range 2-20%) as well as in blood, spleen and liver (Table 2). In 13% of mice transplanted with FBM cells, human cells could also be found in the blood (median 0.4%, range 0-4%). Mice with a high percentage of human cells in the bone marrow also demonstrated engraftment in spleen and, to a lesser extent, in liver (Table 2). No human cells were found in the thymus of the mice. The absolute numbers of human cells were calculated from both femora of each mouse, liver, spleen and peripheral blood (1 mL). The absolute number of human CD45+ cells in two femora were 3.4×10^6 cells (median, range $0.2\text{-}10.4\times10^6$) after transplantation with FL CD34+ cells and 0.8×10^6 cells (median, range $0.1\text{-}1.9\times10^6$) after transplantation with CD34+ FBM cells (Figure 1). Liver, spleen and blood contained lower absolute numbers (Figure 1) and therefore statistical analysis of these absolute numbers was not performed. No differences in engraftment in relation to gestational age (14-22 weeks) were observed (data not shown). Four control mice transplanted with 300,000 CD34- FL or FBM cells did not show any human cells.

Lymphoid, myeloid, and erythroid engraftment of human cells in the bone marrow of NOD/SCID mice

To analyze the differentiation of the engrafted human cells into the various lineages, the bone marrow of NOD/SCID mice was examined further since this compartment showed the highest levels of engraftment. The percentages of myeloid and lymphoid lineages of human cells were determined as the percentages of all human (CD45+) cells. For mice transplanted with cells from FL (Figure 2a) the CD45+ human cells found in bone marrow consisted of 56% (29-88%) B-cells (median and range), 36% (20-87%) myeloid cells and 32% (14-48%) CD34+ cells. These data did not differ statistically from those found in mice transplanted with FBM cells (Figure 2b) which showed 71% (29-95%) B-cells, 34% (19-92%) myeloid cells and 31% (4-56%), CD34+ cells. In addition to the huCD45+ population, significantly higher percentages of human erythroid cells (huGPA+) were found in the bone marrow of mice following FL transplantation than following FBM transplantation, [medians 3.3% and 0.4%, respectively (Table 2)].

Progenitor cell assay to determine human HPC frequencies

To compare the nature of committed progenitor cells derived from FL and FBM, 500 CD34+ cells from each cell suspension were assessed in progenitor cell assays (Figure 3). The total number of clonogenic progenitors derived from 500 CD34+ FL cells was 57 ± 8, and from 500 CD34+ FBM cells 24 ± 10. This difference was statistically significant ($p < 0.03$). In particular, higher numbers of erythroid colonies and mixed colonies were present in the FL CD34+ population than in the FBM CD34+ cell suspension ($p<0.02$). To analyze whether the bone marrow of repopulated animals had the ability to generate human HPC, four control progeni-

Table 2. Percentage white blood cells and nucleated red blood cells of human cells in organs of NOD/SCID mice.

	Fetal liver		*Fetal BM*	
	CD45+	*GPA+*	*CD45+*	*GPA+*
Mice organs				
bone marrow	14% [2-76]	3.3% [0.3-14.2]	4% [2-20]	0.4% [0.1-2.5]
blood	1.8% [0-22]	0% [0-0.6]	0.4% [0-4]	0.1% [0-0.8]
spleen	0.9% [0-13]	0.2% [0-0.2]	0.2% [0-0.6]	0% [0-0.4]
liver	0.3% [0-2]	0 % [0-0.4]	0% [0-0.4]	0% [0-0.1]

Percentage (median and range) of huCD45+ cells and huGPA+ cells in different mice organs after transplantation of equal numbers of CD34+ cells derived from fetal liver or fetal bone marrow.

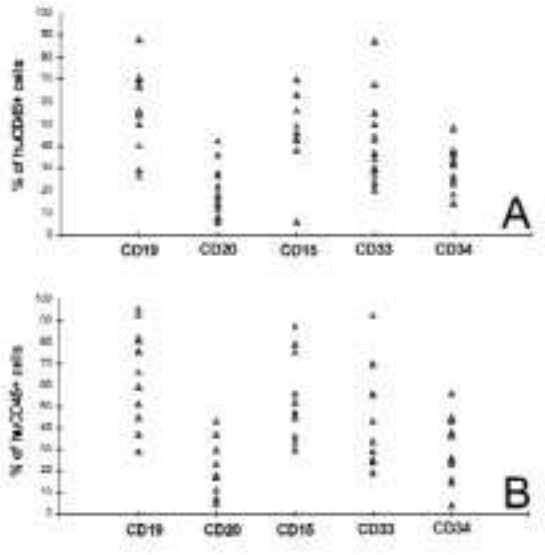

Figure 2. Lymphoid, myeloid, and CD34⁺ human cells in bone marrow of engrafted recipient mice transplanted with A) FL CD34⁺ (n=13) and B) FBM CD34⁺ cells (n=12). Each data point represents for each recipient the lineage differentiation of human cells as percentage of total CD45⁺ cells. All CD45⁺ mice transplanted with CD34⁺ FL cells or CD34⁺ FBM cells were analyzed for the expression of the 5 markers indicated.

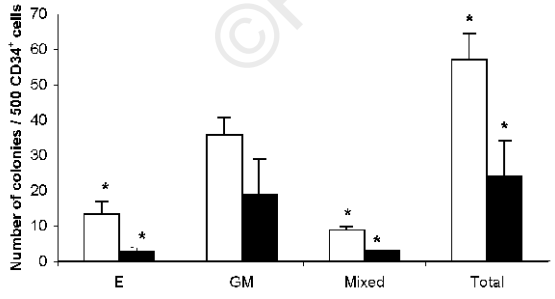

Figure 3. Mean numbers of clonogenic human progenitors ±SEM derived after culture of 500 CD34⁺ FL (white bars) or FBM cells (black bars). From 9 cell suspensions of FL and FBM, 500 CD34⁺ cells were assayed in methylcellulose as described in the *Design and Methods* section. Total numbers of colonies represent the arithmetic sum of E, erythroid colonies; GM, granulocyte-macrophage colonies and GEMM, granulocyte-erythroid-macrophage-megakaryocyte colonies. **Indicates statistically significant differences.*

tor assays in methylcellulose were performed. Quantities of 2,000 CD34⁺CD45⁺ cells derived from the bone marrow of recipient mice were put in culture. The culture conditions had been previously shown to induce colony formation exclusively by human cells, and not mouse precursor cells.[14] After 2 weeks of culture colonies were detected in all cultures. The mean number of colonies was 143 (51-275) for bone marrow cells derived from mice transplanted with FL cells and 245 (174-312) for mice transplanted with FBM cells, illustrating similar colony-forming capacity of CD34⁺ cells after transplantation.

Discussion

Fetal HPC derived from fetal liver have been described to differ quantitatively and qualitatively from postnatal and adult HPC, both *in vitro* and *in vivo* as determined by engraftment into NOD/SCID mice.[15-19] Here, we describe the comparison of the *in vivo* repopulating potential of human HPC from two fetal tissues, i.e. fetal liver and fetal bone marrow cells.

Human SCID repopulating cells (SRCs), defined as HPC capable of engrafting NOD/SCID mice, leading to multilineage differentiation have been reported to be exclusively present in the CD38⁻ fraction of the CD34⁺ progenitor cell population.[12,20,21] As illustrated in this study CD34⁺ FL cells differed phenotypically from CD34⁺ FBM cells in the percentage of CD38⁻ cells with a 4-fold higher frequency of FL CD34⁺ CD38⁻ cells (5.6±0.7%) than of FBM cells (1.4±0.9%). Therefore, assuming similar engraftment potential of the CD34⁺CD38⁻ cells from these sources, a similarly increased repopulation of human cells was expected to be obtained after transplantation of CD34⁺ cells from FL as compared to FBM.

In accordance with this assumption, our results show that transplantation of CD34⁺ cells derived from FL resulted in a statistically significant fourfold higher engraftment of absolute numbers of huCD45⁺ cells as well as percentage of huCD45⁺ cells of total cells per tissue, as compared to transplantation of CD34⁺ cells from FBM. After correction for this 4-fold difference, the experiments shown in Figures 1 and 2 illustrate that the organ distribution and lineage differentiation pattern of the engrafted cells showed no differences. These findings made it unlikely that the HPC responsible for engrafting the NOD/SCID mice differed qualitatively, although a difference in proliferative potential of CD34⁺ cells from these fetal tissues cannot be excluded. Transplantation with fetal cells did not

only lead to lymphoid and myeloid differentiation of engrafted cells but also, to a lesser extent, to erythroid development. Similarly, engraftment of human glycophorin-A positive cells was comparable following transplantation of CD34+ cells from FL and FBM after adjusting for the number of CD34+CD38- cells infused.

After transplantation of similar numbers of CD34+ cells from postnatal bone marrow or mobilized peripheral blood, similar lineage distribution of engrafted cells was found, despite differences in the efficacy of CD34+ cells in engrafting NOD/SCID mice.[18,22,23] These results indicate conservation of CD34+ SRCs capable of engraftment into NOD/SCID mice during ontogeny from the second trimester until after birth. This does not exclude differences in other characteristics such as homing efficiency of CD34+ cells from different sources. We recently demonstrated that whereas from adult sources only CD34+ cells in G_0 phase of the cell cycle were able to engraft, from umbilical cord blood CD34+ cells in both G_0 and G_1 phases were capable of efficient engraftment of NOD/SCID mice.[14] The role of the cell cycle status of fetal cells on engraftment is currently under investigation.

Despite the apparent similarity of the HPC from FL and FBM responsible for engraftment of human cells in NOD/SCID mice, during the second trimester of gestation the two compartments show major differences in the progeny of the CD34+ cells. Whereas lymphoid development is mainly observed in FBM, FL is the major organ responsible for production of myeloid and erythroid cells.

When the functional *in vitro* characteristics of equal numbers of CD34+ cells derived from FL and FBM were compared in semi-solid medium progenitor cell assays, the total number of colonies scored after two weeks of culture was statistically significantly higher for FL. Likewise, the number of erythroid and mixed colonies was significantly higher ($p< 0.02$) for FL, indicating higher potency for differentiation into erythroid lineage and multi lineage for FL CD34+ cells, as has been reported previously.[24] We hypothesize that the differences in FL and FBM in differentiation patterns of more committed progenitor cells is more likely to be due to environmental differences than to major differences in the immature CD34+ SRC-subset. This is supported by the fact that equal numbers of engrafted CD34+ bone marrow cells derived from mice after transplantation with FL or FBM CD34+ cells showed similar colony forming capacity.

In conclusion, we have demonstrated that, after transplantation into NOD/SCID mice, FL CD34+ cells have a statistically significant four-fold higher engraftment level than FBM CD34+ cells. This difference can be explained by the four fold higher number of CD34+CD38- cells in FL CD34+ cells. The similar lineage-specific differentiation pattern of the human cells indicate that the NOD/SCID repopulating HPC show quantitative, but no major qualitative differences, and therefore the fact that FBM is the major site of B-cell development and that FL appears to be responsible for maintaining erythropoiesis and myelopoiesis are more likely to be due to micro-environmental differences of these specific hematopoietic organs.[25,26]

Contributions and Acknowledgments

All authors (JW, WAN, HHHK, RW, JHFF) have participated in conception and design of the study, analysis and interpretation of data as well as in drafting and revising the article for important and intellectual content. All authors gave final approval of the version to be published.

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Disclosures

Conflict of interest: none.

Redundant publications: no overlapping with previous papers.

Manuscript processing

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Potential implications for clinical practice

CD34+ cells derived from fetal liver showed a four-fold higher content of the CD34+CD38- subset, coinciding with a four-fold higher engraftment of CD34+ cells into NOD/SCID mice compared to CD34+ cells derived from fetal bone marrow. Identical organ distribution and lineage differentiation was found after transplantation of CD34+ cells from fetal liver or fetal bone marrow. These results are important for in utero stem cell transplantation and gene therapy, since hematopoietic stem cells may home similarly to fetal liver and bone marrow in the second trimester of gestation.

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