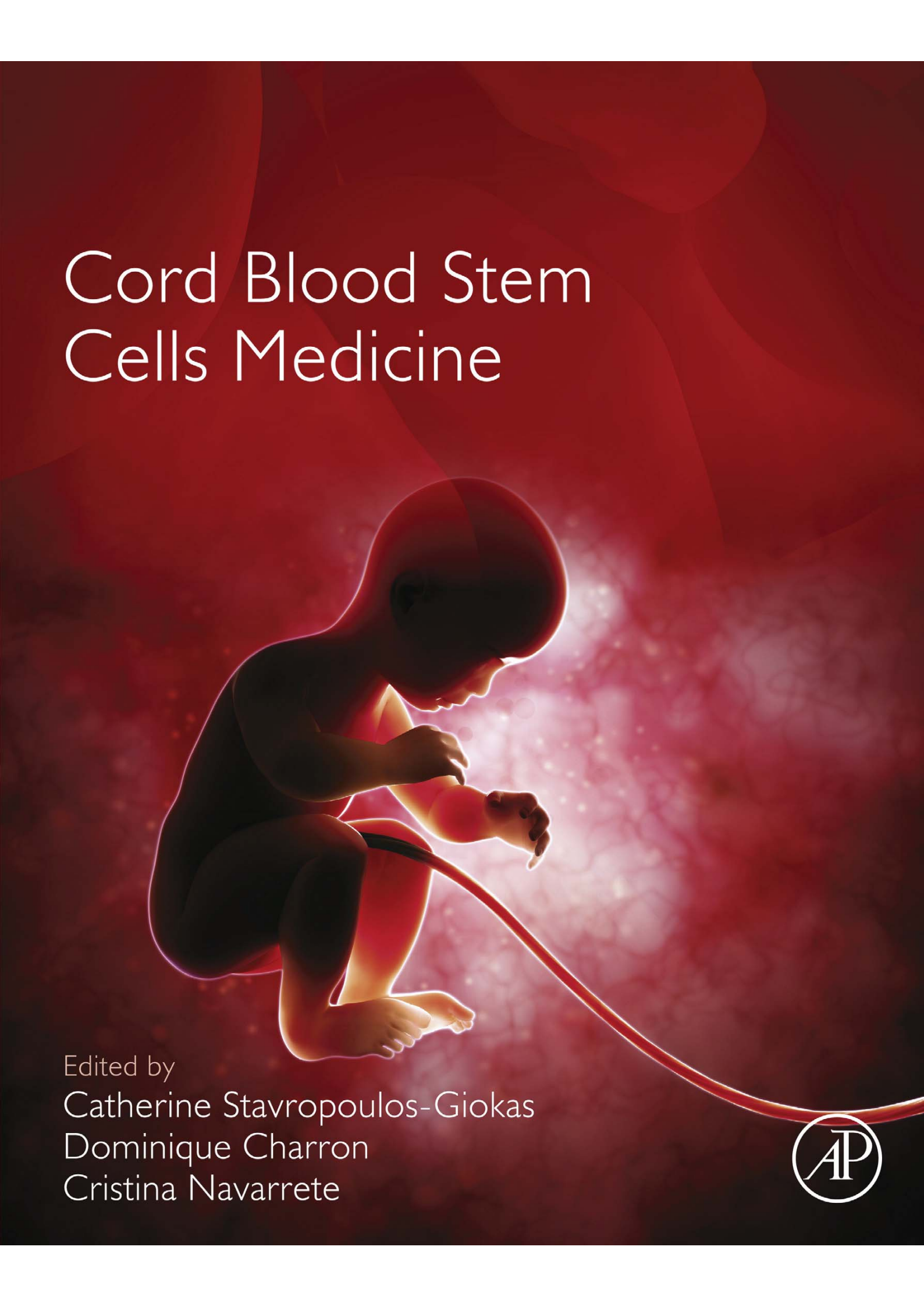
Cord Blood Stem Cells Medicine



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Chapter 2

Cord Blood Content

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Chapter Outline

1. BIOLOGICAL BACKGROUND OF CORD BLOOD CELLS—DEVELOPMENT OF HEMATOPOIETIC AND NONHEMATOPOIETIC CELLS

Cord blood (CB) is characterized by a unique richness in hematopoietic stem and progenitor cells, particularly those "early" cells which are detected in in vitro assays like the long-term culture-initiating cell (LTC-IC) assay and high proliferative potential-colony forming cell (HPP-CFC) assay or in vivo due to their potential to repopulate non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (SRC—SCID repopulating cells). It has been extensively shown that hematopoietic stem cells (HSC) develop during embryogenesis and fetal life in a complex process involving multiple anatomic sites and niches (yolk sac, the aorta–gonad–mesonephros region, placenta, and fetal liver),[1] before they colonize the bone marrow (BM), as summarized recently.[2] As fetal and neonatal hematopoietic cells are markedly different from adult HSC with respect to their proliferative capacity, it was conceivable that different mechanisms and/or niches control engraftment and self-renewal of HSC during fetal and adult life. Since fetal blood is formed in close association with organs, the search for cell functions as niches similar to cell types present in adult BM environment (osteoblasts, endothelial cells, fibroblasts, reticular cells) was a logical consequence.

Although the HSC contained are currently the most relevant cells in CB with regard to clinical application, it also contains nonhematopoietic cell types which bear interesting properties that can potentially be utilized in regenerative medicine.

2. ENDOTHELIAL CELLS IN CB

Endothelial progenitor cells (EPCs) have been investigated as a potential source of cells for vascular repair. First described in 1997,[3] EPCs have been characterized by many investigators based on their morphology and surface antigen expression,[4] but frequently without stringent in vivo analysis of function.[5]

By the group of Mervin Yoder,[6] endothelial cord forming cells (ECFCs) in CB have been shown to be the only circulating cells that possess all the characteristics of an endothelial cell progenitor, including distinct functions. To isolate ECFCs, CB-derived mononuclear cells (MNC) or $CD34^+/CD45^-$ cells are plated on a collagen-coated surface and form adherent colonies with a cobblestone-like morphology between day 7 and 14.[6] ECFCs are rare cells, found at a concentration of about 0.05–0.2 cells/ml in adult

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peripheral blood. They are enriched in human umbilical CB, being found at a concentration of about 2–5 cells/ml. ECFC can be enriched from each CB sample (fresh or cryopreserved) applying the isolated $CD34^+$-subpopulation as a basis. ECFC progeny express the cell surface antigens CD31, CD105, CD144, CD146, von-Willebrand factor, and kinase insert domain receptor (KDR), but do not express the hematopoietic or monocyte/macrophage cell surface antigens CD14, CD45, or CD115.[6] Additionally, they are characterized by uptake of acetylated-low-density lipoprotein (AcLDL). Functionally ECFC progeny form tubes when plated alone and form de novo functionally active human blood vessels in vivo. One potential clinical use of ECFCs is in the treatment of patients with ischemia and defective wound healing due to impaired neoangiogenesis.[7] The authors state that the ability of implanted endothelial cells to form a vascular network when the host's angiogenic response is inhibited suggests that this strategy could be useful in treating patients with impaired wound healing. These and other reports suggest that ECFCs represent an excellent cell source for vascular engineering strategies. While there are not so many data available of the use of ECFCs in human clinical trials, the results with preclinical rodent studies provide some hope for patients who suffer from poor vascular function. Moreover, based on their growth kinetic, they are interesting candidates for tissue engineering in combination with MSC, induced pluripotent stem (iPS) cells, or mature tissue cells.

3. STROMAL CELLS IN CB AND CORD TISSUE AS COMPARED TO BM

The heterotopic transplantation of BM results in the formation of ectopic bone and marrow.[8] This "osteogenic potential" is associated with nonhematopoietic stromal cells coexisting with HSC in the BM.[9,10] Friedenstein and colleagues originally called these cells "osteogenic" or "stromal stem cells,"[10,11] in the following years the terms "mesenchymal stem cells," "mesenchymal stromal cells," or "skeletal stem cells" have been widely used in the literature.[12] In the present article, the BM-derived nonhematopoietic cells are referred to as "bone marrow mesenchymal/multipotent stromal cells" (BM MSC). Several groups proved the in vivo osteogenic potential—including the recruitment of hematopoietic cells of recipient origin—of BM MSC after transplantation on a hydroxyapatite scaffold.[13,14] After the original reports by Friedenstein, many other sources of MSC-like cells were described, for example adipose[15] or fetal tissue. In 2000, the first adherent cells (also termed "MSC") from CB (as summarized in Koegler et al. [16]) or in the umbilical cord tissue,[17,18] which revealed an immunophenotype ($CD45^-$, $CD13^+$, $CD29^+$, $CD73^+$, $CD105^+$) similar to BM MSC, however without any in vivo reconstitution studies. This was accompanied by many other publications, as summarized by Kogler et al.[18] In 2004, our group was able to detect cells in CB with a different proliferative potential, the so-called unrestricted somatic stromal cells (USSC),[19] and in the following years these data were confirmed by other groups (Figure 1).[20–22]

Several markers described in the literature for defined subtypes of MSC from BM, e.g., CD271, CD140b, STRO-1, GD2, or NG2 could not be correlated to functional subpopulations in CB.

In the publication of 2004,[19] we described USSC as a homogenous cell population with respect to their phenotype. During the following years, further detailed characterization in vitro and in vivo applying clonal cell population

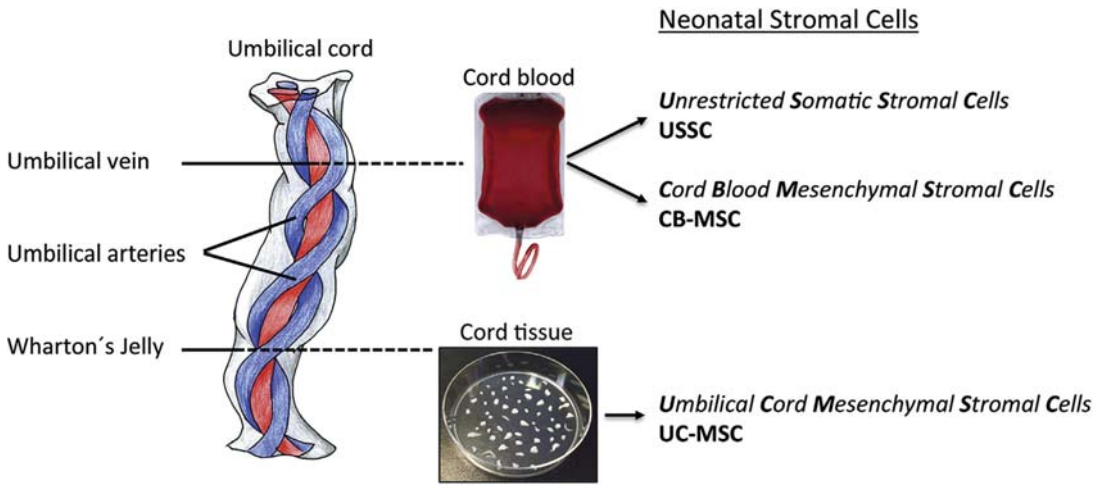

FIGURE 1 **Neonatal stromal cells.** Neonatal stromal cells were isolated from cord tissue/Wharton's Jelly (UC MSC) or cord blood, respectively. In cord blood, two distinct nonhematopoietic stromal cell populations were described so far: USSC and CB MSC. UC MSC, umbilical cord mesenchymal stromal cells; USSC, unrestricted somatic stromal cells; CB MSC, cord blood mesenchymal stromal cells.

isolated and expanded from CB, clearly revealed distinct cell populations.[23,24] We termed them according to the revisited MSC concept:[25] USSC and CB mesenchymal/multipotent stromal cells (CB MSC).[26] We could also define that clonal USSC and CB MSC lines differ most likely in their developmental origin reflected by a distinct homeobox (*HOX*) gene expression and expression of the delta-like 1 homolog (DLK-1), resulting in a completely different differentiation and regeneration in vivo applying specific models for neural, cardial, liver, and mesodermal (skeletal) regeneration. About 20 (out of 39) *HOX*-genes are expressed in CB MSC (*HOX*-positive), whereas native USSC (*HOX*-negative) reveal no *HOX*-gene expression.[27] In addition, USSC display a lineage-specific lack of the adipogenic differentiation potential along with the expression of DLK-1 (Figure 2).[23,24,28]

During recent years more than 300 cell lines were generated, characterized, and cryopreserved. Moreover, it was clearly documented that neonatal CB cells delivered at birth (>36 weeks of gestation) do not contain any OCT4A positive "embryonic-like" cells, and that the majority of data published by other groups were misleading due to artifacts based on OCT4A pseudogenes.[29] Due to the developmental status, it is unlikely that OCT4A is found in fetal, neonatal CB of 36 weeks gestation.[30] Moreover, more than 25,000 allogeneic CB transplantations have been performed to date without any germ cell tumor formation (including teratoma). In contrast to iPS cells (also from CB cells)[31] that form teratomas, the absence of tumor formation in clinically relevant models (nude mice) is one of the most important features indicating why native neonatal CB subpopulations are promising candidates in regenerative cell-based approaches.

Beside the cell populations described above, other cells with "embryonic characteristics" were claimed to be present in CB. These so-called very small embryonic-like (VSEL) cells were described as being pluripotent and positive for expression of OCT4.[32] As of today, when nearly every cell can be reprogrammed to a pluripotent state with a defined set of transcription factors resulting in a true "pluripotent nature," this puts the idea of pluripotency in CB to a break. In addition, recent reports doubt the existence of these cells.[33] This resulted in a report in Nature News, summarizing studies refuting the existence of VSEL cells in tissue, including CB.[34] Since there are many publications about the regenerative ability of CB cells, we will try to focus on the ones that, in addition to the ECFC already highlighted above, could have therapeutic applicability based on cell numbers generated as well as functional data available in vitro and in vivo.

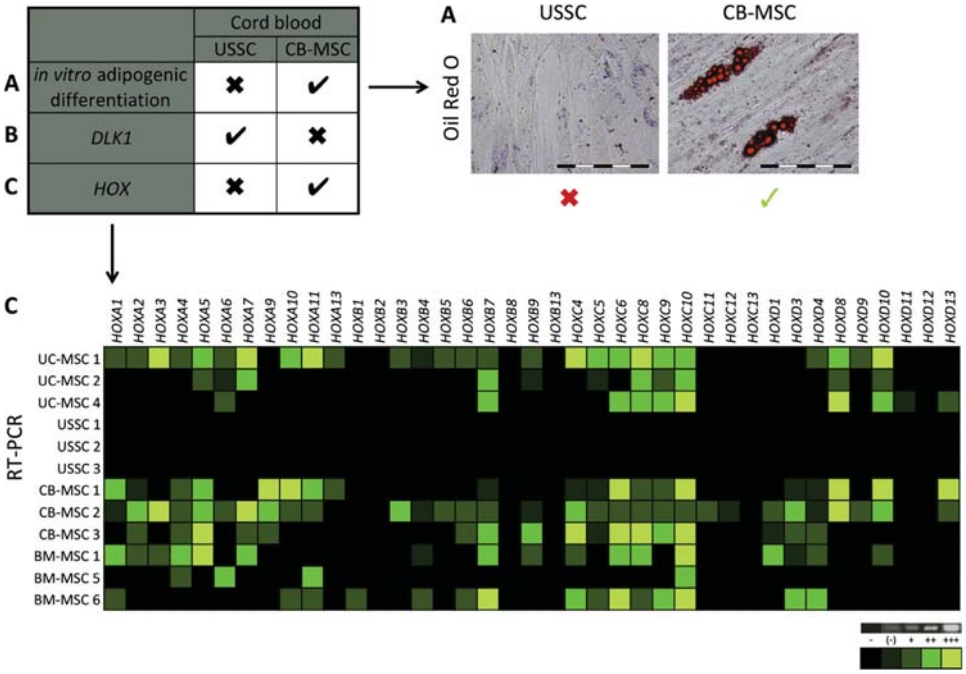

FIGURE 2 **Discrimination of cord blood-derived stromal cell types.** The nonhematopoietic stromal cell types from cord blood—USSC and CB MSC—can be distinguished on the basis of differentiation potential and gene expression. (A) In vitro adipogenic differentiation potential (21 days). Lipid vacuoles were visualized by Oil Red O staining. Scale bar: 100 µm. (B) RT-PCR analyses to evaluate the expression of *DLK-1* in USSC and CB MSC (Kluth et al. 2010, Stem Cells Dev). (C) RT-PCR of *HOX*-genes in the indicated cell types (black: absent (−); green: present, different expression levels are reflected by the color-intensity). UC MSC, umbilical cord mesenchymal stromal cells; USSC, unrestricted somatic stromal cells; CB MSC, cord blood mesenchymal stromal cells; BM MSC, bone marrow mesenchymal stromal cells; DLK-1, delta-like one homolog; HOX, homeobox.

4. ISOLATION, EXPANSION, AND CHARACTERIZATION OF CB-DERIVED ADHERENT CELLS FROM CB

Cultures of USSC and CB stromal cells are generated by the same method. Briefly, blood is collected from the umbilical cord vein directly after clamping, with informed consent of the mother. MNC are obtained by density-based Ficoll gradient separation and are subsequently cultured in plastic culture flasks containing serum-enriched medium supplemented with defined amounts of dexamethasone. As soon as colonies of adherently growing cells are observed, usually after 7–21 days in culture at 37 °C with 5% CO_2 and humidified atmosphere, the dexamethasone is omitted to avoid unwanted triggering of osteogenic differentiation.

Classification of the adherent cells into USSC and CB MSC can be assessed only after generation by determining the adipogenic differentiation potential, expression of *DLK-1* as well as *HOX*-gene expression. Over recent years, we initiated adherent cell cultures from 1009 CB samples, from which 40% ($n = 394$) gave rise to an average of 1–11 colonies per individual blood sample. Due to their very low frequency in CB (1–100 per $1 * 10^{-8}$ MNC),[23,24,28] other fetal sources (cord) were also used,[35,36] however, resulting in stromal cells had a completely different functionality, as discussed below.

5. GENERATION OF CELL CLONES AND CLONAL POPULATIONS

The availability of clones as well as clonal populations is mandatory to characterize stromal populations derived from CB as compared to those derived from BM.

Colony-clonal populations were obtained during generation of cell lines by applying special cloning cylinders. In this case, cell lines were generated as described before and, if distinct, separate colonies were observed, a cloning cylinder was attached on a single colony and cells were trypsinated according to the standard protocol. While these cells could be regarded as clonal in being derived from one single developing colony, expansion of single cells is mandatory for true clonality. Therefore, cells of one colony or of already established cell lines, respectively, were plated at low density into six-well cell culture plates. Employing an AVISO CellCelector™, single cells were picked and transported to a defined destination well of a 96-well cell culture plate. For verification, pictures were taken before and after each picking process to document successful single cell selection. By subsequent cultivation, initially with preconditioned medium, clonal lines were established which then could be expanded under standard conditions. In the case of cells picked from primary colonies, the remaining cells were expanded as bulk culture and referred to as initial cell line.

6. USSC AND CB MSC

USSC and CB MSC exhibit a comparable proliferative potential (Figure 3(A)[36]), and can be distinguished on the basis of gene expression and differentiation potential (Figure 2(A)). Both cell populations can be expanded under conditions that conform to clinical requirements ("good manufacturing practice;" GMP).[37] Here, the target cell number to be reached is more than 1.5×10^9 cells within 30 cumulative population doublings (corresponding to passage 4–5).

Applying in vitro adipogenic differentiation assays, CB MSC, but not USSC, can differentiate into adipocytes (Figure 2(B)). Former results indicated a correlation of this absent adipogenic potential and the expression of *DLK-1* in USSC, since USSC but not CB MSC express *DLK-1*. Therefore, this marker can be applied to discriminate the CB stromal cells on a transcript but not protein level.[23]

Applying microarray- and PCR-analyses, the presence of *HOX*-genes was defined as a distinguishing feature between USSC and CB MSC besides the expression of *DLK-1*: USSC do not express *HOX*-genes, while CB MSC are *HOX*-positive (Figure 2(C)[27]).

In addition to the discriminating features presented in Figure 2, further differences between USSC and CB MSC were detected. USSC possess a higher hematopoiesis-supporting capacity in coculture experiments.[24] With respect to the immunophenotype, CB MSC reveal a stronger expression of CD146 (melanoma adhesion molecule, MCAM) than USSC. During flow cytometric analysis, no endothelial ($CD31^{negative}$), leukocytic ($CD45^{negative}$), epithelial ($CD326^{negative}$), or antigen-presenting ($HLA\text{-}DR^{negative}$) phenotype was detected in any cell type analyzed.[36]

7. GENERATION OF ADHERENT CELLS FROM THE WHARTON'S JELLY (CORD)

The umbilical cord consists of one vein and two arteries, which are embedded in a connective tissue, the so-called "Wharton's Jelly" (Figure 1). Many protocols to isolate stromal cells from Wharton's Jelly (often referred to as umbilical cord mesenchymal stromal cells, UC MSC), with or without an enzymatic digestion step, have been published over time. In our group, UC MSC were isolated as described in[36] by the outgrowth of stromal cells from umbilical cord tissue cut into small pieces and cultivated in standard culture medium. Commonly, in vitro differentiation assays were performed to prove the identity of the isolated cells as being MSC-like with the potential to give rise to cells of skeletal tissues (osteoblasts, chondroblasts, adipocytes).[38] In accordance with our results, further studies reported a restricted differentiation potential of so-called UC MSC concerning in vitro[39,40] and in vivo[41] assays. Compared to USSC and CB MSC from CB as well as to

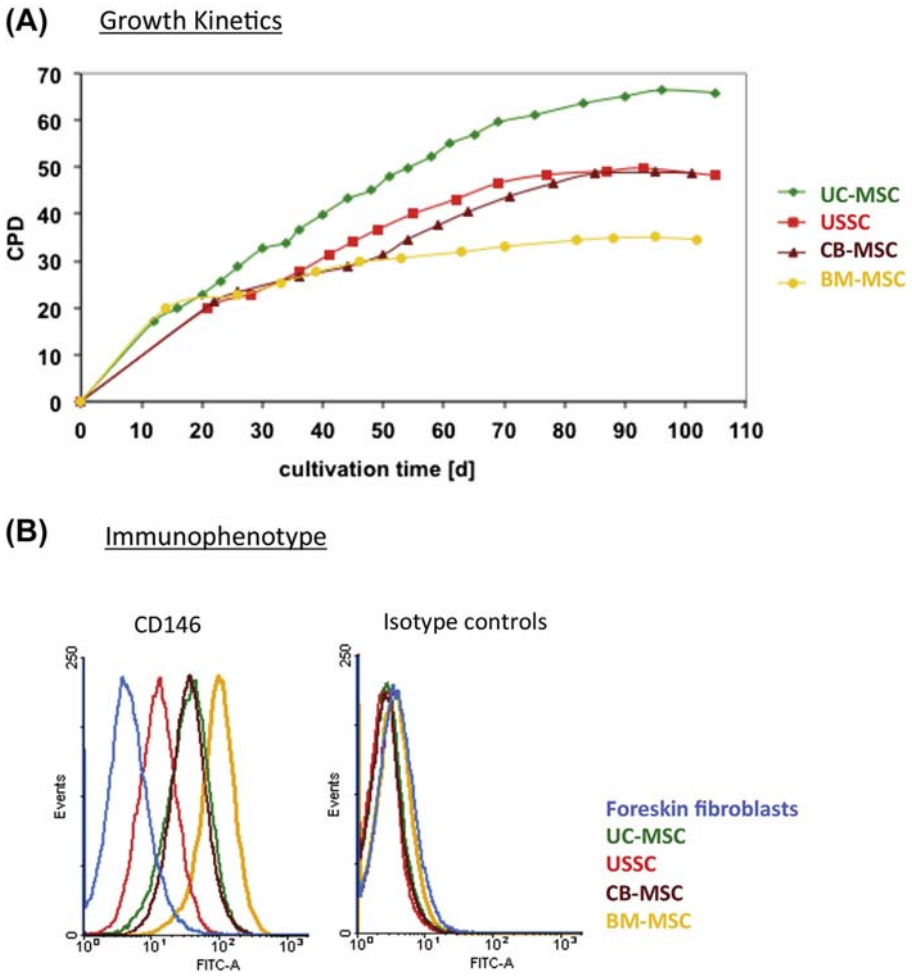

FIGURE 3 Characterization: growth kinetics and immunophenotype. (A) Representative growth kinetics. (B) Flow cytometric analysis of CD146 and the corresponding isotype controls. Foreskin fibroblasts were used as negative control. CPD, cumulative population doublings; UC MSC, umbilical cord mesenchymal stromal cells; USSC, unrestricted somatic stromal cells; CB MSC, cord blood mesenchymal stromal cells; BM MSC, bone marrow mesenchymal stromal cells.

BM MSC, these cells derived from the cord tissue demonstrated the highest proliferation potential (Figure 3(A)), but the lowest (or completely absent) differentiation potential toward the osteogenic, chondrogenic, and adipogenic lineage.[36] Therefore, it also has to be questioned whether the term mesenchymal or multipotent does apply at all to stromal cells from cord tissue.

8. GENE EXPRESSION PROFILES

Microarray gene expression analysis is a useful tool in comparing different cell types and provided further insight into the gene expression profile of CB-derived USSC and CB MSC in comparison to BM MSC.[35]

The expression of the majority of genes was shared by USSC, CB MSC, and BM MSC (Figure 4(A)). The expression pattern of USSC and CB MSC was more similar to each other than to BM MSC, which reflects the common CB origin.

These arrays also gave hints for differences in regard to differentiation capacity, and subsequent analysis of specific gene expressions was carried out. Special attention was paid to genes associated with the process of osteogenesis and in according quantitative PCR experiments, a stronger expression of bone sialoprotein (*BSP*), osterix (*OSX*, correctly *SP7*), bone morphogenetic protein 4 (*BMP4*) and osteocalcin (*OC*) in BM MSC compared to the CB-derived cell types was shown (Figure 4(B)[35]). Unlike BM MSC, the CB stromal cells lacked the typical bone signature.

Thus, USSC and CB MSC exhibit a more immature status than BM MSC with respect to the genetic control of bone formation.

9. CORRELATION OF *HOX*-GENE EXPRESSION AND REGENERATIVE POTENTIAL

The vertebrate skeleton consists of elements which vary regarding their embryonic origin. The craniofacial skeleton is derived from the cranial neural crest, the axial skeleton from the paraxial mesoderm, and the limb skeleton from the lateral plate mesoderm.[42] Depending on

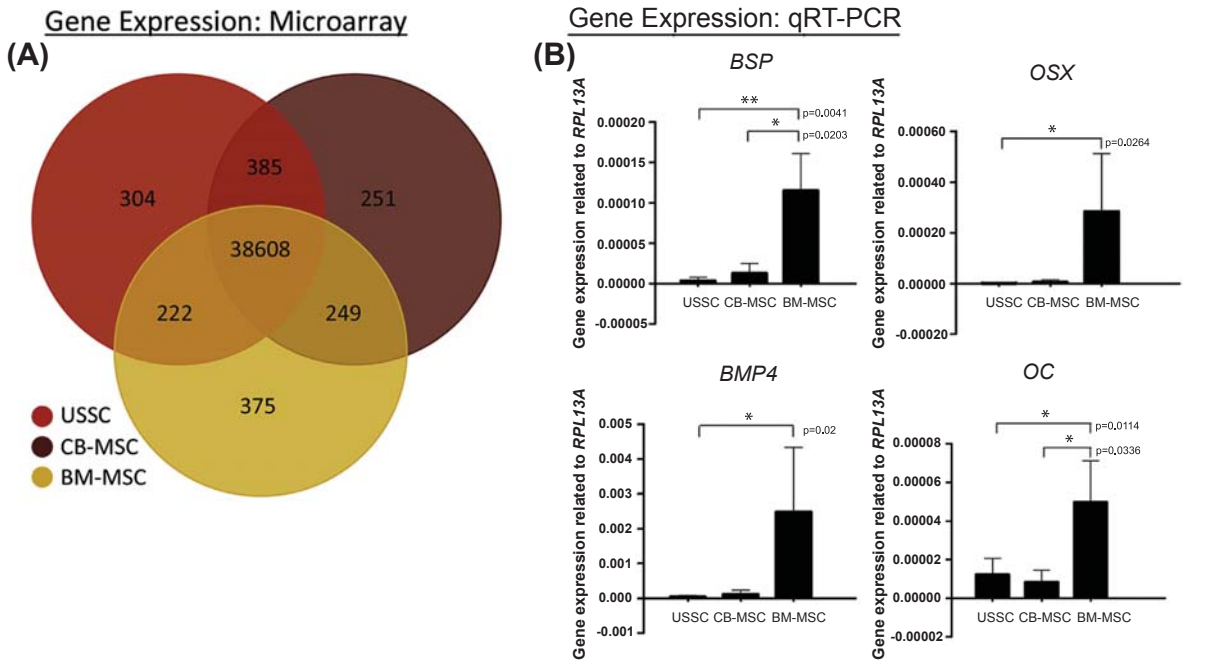

FIGURE 4 **Gene expression profile.** (A) The gene expression of USSC, CB MSC, and BM MSC (each in triplicate) was analyzed applying PrimeView™ Human Gene Expression Arrays. The Venn-diagram illustrates the common and unique expression of genes. The gene expression was filtered for each cell type: A probe set had to be expressed above the background (twentieth and hundredth percentile of the raw signal distribution) in at least two out of three replicates. This resulted in 39,519 transcripts for the "group USSC," 39,493 transcripts for CB MSC, and 39,454 transcripts for BM MSC which were compared. (B) Quantitative RT-PCR analyses of genes expressed differentially in USSC, CB MSC, and BM MSC. Illustrated are the arithmetic means and standard deviations of at least three different cell lines per cell type. *$p = 0.01–0.05$, significant; **$p = 0.001–0.01$, very significant (unpaired *t*-test). RPL13A was used as housekeeping gene. USSC, unrestricted somatic stromal cells; CB MSC, cord blood mesenchymal stromal cells; BM MSC, bone marrow mesenchymal stromal cells; BSP, bone sialoprotein; OSX (SP7), osterix; BMP4, bone morphogenetic protein 4; OC (BGLAP), osteocalcin; RPL13A, ribosomal protein L13A.

the embryonic origin, a different *HOX*-gene expression pattern can be detected. While most parts of the skeleton—including the corresponding tissue-derived progenitor cells—are *HOX*-positive, the craniofacial skeleton is *HOX*-negative.[43,44]

Leucht and coworkers performed transplantation experiments in mice and analyzed the influence of the *Hox*-gene expression.[44] As illustrated in Figure 5(A), skeletal progenitor cells derived from the mesodermal tibia ($Hox^{positive}$) were transplanted in defects of the ectodermal mandible ($Hox^{negative}$), which resulted in cartilage formation instead of bone regeneration. On the contrary, the transplantation of mandibular cells ($Hox^{negative}$) in a defect of the *Hox*-positive tibia resulted in effective bone repair (Figure 5(A)). The *Hox*-positive cells retained their *Hox*-gene expression after transplantation in a *Hox*-negative tissue. In contrast, *Hox*-gene expression was adapted in the previously *Hox*-negative cells following transplantation in a *Hox*-positive tissue. These results led to the conclusion, that the *Hox*-negative status of the mandibular cells and the potential to adapt a *Hox*-gene expression after transplantation may be regarded as beneficial concerning the regenerative potential.

As described above, CB-derived USSC lack the expression of *HOX*-genes, while CB MSC are *HOX*-positive (Figure 2(C)). As illustrated in Figure 5(B), we transferred the in vivo model described by Leucht et al. to an in vitro coculture model.[26] USSC were tagged using the green fluorescent protein (GFP) and cocultivated with *HOX*-positive cells (UC MSC, CB MSC, or BM MSC) for 5 days. After coculture and cell sorting, the previously *HOX*-negative USSC expressed *HOX*-genes (Figure 6(A)). Analogous to the *Hox*-negative mandibular cells, which started to express *Hox*-genes after transplantation in the tibial defect (Figure 5(A)), USSC were able to adapt a *HOX*-gene expression after cocultivation with *HOX*-positive stromal cells. Besides the *HOX*-gene expression, the adipogenic differentiation potential was affected by the direct coculture. USSC, which failed to differentiate into adipocytes, exhibited an adipogenic potential after coculture with CB MSC (Figure 6(B)). The direct coculture represents a simple way to modulate the cell fate decision of neonatal stromal cells. Due to their ability to adapt to the surrounding tissue, USSC are a promising cell type for the potential clinical application in the future, if the mechanisms are enlightened and proven in vivo.

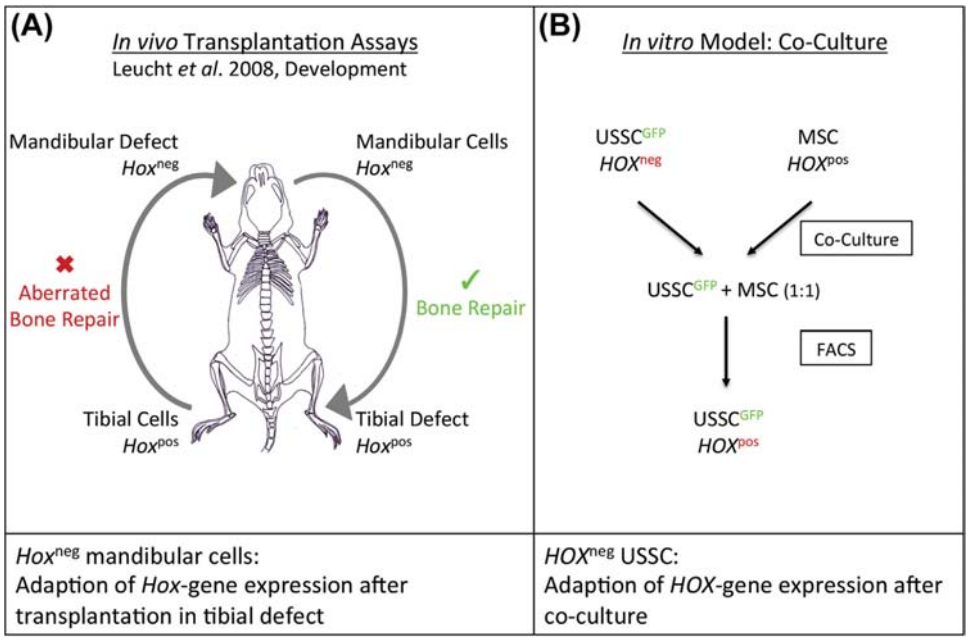

FIGURE 5 **Influence of *HOX*-gene expression on regenerative potential.** (A) Schematic illustration of in vivo experiments by Leucht et al. (2008, Development) analyzing the influence of *HOX*-gene expression of transplanted cells on bone repair. (B) Scheme of the in vitro coculture (5 days) of *HOX*-negative USSC with other *HOX*-positive cells. *HOX*, homeobox; USSC, unrestricted somatic stromal cells; MSC, mesenchymal stromal cells; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorting.

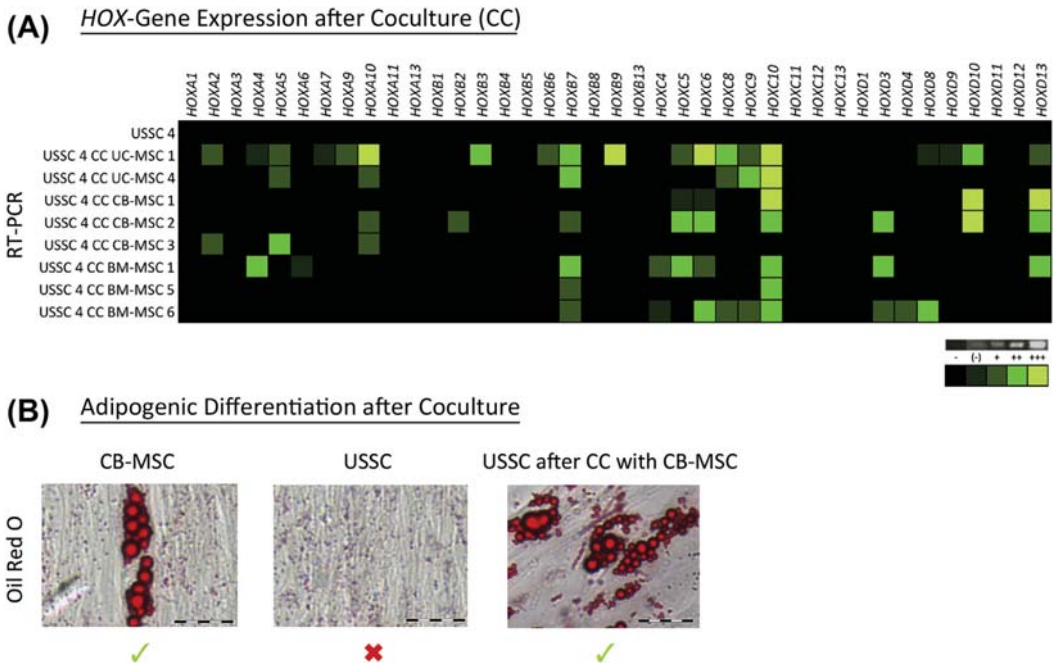

FIGURE 6 **Influence of coculture on *HOX*-gene expression and differentiation potential.** *HOX*-negative USSC were cocultivated (CC) with *HOX*-positive cells (UC MSC, CB MSC, or BM MSC) for 5 days, followed by GFP-mediated cell sorting. (A) RT-PCR of *HOX*-genes in the indicated cell types (black: absent (−), green: present, different expression levels are reflected by the color-intensity). (B) After FACS, USSC were cultivated for one further passage before adipogenic differentiation and Oil Red O staining (d21). Scale bar: 25 µm. *HOX*, homeobox; UC MSC, umbilical cord mesenchymal stromal cells; USSC, unrestricted somatic stromal cells; CB MSC, cord blood mesenchymal stromal cells; BM MSC, bone marrow mesenchymal stromal cells; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorting.

10. BONE AND CARTILAGE FORMING POTENTIAL OF CORD BLOOD STROMAL CELLS

For the purpose of bone and cartilage regeneration (lost due to trauma, surgical resection of tumors, skeletal disorders, and aging), cell-based strategies are currently the gold standard of treatment. The use of freshly isolated CD146-positive BM MSC, in contrast to the extensively expanded counterpart, provides an important therapeutic tool for bone regeneration, although not for cartilage.

Bone is a highly vascularized connective tissue undergoing continuous remodeling and regeneration processes. The intrinsic regeneration potential is initiated in response to injury, as well as during normal skeletal development reflected by continuous remodeling throughout adult life.[45] Many bone and cartilage associated diseases require regeneration in large scale, e.g., large bone defects also known as "critical size" defects due to trauma, surgical resection of tumor, infection, or skeletal disorders. Especially for treatment of these defects, a cell-based strategy is the most promising approach as long as a sufficient number of cells can be supplied. Clinically, stromal cells can be used as cell suspension expanded by culture or simply as BM concentrate.[46] In this context, ex vivo expanded mesenchymal stromal cells (BM MSC) have demonstrated their ability to function as a tissue repair model in manifold therapeutic applications investigated in clinical trials.[47] However, the outcome of tissue repair is strongly associated with the applied cell concentration, which is lower in BM transplants compared to cultured cells. Moreover, large-scale cell amplification by ex vivo expansion harbors the risks of dilution of the relevant osteogenic clones, xenogenic incompatibility, and cellular transformation.[10,48] For de novo cartilage repair there are no established methods available, simply based on the fact that adult BM does not contain the early chondrogenic progenitors in sufficient amounts to regenerate large areas of defects. For all clinical applications one should choose the best characterized cells for a directed and specific tissue repair. As shown in Figure 4 and summarized in Bosch et al.,[35] Kluth et al.,[23] Liedtke et al.,[26] and many other publications in vitro and in vivo, fetal stromal cells (both USSC and CB MSC) have specific signatures for bone and cartilage formation.

11. WHY DO WE HAVE THESE PROGENITORS OR ELUSIVE CELLS IN CB?

The correct formation of the skeleton during embryogenesis and fetal development, and its preservation during adult life is essential and is maintained through the complementary activities of bone-forming osteoblasts and bone-resorbing osteoclasts.[49] The stability and strength of bones is accomplished by mineralization of the extracellular matrix, leading to a deposition of calcium hydroxyapatite.[50] The osteogenesis can be split into two different processes: intramembranous and endochondral ossification.[51] The intramembranous ossification is characterized by mesenchymal cells that condense and directly differentiate into osteoblasts and thereby deposit bone matrix. This process of bone formation is limited to certain parts of the skull as well as part of the clavicle. All other bones of the skeleton are formed by endochondral ossification. The formation of skeletal elements by endochondral ossification begins with the migration of undifferentiated mesenchymal cells to the zones that are destined to become bone. The undifferentiated cells condense, resulting in an increase in cell packing and forming of the cartilaginous anlagen. This process is regulated by mesenchymal–epithelial cell interactions. The next step, the aggregation of chondrogenic progenitor cells into precartilage condensations, is dependent on cell–cell and cell–matrix interactions.[51] The following transition from a chondrogenic progenitor cell to a chondrocyte is marked by a change in the extracellular matrix composition. The chondrocytes thereby acquire a rounded morphology and undergo hypertrophy (substantial increase in size). This chondrocyte hypertrophy triggers the initial osteoblast differentiation from perichondrial cells. Blood vessels start to invade the cartilage from the perichondrium and thereby transport osteoclast cells into the bone to degrade the existing cartilage matrix producing marrow cavity. Additionally, the blood vessels transport perichondrial cells to nascent BM, where they differentiate into osteoblasts. Many different transcription factors and regulatory signals are involved in the endochondral ossification, such as the transcription factor of the sex-determining region Y (SRY)-related high mobility group box, SOX9,[50] the Runt domain-containing transcription factor 2 (RUNX2),[52] Osterix (OSX),[50] BSP,[53] and parathyroid hormone-related protein (PTHLH).[52] All of the transcription factors are regulated by a variety of developmental signals, including Hedgehog (HH) proteins, NOTCH signaling, WNT signaling, BMP signaling, and fibroblast growth factor (FGF)-signaling.[50] It has been shown already that distinct populations can be defined, each of them representing progenitors of skeletal development during fetal life.[27,54] The transcription factor analysis of the subpopulation suggests that the stromal components in CB are elusive cells circulating from different stages of fetal development.

For bone- and cartilage-forming cells it can be concluded that CB contains natural progenitors, however with a different signature in vitro and a distinct in vivo regenerative capacity as compared to BM MSC.

12. USSC AND MSC FROM CB SUPPORT HEMATOPOIETIC CELLS

Another potential application of neonatal stromal cells is as a supportive mean for hematopoietic cells in general and for HSC in particular. Although technical procedures and protocols have been optimized since Eliane Gluckman's first transplantation in 1988,[55] the limited number of cells available from one single CB still is one of the major hurdles for transplantation purposes. With a harvested volume of usually between 50 and 150 ml, total nucleated cell (NC) counts and numbers of HSC rarely exceed $2 * 10^9$ or $1 * 10^7$, respectively. With requirements of at least $3.7 * 10^7$ cells per kilogram body weight for NC, and a desired amount of more than $1 * 10^5$/kg for HSC, the utilization in adults is highly restricted.

Accordingly, great efforts were made to find appropriate ways to overcome this problem and to provide cell counts sufficient even for heavy-weight patients. Among the ideas pursued, two basic principles can be distinguished: the expansion of the hematopoietic cells prior to transplantation in vitro, and the improvement of engraftment during or after transplantation in vivo.

Both approaches have their own advantages and disadvantages which have to be taken into consideration. For example, in vitro expansion not only requires GMP-grade equipment and reagents, but also requires development a certain time ahead of transplantation. Additionally, exhaustion of stem cells might occur and result in impaired capability of long-term reconstitution. On the other hand, manipulating engraftment in vivo is much more complicated and it goes without saying that any possible negative side effect on the patient has to be thoroughly excluded.

Double cord blood transplantation (DCBT), since being described in 2005 by the group of John E. Wagner,[56] doubtlessly has become a standard method in clinics. Here, the property of CB to be less restricted in regard to HLA-matching is utilized to successfully combine two individual CB units for treatment of a single patient. However, this still might not be sufficient for heavy-weight patients and/or patients with rare HLA-type.

Cytokine-driven expansion is performed in vitro by addition of specific hematopoietic cytokines. These cytokines, namely stem cell factor (SCF) as the most prominent one, lead to an extensive proliferation already at low concentration and multiple protocols for cytokine cocktails are described in literature. While this expansion results in increased numbers of hematopoietic stem and progenitor cells, the influence on the stemness of these cells has to be evaluated. For example, already short expansion in medium enriched with SCF, thrombopoietin (THPO), FMS-related tyrosine kinase 3 ligand (FLT3LG), and interleukin 6 (IL-6) leads to a strong expansion of cells isolated by fluorescence-activated cell sorting (FACS). On day 3, nearly all resulting cells are positive for surface expression of CD34, but analysis has already revealed distinct changes on protein level.[57] Since cytokines are very potent, fine tuning can be considered as most critical in order to prevent cells from exhaustion and loss of long-term repopulating cells. Novel protocols target the NOTCH-pathway and might help in finally achieving efficient expansion of early stem and progenitors cells,[58] but currently the clinical application is restricted to partial expansion only (either of half a CB unit or, in case of DCBT, of one complete unit). Another aspect might also be the manipulation of not the total cell number, but the engraftment itself. Here, the application of prostaglandin ex vivo prior to transplantation has recently been reported as resulting in a more rapid engraftment in a clinical trial.[59]

Finally, as a third option, nonhematopoietic stromal cells are used as a supportive tool, either in vitro or in vivo. Here, CB-derived stromal cells demonstrate a promising effect which also bears potential clinical importance. Therefore, the following section will comprise and discuss data from cocultivation as well as cotransplantation in an animal model.

Multipotent/mesenchymal stromal cells, such as BM MSC, CB MSC, or USSC, secrete various cytokines. Among these cytokines, many are known for their influence on hematopoietic cells, either as inducer of proliferation, as chemoattractor, or for differentiation. In the hematopoietic niche located in the adult BM, stromal cells are involved in maintaining the quiescence of HSC but they also are responsible for the mobilization and chemotaxis of HSC after injury (e.g., through secretion of stromal-derived factor 1, which strongly attracts HSC via the chemokine receptor CD184, also know as CXCR4).

Assuming that these cells do not only provide a cytokine composition which is well tuned to HSC, but also provide cell–cell contact, which might be an additional need, cocultivation of stromal cells and HSC in order to mimic the natural hematopoietic environment is a sound approach for in vitro expansion prior to transplantation.

Since stromal cells do grow adherently on plastic surfaces, as in culture flasks or well plates, they form a so-called feeder layer without the need of further manipulation. Though, to prevent these cells from overgrowing, they have to be stopped from proliferating in long-term expansion. This can be achieved either by treatment with mitomycin C or by irradiation. Subsequently, either the total fraction of MNC or isolated CD34-positive HSC from CB can be seeded on these layers in medium, e.g., Dulbecco's modified Eagle medium (DMEM) with 30% fetal calf serum (FCS).

Within a few days, while controls of HSC in FCS-enriched medium without stroma results in maintenance or even loss of cells, a definite expansion of the nonadherent cells can be observed on various feeder cells, such as USSC, CB MSC, or BM MSC.

However, flow cytometric analysis of these cells after staining against human CD34 and human CD45 (pan-leukocyte marker) will reveal that expression of CD34 is not detectable on the surface of all cells and that the percentage of CD34-positive cells decreases with days in culture. For estimation of HSC-expansion, this percentage has to be set off against the total cell counts. Further analysis comprises the assessment of colony-forming cells (CFC), which is a more functional test. In this assay, defined numbers of cells are seeded in growth factor-enriched semisolid media that induces strong proliferation and differentiation in HSC. This leads to the formation of clearly distinguishable erythroid (red) or myeloid (white) colonies within 14 days of culture and correlates with the presence of stem and progenitor cells in the sample (a complete scheme of coculture and respective analysis is depicted in Figure 7(A)).

For CB-derived stromal cells, it was already published in 2005 that these are capable of expanding hematopoietic cells in coculture in specific medium (Myelocult H5100) more than 100-fold within 4 weeks,[60] which was distinctly higher than on BM MSC. Interestingly, while total cell count constantly increased, expansion of CD34-positive cells and CFC reached a maximum by day 14 and decreased with further coculture. This might indicate that an exaggerated expansion here also leads to exhaustion of early stem and progenitor cells.

However, more recent data demonstrate that this ability to support HSC is much stronger in USSC than in CB MSC. Even in short time expansions for a maximum of 14 days and using DMEM with FCS as medium, which is less supportive to HSC than Myelocult H5100, expansion of total cells, CD34-positive cells as well as of CFC was clearly detectable on USSC-feeder. Under these conditions, cocultivation on CB MSC also resulted in expansion of total cell count, but only a maintenance of CD34-positive cells and CFC could be achieved[24] (see Figure 7(B)), similar to stromal cells derived from BM (data not published).

While cocultivation on neonatal stromal cells might provide a gentle expansion of HSC in vitro, application as supportive cells in vivo is another promising approach. Since stromal cells from CB can be generated, expanded, and cryopreserved under GMP-conform conditions,[37] they are potentially directly applicable, e.g., in transplantation.

First of all, it had to be verified that these cells do not bear the risk of intrinsic tumorigenicity. According to experiments in which CB- and BM-derived stromal cells were injected subcutaneously in the flank of nude mice, no tumor formation related to the human cells was demonstrated.[61]

In a further step to evaluate effects on engraftment and hematopoiesis, USSC cells were cotransplanted intravenously with CB-derived HSC, and isolated by magnetic-activated cell sorting (MACS) into immune-deficient mice (NOD/SCID) after sublethal irradiation. Additionally, a group of animals received stromal cells

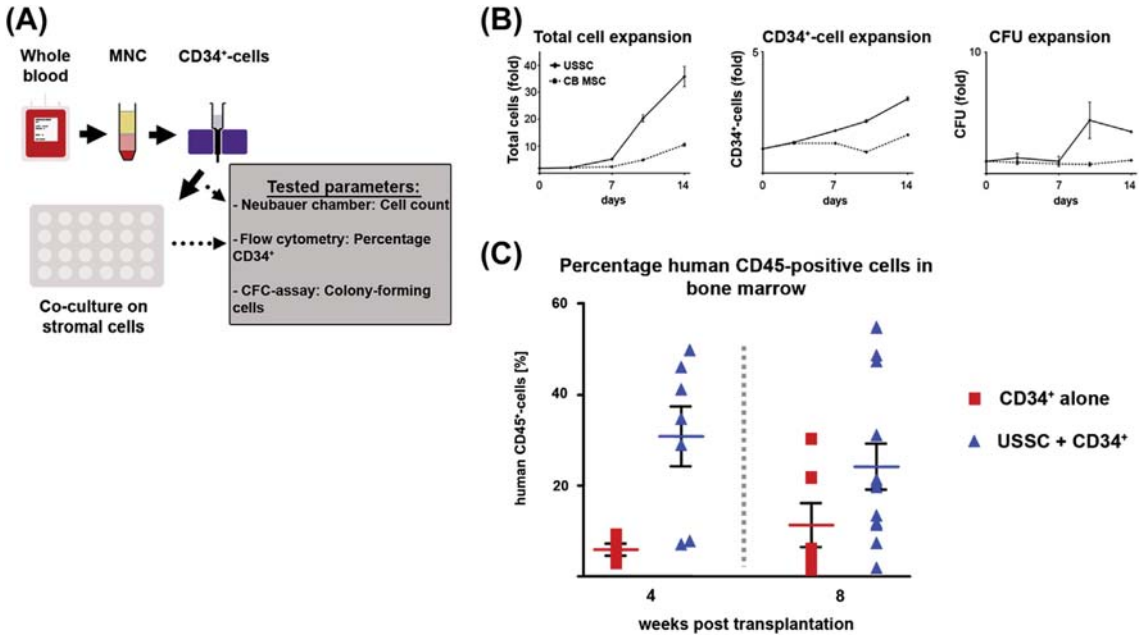

FIGURE 7 **Hematopoietic support of cord blood-derived stromal cells.** (A) Schematic illustration of coculture experiments with MACS-isolated CD34-positive cells on stromal feeder layers. (B) Under identical conditions, coculture of cord blood-derived HSC resulted in a higher expansion of total cells, CD34-positive stem/progenitor cells and colony-forming cells on USSC than on CB MSC. (C) In NOD/SCID-mice, cotransplantation of USSC resulted in higher percentages of human leukocytes in the bone marrow after 4–8 weeks. MNC, mononucleated cells; MACS, magnetic-activated cell sorting; CFC, colony forming cells; USSC, unrestricted somatic stromal cells; CB MSC, cord blood mesenchymal stromal cells.

only and another group HSC only. After 4 and 8 weeks, respectively, animals were sacrificed and analyzed. For assessment of reconstitution, occurrence of human hematopoietic cells in peripheral blood, spleen, and BM was analyzed by flow cytometry while solid organs (brain, heart, kidney, liver) were tested in immunohistochemistry by staining against human nuclei.

Albeit animals that received stromal cells only were completely negative for the presence of human cells in all organs tested and no difference could be observed in the percentage of engrafted human HSC between animals that were transplanted with CD34-positive cells alone or cotransplanted with stromal cells, the latter ones had a significantly higher percentage of human leukocytes (detected by expression of human CD45 in flow cytometry) in the BM.[61] While after 4 weeks cotransplantation resulted in a 5.2-fold higher amount, this difference was less marked after 8 weeks (2.1-fold higher in cotransplanted animals). This leads to the conclusion that probably a paracrine effect enhances the initial engraftment of the HSC and results in faster generation of more differentiated cells within the first period after transplantation. The impact observed was higher than that reported for other cotransplanted cells and also could be beneficial for human patients in regard to a faster reconstitution. Apart from this effect, CB-derived stromal cells are also reported to be more immunosuppressive than BM MSC and might help in avoidance of severe acute graft-versus-host-disease (GvHD). So far, clinical results are still rare but first reports are promising, including the combination of cotransplantation with the already established DBCT.[62]

Despite these clearly demonstrated effects on hematopoietic cells in vitro and in vivo, the mechanisms are yet not fully understood. However, it will at least in part be related to cytokines secreted by the stromal cell type utilized. For example, CB-derived stromal cells in general demonstrated at least a two-fold higher expression of SCF in quantitative real-time PCR than BM stromal cells, while vice versa, IGF-2 was expressed 10-fold more in BM MSC than in USSC or CB MSC. The findings that CB MSC do express a higher level of SCF, known as the most potent inducer of proliferation in hematopoietic stem and progenitor cells, is somewhat surprising since capacity for hematopoietic support in coculture expansion was comparable to that of BM MSC. Either the positive effect of SCF is counteracted by an inhibiting factor in CB MSC and BM MSC or enhanced in USSC. Analysis of additional cytokines revealed further differences between USSC and other stromal cells: IGFB-1 is expressed 10-fold higher than in CB MSC or BM MSC, while SDF-1 expression is five-fold lower (Figure 8(A)). Especially the lower expression of stromal-cell-derived factor 1 (SDF-1) might explain the differences in hematopoietic support, since this cytokine is known not only as a potent chemoattractant (in its secreted form[63]) for HSC, but also functions as an inducer of quiescence (if presented membrane-bound by stromal cells[64]).

Taken together, these results not only confirm that USSC are distinctly different from CB MSC or BM MSC, but also might indicate that USSC are of different origin than CB MSC. Chou and Lodish[65] reported a DLK-1-positive subpopulation in murine fetal liver cells with an increased capacity of hematopoietic support. With USSC being positive for DLK-1 on the transcript level, showing a hematopoietic support superior to comparable stromal cells and expressing some cytokines described by Chou and Lodish (Figure 8(B)), it can be speculated that these cells are descendants of the fetal liver while CB MSC are the progeny the of fetal BM cells.

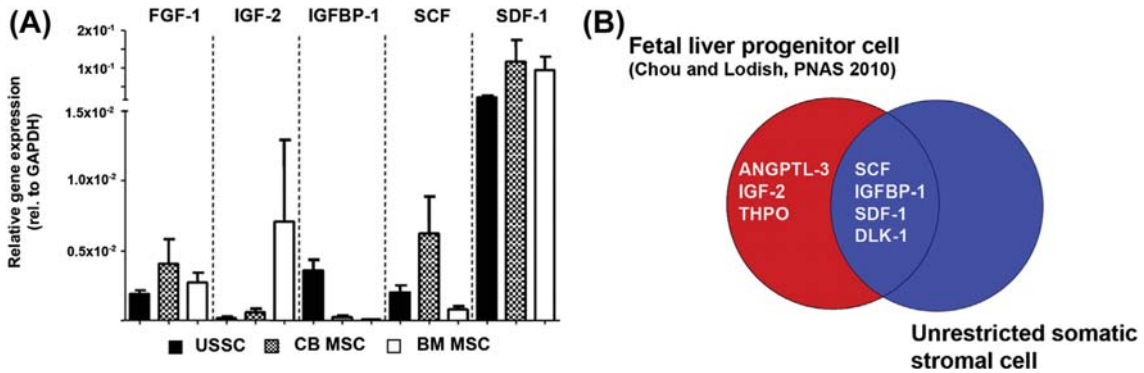

FIGURE 8 **Hematopoietic cytokines expressed by stromal cells.** (A) USSC, CB MSC, and BM MSC demonstrate distinctly different expression profiles for selected hematopoietic cytokines in real-time PCR analysis. (B) Comparing USSC with a subpopulation of murine fetal liver cell described by Chou and Lodish in 2010[65] reveals similarities with regard to expression of specific cytokines. GAPDH, glycerinaldehyde 3-phosphat dehydrogenase; FGF-1, fibroblast growth factor 1; IGF-2, insulin-like growth factor 2; IGFBP1, insulin-like growth factor-binding protein 1; SCF, stem cell factor; SDF-1, stromal cell-derived factor 1; ANGPTL-3, angiopoietin-like 3; THPO, thrombopoietin; USSC, unrestricted somatic stromal cells; CB MSC, cord blood mesenchymal stromal cells; BM MSC, bone marrow mesenchymal stromal cells.

13. LIVER REGENERATION AND POTENTIAL OF CB-DERIVED STEM CELLS TO UNDERGO HEPATIC DIFFERENTIATION

To date the clinical importance of cellular therapies for hepatic surgery is demonstrated by application of mature donor hepatocytes in certain disease forms. The marginal number of available donor tissues, and thus donor cells, determines the global restriction for therapeutic hepatocyte-based applications. Thus, appliance of differentiated stem cells could increase availability of hepatic cells for transplantation purposes.

The consideration of nonhepatic stem cells as sources for hepatocytes or hepatocyte-like cells in rodents rose after identification of donor cells in livers after stem cell transplantation.[66] Further studies revealed that a broad spectrum of extrahepatic stem cells repopulate rodent livers.[67] Nevertheless, it has been discussed controversially whether extrahepatic stem cells retain nonhepatic features after transplantation and whether the hepatic transformation is caused by cell fusion with hepatic cells or is committed intrinsically.[68] Other groups reported that transplanted CB stem cells express some liver markers but differ in many aspects from definitive hepatocytes.[69] A principal proof of the ability of extrahepatic stem cells to undergo hepatic differentiation is performed by in vitro differentiation analysis. Many scientists focused on differentiation of umbilical CB stem cells into the hepatic lineage. Different cell culture strategies were applied to induce differentiation. Mostly the authors described protocols reflecting embryonic development of the liver to achieve proper differentiation. For stromal cells from CB it could be shown to respond to signaling molecules of hepatic differentiation, followed by induction of hepatic gene expression, performance of hepatic functions, and in vivo liver repopulation capacity.[70]

First reports for USSC of hepatic differentiation could be reported after transplantation of USSC in a noninjury model, the preimmune fetal sheep model. USSC engrafted into host livers and revealed over 20% cells of total livers.[19] These cells could be characterized by human albumin and expression of hepatocyte paraffin 1 (HepPar1), further serum of born sheep showed human albumin content demonstrating the functionality of engrafted USSC to produce and release serum proteins.[19] In in vitro approaches it was reported that USSC differentiate into cells expressing liver markers like albumin, hepatocyte nuclear factor 4 α (HNF4α), and glycogen synthase 2 (GYS2) to different protocols[71] were applied. Ghodsizad et al.[72] could demonstrate the engraftment of USSC into mature sheep livers after portal injection. In this noninjury model over 80% of total cells around portal veins contributed to a human phenotype. Technically an adult sheep model was applied where portal vein cannulation was performed under systemic administration of heparin, while a port access sheet was placed into the portal vein. An angiography catheter was placed into the left portal vein and acute hepatic ischemia was induced by selective injection of micro beads. Subsequently, USSC were injected into the right portal vein. Sheep were sacrificed after 1 month and livers were analyzed by HepPar1 and human albumin staining of formalin-fixed sections. A majority of chimeric liver parenchymal cells showed HepPar1 and human albumin expression. In order to demonstrate hepatic-like phenotype resulting from differentiation and not from fusion events, single HepPar1 positive and negative cells were microdissected from 2-μm liver sections for single cell PCR analysis. Coamplification of species-specific genes, such as human VH1, human TCRVβ7.2, ovine VH7, and TCRCδ loci from single cells allowed selective detection of human DNA in HepPar1 positive and ovine DNA in HepPar1 negative cells, thereby demonstrating that USSC differentiation in vivo was not caused by cell fusion.

The differentiation capacity of USSC into endodermal tissue was further proven by in vitro assays, although the in vitro testing is always more "artificial" as compared to in vivo regenerative or injury models.

It could be demonstrated that induction of hepatic markers in USSC is possible upon treatment with growth factors, retinoic acid, and administration of different coculture assays. Hepatic-like identity of differentiated USSC was confirmed by functional assays for glycogen synthesis (periodic acid-Schiff staining) and albumin secretion (enzyme-linked immunosorbent assay; ELISA).[71,73] Assessment of the hepatic differentiation was also analyzed applying a novel three-stage protocol resembling embryonic developmental processes of hepatic endoderm. Hepatic preinduction was performed by Activin A and FGF4 resulting in enhanced expression of SOX17 and Forkhead box protein A2 (FOXA2) as demonstrated by real-time PCR and immunohistochemical analysis. Differentiation into mature hepatic cells was achieved sequentially by retinoic acid, FGF4, hepatocyte growth factor (HGF), epidermal growth factor (EGF), and oncostatin M (OSM) resulting in gene expression of GYS2, glucose 6-phoshatase (G6PC), fructose 1,6-bisphosphatase (FBP1), arginase1 (ARG1), and HNF4α after differentiation, thus indicating a more mature state. Functional testing specified the hepatic-like nature of differentiated USSC by albumin secretion, urea formation, and cytochrome-p450-3A4 (CYP3A4) enzyme activity.[74] In order to characterize the differentiated cells at a metabolic level, USSC were incubated with [1–13C] glucose, and neutralized perchloric acid extracts were analyzed by nuclear magnetic resonance spectroscopy. Corresponding to GYS2, G6PC, and FBP1 expression, formation of both glycogen and some gluconeogenetic activity could be observed providing evidence of a hepatocyte-like glucose metabolism in differentiated USSC.[74] Since USSC already express transcription factors

of the early hepatic endoderm development, such as GATA binding protein 6 (GATA6), hematopoietically expressed homeobox gene (HEX), or prospero-related homeobox 1 (PROX), they are also an attractive source for reprogramming.[74]

Since USSC resemble in their expression status (*DLK-1*-positive, *HOX*-negative) fetal liver cells, their regenerative potential toward endoderm is logical biological evidence, however, the potential so far is much lower as compared to mature liver cells and reprogrammed cells (iPS cells) differentiated toward the endodermal lineage.

14. CARDIAL REGENERATION IN VIVO

Human heart infarction involves the dramatic loss of cardiomyocytes. Therefore investigators sought to identify endogenous cells or stem cells with the ability to differentiate into committed cardiomyocytes and to regenerate the myocardium.[75,76] Dozens of stem cell types have been reported to have cardiac potential, including pluripotent embryonic stem cells, iPS cells and adult progenitor cells from BM, peripheral blood, or intrinsic cardiac cells.[77] For donor cell types as BM MSC, MNC from BM, and endothelial cells from different sources already in clinical studies, the dominant in vivo effect may be neoangiogenesis and not cardiac specification. Moreover, despite some encouraging results from clinical trials, disagreement exists about the efficiency of the treatment, making this issue controversial in the field of stem cell therapy. Moreover for skeletal myoblasts, despite integration and survival, arrhythmia was found in clinical trials.

These obstacles provided the basis why researchers were interested in CB subpopulations, since CB exhibit great functional plasticity and can adapt in a new environmental niche to give rise to cell lineages for the new tissue site as described above for the adaptation of *HOX*-negative cells to *HOX*-positive tissue.[26] In two early reports, surgically implanted USSC into the infarcted heart in a porcine model revealed improvement of the regional and global left ventricular function, but only very few cells were detected in the myocardium after 4–8 weeks, and it could not be documented whether the effects were a result of de novo formation of cardiomyocytes together with a vascular restoration, or a paracrine effect.[20,78] USSC transplantation in a rat model resulted in functional improvement after myocardial infarction, however the observed functional effects could not be confirmed in two reports.[79,80] Besides minor variations in terms of isolation and cultivation of CB-derived adherent cells, the reason for the "negative" results remained unclear.[80] Mechanistic insights are required to evaluate the route of application of cells and the discrimination between differentiation and paracrine mechanisms. Applying a Wistar rat model with cyclosporin A for immunosuppression and an additional model of nude rats, Ding et al.[81] recently analyzed the fate of cells directly after coronary delivery. In detail, the differentiation of cells into vascular cells and cardiomyocytes was assessed. A major finding of the study was that about 80% of the initially infused USSC were retained in the heart directly after transplantation, however, the retained USSC underwent apoptosis so that the long-term engraftment was very low (0.13%). This small fraction adopted a cardiomyocytic phenotype (morphologically, positive for alpha-actinin and the mitochondrial human protein, hMITO as well as human nuclei). Some cells were incorporated into the vascular wall. The major observation of the study of Ding et al. is that the majority of USSC disappeared over time, independent of the applied model (also in nude rats lacking T cells). The reason for this observation was based on a high apoptosis activity analyzed by active-caspase 3 in the USSC. In addition, a substantial infiltration of CD11b positive cells into the myocardium 7 days after transplantation was noticed. In summary, the functional implications of the study clearly document that the number of surviving and differentiating USSC after transplantation was very small, so that reported beneficial effects observed in different models were unlikely to be a result of functional replacement of cardiomyocytes, but of paracrine factors stimulating the repair of resident cardiac progenitors in the heart. Moreover, the integration of USSC into the endothelium may support vasculogenic regeneration.

15. IN VITRO DIFFERENTIATION POTENTIAL TOWARD CARDIOMYOCYTES

In 2007, Nishiyama et al. described an MSC population in CB expressing GATA4 and Nkx2.5, cardiac actin as well as troponin T at default state, and termed these cells "cardiac progenitors." They had a very limited proliferation potential and were genetically manipulated for expansion.[82] In order to critically evaluate and confirm published data, eGFP-labeled USSC were cocultivated with neonatal rat cardiomyocytes. A significant number of USSC were stained positive for the cardiac markers alpha-actinin and cardiac troponin T, respectively, 3 and 7 days after cocultivation. A clear striation pattern of cardiac troponin T could be observed together with a spontaneous contraction of the cells. Gene expression profile of the isolated cells revealed the expression of GATA4 and the cardiac markers cTnT and cardiac actin, but only after cocultivation with neonatal cardiomyocytes.[81]

In summary, the application of USSC or MSC from CB in cardiac regeneration may involve both paracrine mechanisms, inflammatory mediators that support cardiomyogenesis as well as support of neovascularization. It will be of critical importance to understand the mechanisms at the cellular level ongoing in vivo. In vitro differentiation

experiments can never reflect the in vivo situation, however, they can help to compare the distinct progenitor cells to the adult mature cardiomyocyte and differentiated embryonic or reprogrammed stem cells.

16. CB SUBPOPULATIONS FOR NEURONAL REGENERATION

During the past 10 years CB has created great interest as a valuable source for neural stem cells or scientifically more precisely for the support of neural regeneration.

Although studies including our own have shown how subsets of CB cells differentiate under defined conditions into neurons, astrocytes, and microglia in vitro by more or less "artificial methods and substances,"[83] which do not reflect the in vivo situation, it is as of today common knowledge that CB stem cells secrete trophic factors that initiate and maintain the process of repair toward neurons in vivo.[84] In the meantime this resulted in the first clinical trials in treatment of neurological disorders such as cerebral palsy in children. Here, application of autologous or allogeneic matched CB resulted in amelioration of the motor skills and cognitive functions.[85]

In 2001, Chen et al.[86] were the first to demonstrate that the infusion or intracerebral transplantation of CB stem cells into rats that had been stroke-induced by occlusion of the middle cerebral artery displayed beneficial effects. The underlying mechanisms of these observations have not yet been elucidated, though the most straightforward idea is that stem cells differentiate into mature cell types and simply replace the lost tissue. However, there is increasing evidence that transplanted cells may secrete neurotrophic or neuroprotective factors[87] that can counteract degeneration or promote regeneration. It was demonstrated that USSC isolated from human CB are strongly attracted by HGF that is secreted by ischemia-damaged brain tissue and by apoptotic neurons in vitro and in vivo.[87] In opposition, necrotic neurons do not secrete hepatocyte growth factor and therefore have no potential to initiate migration of USSC. In all paradigms used in this study the secretion of HGF by neural target tissue and the expression of the HGF receptor c-MET in USSC directly correlated to migrational potency of USSC but also MSC, indicating that the HGF/c–MET axis is the driving force for migration toward neuronal injury.

Besides stroke and cerebral palsy, the treatment of spinal cord injury with stem cells is in the focus of many researchers. The major problem in spinal cord injury is the breakdown of the blood–spinal cord barrier associated with invasion of inflammatory cells, the activation of the glia, and subsequent axonal degeneration.[84] Stem cell populations derived from BM or peripheral blood have been transplanted in animal models and in pilot clinical studies as also summarized. Reports on the functional recovery were frequently only based on a single behavioral test. Although improvement of sensory and motor activity was reported in some studies,[88] no recovery was observed in others. Preclinical studies with MSC isolated from BM or CB in rodent spinal cord injury suggested variable mechanisms underlying the observed effect as differentiation into oligodendroglia. Here, Schira et al. transplanted USSC into a rodent model of acute spinal cord injury and investigated their survival, migration, and neural differentiation potential as well their influence on axonal regrowth, lesion size, and protection from spinal tissue loss. Moreover three different locomotor tasks (open field Basso–Beattie–Bresnahan locomotion score, horizontal ladder walking test, and CatWalk gait analysis) were applied. In the report, immunesuppressed adult rats received a highly reproducible dorsal hemisection injury at thoracic level Th8. Immediately after hemisection USSC were transplanted close to the site of the injury. Two days after transplantation grafted cells were located at the injection site, and one week after transplantation in the lesion center but without revealing immunoreactivity for the axon marker neurofilament. Three weeks after transplantation, USSC were mainly confined to the injury site, but in close proximity to the grafted USSC, neurofilament positive host cells were present in the lesion center. Although the USSC itself did not differentiate toward neurons or glia cells, they reduced the tissue loss significantly. This leads to improved locomotor function 16 weeks after transplantation. In three different test systems the grafting of USSC into the traumatic spinal cord injury significantly reduced the lesion size and enhanced the amount of spared tissue, indicating a strong neuroprotective function of USSC similar to BM MSC. USSC release a wide range of cytokines including stromal cell-derived factor 1 (SDF-1),[60] which induces homing of HSC and neural stem cells in ischemic and injured brain, and HGF, which is a known survival factor of neural development. The different growth factors or the combination of several as in other models of tissue regeneration are likely to participate in the positive regeneration effects observed.

Although most studies using CB stem cells for the treatment of neurological disorders in animal models, but also already in the clinic are very promising, several questions such as the route of administration, the amount of cells and the mode of action, the observed side effects, and mainly the *scientific* mechanisms have to be addressed before stem cells from CB can be brought into the clinical arena as a therapeutic strategy to treat neurological disorders.

17. REPROGRAMMED SUBPOPULATIONS FROM CB

Since their first description by the group of Yamanaka in 2007[89] iPS including CB-derived cells have received major attention by the scientific CB community and two distinct fields of interest were established: the application

in regenerative medicine to deliver specific cells or tissue on demand, and the use in diagnostic systems. While the original protocol described lentiviral insertion of genes for the transcription factors Oct4, Sox2, Klf4, and c-Myc, this method bears the potential risk of disrupting normal genes or of activating oncogenes in close proximity to the integration site. This can be avoided by using more sophisticated integration-free methods, such as appliance of episomal plasmids[90] Sendai-virus,[91] mRNA[92] or direct addition of the according proteins,[93] and small molecules. Although generation frequencies reported for different reprogramming systems vary, they are still generally low, ranging from 0.001% to 0.1%. Furthermore, especially the protooncogene c-Myc is seen critically, therefore multiple different combinations of factors, including L-Myc and Lin28, are currently proposed for generation, with only Oct4 and Sox2 being overexpressed in the most minimalist protocols.[94] In regard to cell origin, publications demonstrate that cells from CB in general, and in particular CD34-positive HSC, are a better choice than adult cells. Not only that generation frequencies for iPS are higher, these biologically younger cells also bear a lower risk of acquired genetic alterations. In addition, isolation and cryopreservation of these cells is well established, making CB cells an easily available source. Although, as a disadvantage, it has to be taken into consideration that innate genetic mutations of the donor might not be obvious directly at birth,[95] CB-derived cells are the most promising candidates for standardized generation of iPS. Nearly all cell subpopulations, including MSC, USSC, ECFC, and CD34-positive cells from CB were analyzed in reprogramming using lentivirus- and Sendai-virus-based protocols, respectively. Both approaches resulted in cells with embryonal stem cell-like morphology with no detectable difference in growth or expression of pluripotency markers. However, for standardization CB-derived cells, which have a constant frequency both in fresh and cryopreserved CB, are among the most interesting candidates. iPS are a promising tool for disease modeling, toxicological tests, and basic science, and further improvements of generation (integration-free), maintenance (serum-/feeder-free), and differentiation (new differentiation pathways, higher efficiency) are likely to further extend their applicability. Therefore, although iPS are already becoming a laboratory standard, it is very likely that it will still take some years until they are finally transferable into the clinic, since it is mandatory that the technology needs to be improved first before CB-IPS banking is routinely feasible.

18. CONCLUSION

CB contains valuable nonhematopoietic progenitor cells from different stages of fetal development circulating in the CB as "elusive" cells. In order to characterize them with regard to their true differentiation potential, for each cell population clonal cells and in vivo experimental design is required. The results summarized here clearly show that USSC are different from CB MSC. The difference exists both for *HOX*-genes, playing an important role in skeletal formation as well as for transcription factors detecting differences between these cell populations, but also in differences to BM MSC. Although USSC and MSC have no clear "bone signature" they are able to differentiate under appropriate conditions toward bone and cartilage. USSC share many markers with early liver development and are therefore able to reconstitute the liver upon injury. Although there were in vitro differentiation data available to show differentiation of CB-derived cells toward neurons (ectodermal tissue) and cardiomyocytes, all in vivo experiments clearly indicate that no transdifferentiation occurred. Though, as recently also described by Bianco et al. for cultivated mesenchymal cells from BM,[25,96] it has to be considered that the positive data published in different models are probably mainly related to trophic factors, immune modulation, or antiinflammatory effects. Based on the developmental advantage of CB subpopulations they might be ideal tools to analyze the fate of the distinct population in extensive preclinical models and define the mechanisms behind the improvements observed in beginning clinical trials.

LIST OF ACRONYMS AND ABBREVIATIONS

AcLDL Acetylated-low density lipoprotein
ARG1 Arginase1
BM MSC Bone marrow mesenchymal/multipotent stromal cells
BMP4 Bone morphogenetic protein 4
BSP Bone sialoprotein
c-MET MET or MNNG HOS transforming gene
CB Cord blood
CB MSC Cord blood mesenchymal/multipotent stromal cells
CB-SC Cord blood stromal cells
CD Cluster of differentiation
CFC Colony-forming cell
CPD Cumulative population doubling
CYP3A4 Cytochrome-p450-3A4
DCBT Double cord blood transplantation
DLK-1 Delta-like 1 homolog
DMEM Dulbecco's modified Eagle medium
ECFC Endothelial cord forming cell
EGF Epidermal growth factor
EPC Endothelial progenitor cell
FACS Fluorescence-activated cell sorting
FBP1 Fructose 1,6-biphosphatase
FCS Fetal calf serum
FGF Fibroblast growth factor
FLT3LG Fms-related tyrosine kinase 3 ligand
FOXA2 Forkhead box protein A2
G6PC Glucose 6-phosphatase
GATA4 GATA binding protein 4
GATA6 GATA binding protein 6

GFP Green fluorescent protein
GMP Good manufacturing practice
GvHD Graft-versus-host-disease
GYS2 Glycogen synthase 2
HepPar1 Hepatocyte paraffin 1
HEX Hematopoietically expressed homeobox gene
HGF Hepatocyte growth factor
HH Hedgehog protein
HNF4α Hepatocyte nuclear factor 4 alpha
HOX Homeobox
HPP-CFC High proliferative potential-colony-forming cell
HSC Hematopoietic stem cells
IGF Insulin-like growth factor
IGFBP Insulin-like growth factor-binding protein
IL-6 Interleukin 6
iPS Induced pluripotent cells
KDR Kinase insert domain receptor
LTC-IC Long-term culture-initiating cell
MNC Mononuclear cells
MSC Mesenchymal/multipotent stromal cells
NC Nucleated cells
Nkx2.5 NK2 homeobox 5
NOD/SCID Nonobese diabetic/severe combined immunodeficiency
OC Osteocalcin
OCT4A Octamer binding transcription factor 4
OSM Oncostatin M
OSX (SP7) Osterix
PCR Polymerase chain reaction
PROX Prospero-related homeobox 1
PTHLH Parathyroid hormone-related protein
RPL13A Ribosomal protein L13A.
RUNX2 Runt domain-containing transcription factor 2
SCF Stem cell factor
SCID Severe combined immunodeficiency
SDF-1 Stromal cell-derived factor 1
SOX9 Sex-determining region Y-related high mobility group box 9
SOX17 SRY (sex-determining region Y)-box 17
SRC SCID-repopulating cells
TCR T-cell receptor
THPO Thrombopoietin
UC MSC Umbilical cord mesenchymal stromal cells
USSC Unrestricted somatic stromal cells
VSEL Very small embryonic-like cells

REFERENCES

1. Cumano A, Ferraz JC, Klaine M, Di Santo JP, Godin I. Intraembryonic, but not yolk sac hematopoietic precursors, isolated before circulation, provide long-term multilineage reconstitution. *Immunity* 2001;**15**:477–85.
2. Ciriza J, Thompson H, Petrosian R, Manilay JO, Garcia-Ojeda ME. The migration of hematopoietic progenitors from the fetal liver to the fetal bone marrow: lessons learned and possible clinical applications. *Exp Hematol* 2013;**41**:411–23.
3. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997;**275**:964–7.
4. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* 2003;**348**:593–600.
5. Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, et al. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. *Blood* 2000;**95**:952–8.
6. Ingram DA, Mead LE, Tanaka H, Meade V, Fenoglio A, Mortell K, et al. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood* 2004;**104**:2752–60.
7. Shepherd BR, Enis DR, Wang F, Suarez Y, Pober JS, Schechner JS. Vascularization and engraftment of a human skin substitute using circulating progenitor cell-derived endothelial cells. *FASEB J* 2006;**20**:1739–41.
8. Tavassoli M, Crosby WH. Transplantation of marrow to extramedullary sites. *Science* 1968;**161**:54–6.
9. Friedenstein AJ. *Osteogenic stem cells in bone marrow*. New York: Elsevier; 1990.
10. Owen M, Friedenstein AJ. Stromal stem cells: marrow-derived osteogenic precursors. *Ciba Found Symp* 1988;**136**:42–60.
11. Friedenstein AJ, Chailakhyan RK, Gerasimov UV. Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers. *Cell Tissue Kinet* 1987;**20**:263–72.
12. Bianco P, Robey PG, Saggio I, Riminucci M. "Mesenchymal" stem cells in human bone marrow (skeletal stem cells): a critical discussion of their nature, identity, and significance in incurable skeletal disease. *Hum Gene Ther* 2010;**21**:1057–66.
13. Kuznetsov SA, Krebsbach PH, Satomura K, Kerr J, Riminucci M, Benayahu D, et al. Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo. *J Bone Min Res* 1997;**12**:1335–47.
14. Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I, et al. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* 2007;**131**:324–36.
15. Gronthos S, Franklin DM, Leddy HA, Robey PG, Storms RW, Gimble JM. Surface protein characterization of human adipose tissue-derived stromal cells. *J Cell Physiol* 2001;**189**:54–63.
16. Kogler G, Critser P, Trapp T, Yoder M. Future of cord blood for non-oncology uses. *Bone Marrow Transpl* 2009;**44**:683–97.
17. Weiss ML, Medicetty S, Bledsoe AR, Rachakatla RS, Choi M, Merchav S, et al. Human umbilical cord matrix stem cells: preliminary characterization and effect of transplantation in a rodent model of Parkinson's disease. *Stem Cells* 2006;**24**:781–92.
18. Reinisch A, Strunk D. Isolation and animal serum free expansion of human umbilical cord derived mesenchymal stromal cells (MSCs) and endothelial colony forming progenitor cells (ECFCs). *J Vis Exp* 2009;32:pii.
19. Kogler G, Sensken S, Airey JA, Trapp T, Muschen M, Feldhahn N, et al. A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. *J Exp Med* 2004;**200**:123–35.
20. Kim BO, Tian H, Prasongsukarn K, Wu J, Angoulvant D, Wnendt S, et al. Cell transplantation improves ventricular function after a myocardial infarction: a preclinical study of human unrestricted somatic stem cells in a porcine model. *Circulation* 2005;**112**:I96–104.
21. Kern S, Eichler H, Stoeve J, Kluter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* 2006;**24**:1294–301.
22. Chang YJ, Shih DT, Tseng CP, Hsieh TB, Lee DC, Hwang SM. Disparate mesenchyme-lineage tendencies in mesenchymal stem cells from human bone marrow and umbilical cord blood. *Stem Cells* 2006;**24**:679–85.

23. Kluth SM, Buchheiser A, Houben AP, Geyh S, Krenz T, Radke TF, et al. DLK-1 as a marker to distinguish unrestricted somatic stem cells and mesenchymal stromal cells in cord blood. *Stem Cells Dev* 2010;**19**:1471–83.
24. Kluth SM, Radke TF, Kogler G. Increased haematopoietic supportive function of USSC from umbilical cord blood compared to CB MSC and possible role of DLK-1. *Stem Cells Int* 2013;**2013**:985285.
25. Bianco P, Cao X, Frenette PS, Mao JJ, Robey PG, Simmons PJ, et al. The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. *Nat Med* 2013;**19**:35–42.
26. Liedtke S, Freytag EM, Bosch J, Houben AP, Radke TF, Deenen R, et al. Neonatal mesenchymal-like cells adapt to surrounding cells. *Stem Cell Res* 2013;**11**:634–46.
27. Liedtke S, Buchheiser A, Bosch J, Bosse F, Kruse F, Zhao X, et al. The HOX Code as a "biological fingerprint" to distinguish functionally distinct stem cell populations derived from cord blood. *Stem Cell Res* 2010;**5**:40–50.
28. Kluth SM, Radke TF, Kogler G. Potential application of cord blood-derived stromal cells in cellular therapy and regenerative medicine. *J Blood Transfus* 2012:2012.
29. Liedtke S, Enczmann J, Waclawczyk S, Wernet P, Kogler G. Oct4 and its pseudogenes confuse stem cell research. *Cell Stem Cell* 2007;**1**:364–6.
30. Liedtke S, Stephan M, Kogler G. Oct4 expression revisited: potential pitfalls for data misinterpretation in stem cell research. *Biol Chem* 2008;**389**:845–50.
31. Zaehres H, Kogler G, Arauzo-Bravo MJ, Bleidissel M, Santourlidis S, Weinhold S, et al. Induction of pluripotency in human cord blood unrestricted somatic stem cells. *Exp Hematol* 2010;**38**:809–18. 818 e1-2.
32. Kucia M, Reca R, Campbell FR, Zuba-Surma E, Majka M, Ratajczak J, et al. A population of very small embryonic-like (VSEL) CXCR4(+) SSEA-1(+)Oct-4+ stem cells identified in adult bone marrow. *Leukemia* 2006;**20**:857–69.
33. Miyanishi M, Mori Y, Seita J, Chen JY, Karten S, Chan CKF, et al. Do pluripotent stem cells exist in adult mice as very small embryonic stem cells? *Stem Cell Rep* 2013;**1**:198–208.
34. Abbott A. Doubt cast over tiny stem cells. *Nature* 2013;**499**:390.
35. Bosch J, Houben AP, Hennicke T, Deenen R, Köhrer K, Liedtke S, et al. Comparing the gene expression profile of stromal cells from human cord blood and bone marrow: lack of the typical "bone" signature in cord blood cells. *Stem Cells Int* 2013;**2013**:631984.
36. Bosch J, Houben AP, Radke TF, Stapelkamp D, Bunemann E, Balan P, et al. Distinct differentiation potential of "MSC" derived from cord blood and umbilical cord: are cord-derived cells true mesenchymal stromal cells? *Stem Cells Dev* 2012;**21**:1977–88.
37. Aktas M, Buchheiser A, Houben A, Reimann V, Radke T, Jeltsch K, et al. Good manufacturing practice-grade production of unrestricted somatic stem cell from fresh cord blood. *Cytotherapy* 2010;**12**:338–48.
38. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**:315–7.
39. Capelli C, Gotti E, Morigi M, Rota C, Weng L, Dazzi F, et al. Minimally manipulated whole human umbilical cord is a very rich source of clinical-grade human mesenchymal stromal cells expanded in human platelet lysate. *Cytotherapy* 2011;**13**:786–801.
40. Sudo K, Kanno M, Miharada K, Ogawa S, Hiroyama T, Saijo K, et al. Mesenchymal progenitors able to differentiate into osteogenic, chondrogenic, and/or adipogenic cells in vitro are present in most primary fibroblast-like cell populations. *Stem Cells* 2007;**25**:1610–7.
41. Kaltz N, Funari A, Hippauf S, Delorme B, Noel D, Riminucci M, et al. In vivo osteoprogenitor potency of human stromal cells from different tissues does not correlate with expression of POU5F1 or its pseudogenes. *Stem Cells* 2008;**26**:2419–24.
42. Olsen BR, Reginato AM, Wang W. Bone development. *Annu Rev Cell Dev Biol* 2000;**16**:191–220.
43. Creuzet S, Couly G, Vincent C, Le Douarin NM. Negative effect of Hox gene expression on the development of the neural crest-derived facial skeleton. *Development* 2002;**129**:4301–13.
44. Leucht P, Kim JB, Amasha R, James AW, Girod S, Helms JA. Embryonic origin and Hox status determine progenitor cell fate during adult bone regeneration. *Development* 2008;**135**:2845–54.
45. Dimitriou R, Jones E, McGonagle D, Giannoudis PV. Bone regeneration: current concepts and future directions. *BMC Med* 2011;**9**:66.
46. Krampera M, Marconi S, Pasini A, Galie M, Rigotti G, Mosna F, et al. Induction of neural-like differentiation in human mesenchymal stem cells derived from bone marrow, fat, spleen and thymus. *Bone* 2007;**40**:382–90.
47. Tuan RS, Boland G, Tuli R. Adult mesenchymal stem cells and cell-based tissue engineering. *Arthritis Res Ther* 2003;**5**:32–45.
48. Prockop DJ, Brenner M, Fibbe WE, Horwitz E, Le Blanc K, Phinney DG, et al. Defining the risks of mesenchymal stromal cell therapy. *Cytotherapy* 2010;**12**:576–8.
49. DeLise AM, Fischer L, Tuan RS. Cellular interactions and signaling in cartilage development. *Osteoarthritis Cartilage* 2000;**8**:309–34.
50. Long F. Building strong bones: molecular regulation of the osteoblast lineage. *Nat Rev Mol Cell Biol* 2011;**13**:27–38.
51. Goldring MB, Tsuchimochi K, Ijiri K. The control of chondrogenesis. *J Cell Biochem* 2006;**97**:33–44.
52. Adams SL, Cohen AJ, Lassova L. Integration of signaling pathways regulating chondrocyte differentiation during endochondral bone formation. *J Cell Physiol* 2007;**213**:635–41.
53. Ogbureke KU, Fisher LW. SIBLING expression patterns in duct epithelia reflect the degree of metabolic activity. *J Histochem Cytochem* 2007;**55**:403–9.
54. Buchheiser A, Houben AP, Bosch J, Marbach J, Liedtke S, Kogler G. Oxygen tension modifies the "stemness" of human cord blood-derived stem cells. *Cytotherapy* 2012;**14**:967–82.
55. Gluckman E, Broxmeyer HA, Auerbach AD, Friedman HS, Douglas GW, Devergie A, et al. Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. *N Engl J Med* 1989;**321**:1174–8.
56. Barker JN, Weisdorf DJ, DeFor TE, Blazar BR, McGlave PB, Miller JS, et al. Transplantation of 2 partially HLA-matched umbilical cord blood units to enhance engraftment in adults with hematologic malignancy. *Blood* 2005;**105**:1343–7.
57. Falkenberg H, Radke TF, Kogler G, Stuhler K. Proteomic profiling of ex vivo expanded CD34-positive haematopoetic cells derived from umbilical cord blood. *Stem Cells Int* 2013;**2013**:245695.
58. Dahlberg A, Delaney C, Bernstein ID. Ex vivo expansion of human hematopoietic stem and progenitor cells. *Blood* 2011;**117**:6083–90.
59. Cutler C, Multani P, Robbins D, Kim HT, Le T, Hoggatt J, et al. Prostaglandin-modulated umbilical cord blood hematopoietic stem cell transplantation. *Blood* 2013;**122**(17):3074–81.
60. Kogler G, Radke TF, Lefort A, Sensken S, Fischer J, Sorg RV, et al. Cytokine production and hematopoiesis supporting activity of cord blood-derived unrestricted somatic stem cells. *Exp Hematol* 2005;**33**:573–83.
61. Jeltsch KS, Radke TF, Laufs S, Giordano FA, Allgayer H, Wenz F, et al. Unrestricted somatic stem cells: interaction with CD34(+) cells in vitro and in vivo, expression of homing genes and exclusion of tumorigenic potential. *Cytotherapy* 2010;**13**(3):357–65.

62. Lee SH, Lee MW, Yoo KH, Kim DS, Son MH, Sung KW, et al. Co-transplantation of third-party umbilical cord blood-derived MSCs promotes engraftment in children undergoing unrelated umbilical cord blood transplantation. *Bone Marrow Transpl* 2013;**48**:1040–5.
63. Mohle R, Bautz F, Rafii S, Moore MA, Brugger W, Kanz L. The chemokine receptor CXCR-4 is expressed on CD34+ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1. *Blood* 1998;**91**:4523–30.
64. Nie Y, Han YC, Zou YR. CXCR4 is required for the quiescence of primitive hematopoietic cells. *J Exp Med* 2008;**205**:777–83.
65. Chou S, Lodish HF. Fetal liver hepatic progenitors are supportive stromal cells for hematopoietic stem cells. *Proc Natl Acad Sci USA* 2010;**107**:7799–804.
66. Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, et al. Bone marrow as a potential source of hepatic oval cells. *Science* 1999;**284**:1168–70.
67. Ishikawa F, Drake CJ, Yang S, Fleming P, Minamiguchi H, Visconti RP, et al. Transplanted human cord blood cells give rise to hepatocytes in engrafted mice. *Ann NY Acad Sci* 2003;**996**:174–85.
68. Wang X, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al-Dhalimy M, et al. Cell fusion is the principal source of bone-marrow-derived hepatocytes. *Nature* 2003;**422**:897–901.
69. Hengstler JG, Brulport M, Schormann W, Bauer A, Hermes M, Nussler AK, et al. Generation of human hepatocytes by stem cell technology: definition of the hepatocyte. *Expert Opin Drug Metab Toxicol* 2005;**1**:61–74.
70. Lee KD, Kuo TK, Whang-Peng J, Chung YF, Lin CT, Chou SH, et al. In vitro hepatic differentiation of human mesenchymal stem cells. *Hepatology* 2004;**40**:1275–84.
71. Sensken S, Waclawczyk S, Knaupp AS, Trapp T, Enczmann J, Wernet P, et al. In vitro differentiation of human cord blood-derived unrestricted somatic stem cells towards an endodermal pathway. *Cytotherapy* 2007;**9**:362–78.
72. Ghodsizad A, Fahy BN, Waclawczyk S, Liedtke S, Gonzalez Berjon JM, Barrios R, et al. Portal application of human unrestricted somatic stem cells to support hepatic regeneration after portal embolization and tumor surgery. *ASAIO J* 2012;**58**:255–61.
73. Kogler G, Sensken S, Wernet P. Comparative generation and characterization of pluripotent unrestricted somatic stem cells with mesenchymal stem cells from human cord blood. *Exp Hematol* 2006;**34**:1589–95.
74. Waclawczyk S, Buchheiser A, Flogel U, Radke TF, Kogler G. In vitro differentiation of unrestricted somatic stem cells into functional hepatic-like cells displaying a hepatocyte-like glucose metabolism. *J Cell Physiol* 2010;**225**:545–54.
75. Lee J, Terracciano CM. Cell therapy for cardiac repair. *Br Med Bull* 2010;**94**:65–80.
76. Wollert KC, Drexler H. Cell therapy for the treatment of coronary heart disease: a critical appraisal. *Nat Rev Cardiol* 2010;**7**:204–15.
77. Passier R, van Laake LW, Mummery CL. Stem-cell-based therapy and lessons from the heart. *Nature* 2008;**453**:322–9.
78. Ghodsizad A, Niehaus M, Kogler G, Martin U, Wernet P, Bara C, et al. Transplanted human cord blood-derived unrestricted somatic stem cells improve left-ventricular function and prevent left-ventricular dilation and scar formation after acute myocardial infarction. *Heart* 2009;**95**:27–35.
79. Moelker AD, Baks T, Wever KM, Spitskovsky D, Wielopolski PA, van Beusekom HM, et al. Intracoronary delivery of umbilical cord blood derived unrestricted somatic stem cells is not suitable to improve LV function after myocardial infarction in swine. *J Mol Cell Cardiol* 2007;**42**:735–45.
80. Rabald S, Marx G, Mix B, Stephani C, Kamprad M, Cross M, et al. Cord blood cell therapy alters LV remodeling and cytokine expression but does not improve heart function after myocardial infarction in rats. *Cell Physiol Biochem* 2008;**21**:395–408.
81. Ding Z, Burghoff S, Buchheiser A, Kogler G, Schrader J. Survival, integration, and differentiation of unrestricted somatic stem cells in the heart. *Cell Transpl* 2013;**22**:15–27.
82. Nishiyama N, Miyoshi S, Hida N, Uyama T, Okamoto K, Ikegami Y, et al. The significant cardiomyogenic potential of human umbilical cord blood-derived mesenchymal stem cells in vitro. *Stem Cells* 2007;**25**:2017–24.
83. Goodwin HS, Bicknese AR, Chien SN, Bogucki BD, Quinn CO, Wall DA. Multilineage differentiation activity by cells isolated from umbilical cord blood: expression of bone, fat, and neural markers. *Biol Blood Marrow Transpl* 2001;**7**:581–8.
84. Schira J, Gasis M, Estrada V, Hendricks M, Schmitz C, Trapp T, et al. Significant clinical, neuropathological and behavioural recovery from acute spinal cord trauma by transplantation of a well-defined somatic stem cell from human umbilical cord blood. *Brain* 2012;**135**:431–46.
85. Min K, Song J, Kang JY, Ko J, Ryu JS, Kang MS, et al. Umbilical cord blood therapy potentiated with erythropoietin for children with cerebral palsy: a double-blind, randomized, placebo-controlled trial. *Stem Cells* 2013;**31**:581–91.
86. Chen J, Sanberg PR, Li Y, Wang L, Lu M, Willing AE, et al. Intravenous administration of human umbilical cord blood reduces behavioral deficits after stroke in rats. *Stroke* 2001;**32**:2682–8.
87. Trapp T, Kogler G, El-Khattouti A, Sorg RV, Besselmann M, Focking M, et al. Hepatocyte growth factor/c-MET axis-mediated tropism of cord blood-derived unrestricted somatic stem cells for neuronal injury. *J Biol Chem* 2008;**283**:32244–53.
88. Kang KS, Kim SW, Oh YH, Yu JW, Kim KY, Park HK, et al. 37-year-old spinal cord-injured female patient, transplanted of multipotent stem cells from human UC blood, with improved sensory perception and mobility, both functionally and morphologically: a case study. *Cytotherapy* 2005;**7**:368–73.
89. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007;**131**:861–72.
90. Yu J, Hu K, Smuga-Otto K, Tian S, Stewart R, Slukvin II, et al. Human induced pluripotent stem cells free of vector and transgene sequences. *Science* 2009;**324**:797–801.
91. Fusaki N, Ban H, Nishiyama A, Saeki K, Hasegawa M. Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *Proc Jpn Acad Ser B Phys Biol Sci* 2009;**85**:348–62.
92. Warren L, Manos PD, Ahfeldt T, Loh YH, Li H, Lau F, et al. Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell* 2010;**7**:618–30.
93. Zhou H, Wu S, Joo JY, Zhu S, Han DW, Lin T, et al. Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell* 2009;**4**:381–4.
94. Kim JB, Zaehres H, Wu G, Gentile L, Ko K, Sebastiano V, et al. Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. *Nature* 2008;**454**:646–50.
95. Takahashi K, Yamanaka S. Induced pluripotent stem cells in medicine and biology. *Development* 2013;**140**:2457–61.
96. Bianco P. Reply to MSCs: science and trials. *Nat Med* 2013;**19**:813–4.