

## The potential of umbilical cord blood multipotent stem cells for nonhematopoietic tissue and cell regeneration

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Stem cells have been isolated from human embryos, fetal tissue, umbilical cord blood (UCB), and also from “adult” sources. Adult stem cells are found in many tissues of the body and are capable of maintaining, generating, and replacing terminally differentiated cells. A source of pluripotent stem cells has been recently identified in UCB that can also differentiate across tissue lineage boundaries into neural, cardiac, epithelial, hepatocytic, and dermal tissue. Thus, UCB may provide a future source of stem cells for tissue repair and regeneration. Its widespread availability makes UCB an attractive source for tissue regeneration. UCB-derived stem cells offer multiple advantages over adult stem cells, including their immaturity, which may play a significant role in reduced rejection after transplantation into a mismatched host and their ability to produce larger quantities of homogenous tissue or cells. While research with embryonic stem cells continues to generate considerable controversy, human umbilical stem cells provide an alternative cell source that has been more ethically acceptable and appears to have widespread public support. This review will summarize the *in vitro* and *in vivo* studies examining UCB stem cells and their potential use for therapeutic application for non-hematopoietic tissue and cell regeneration. © 2007 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.

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A decade has past since we last reviewed the state of the science of umbilical cord blood (UCB) banking and the potential of cryopreserved unrelated UCB as an alternative source of allogeneic stem cells following myeloablative therapy for malignant and nonmalignant hematopoietic and immunological diseases [1]. Our original investigations and those of Kurtzberg et al. demonstrated the successful trilineage hematopoietic reconstitution following myeloablative unrelated cord blood transplantation [2,3]. The National Heart Lung and Blood Institute subsequently provided resources to establish three public cord blood banks to increase the supply of cryopreserved cord blood, develop standard operating procedures for maternal donor screening and consenting, cord blood collection, separation, cryopreservation, and characterization of early and mature cord blood progenitor cells [3,4]. More recently Escolar et al. demonstrated the success of myeloablative unrelated

cord blood transplantation in nonhematopoietic diseases, such as infantile Krabbe's disease [5]. However, there is considerable debate over the value of directed donor cord blood collection and cryopreservation for potential use for future nonhematopoietic tissue and cell regeneration [6].

### Pluripotent stem cells

Cells that are undifferentiated and capable of proliferation, self-renewal, differentiation, and regeneration of tissue are considered pluripotent stem cells. Human pluripotent stem cells are described as having clonogenic and self-renewing capabilities that can differentiate into multiple cell lineages and tissues of the three primary germ layers [7]. Embryonic stem (ES) cells contained in the blastocyst were once considered the sole reserve of pluripotent stem cells, while adult stem cells were considered lineage/tissue-restricted. However, current studies have suggested that adult stem cells may have the potential to contribute to the homeostasis and repair of a host of tissues, including muscle, brain, liver, heart, and the vascular endothelium. Quesenberry et al. [8] recently proposed a unified stem cell theory suggesting that cell-cycle transit is associated

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with continually changing stem cell phenotypes. Thus, at a more primitive stem/progenitor level, a fluctuating continuum rather than a hierarchy may exist where outcomes are determined by the changing surface phenotype and changing environmental stimuli of the stem cell.

### Sources of stem cells

Stem cells have been isolated from human embryos, fetal tissue, amniotic fluid, UCB, and from “adult” sources (Fig. 1) [9,10]. Adult stem cells are found in many tissues of the body, including eye, brain, skeletal muscle, adipose tissue, dental pulp, liver, pancreas, skin, bone marrow, and gastrointestinal tract (Table 1), and are capable of maintaining, generating, and replacing terminally differentiated cells [11].

A source of pluripotent stem cells has been recently identified in UCB. UCB has been reported to be a source of hematopoietic stem cells (HSC), endothelial cell precursors, mesenchymal (MSC) progenitors, and multipotent/pluripotent lineage stem cells (MLSC) [12–14]. Interestingly, MSC are reported to occur in 30% to 60% of low-volume UCB units with a frequency of up to 2.3 MSC clones/ $10^8$  mononuclear cells [15]. Further, investigators have demonstrated that UCB stem cells can differentiate across tissue-lineage boundaries into neural, cardiac, epithelial, hepatocytic, and dermal tissue.

### UCB as a source for multipotent stem cells for tissue repair or regeneration

UCB contains MLSC and endothelial cell precursors in addition to MSCs [16–18]. The multilineage differentiation capacity of UCB suggests that they may have the ability to transdifferentiate to become nonhematopoietic cells of various tissue lineages. Naïve freshly isolated UCB mononuclear cells, as well as the population obtained after negative sorting of HSCs, express substantial levels of genes that denote the molecular signature of stem cell pluripotency including Oct-3/4, Sox2, and Rex-1. Recent studies have demonstrated that these precursors are capable of improving revascularization of organs and tissues affected by ischemic disease and generating bone, cartilage, muscle, liver, and neural cells [19–22].

With the annual global birth rate of over 100 million per year, UCB is a large underutilized stem cell source with

many innate advantages. One paramount advantage of UCB stem cells is that they possess a primitive ontogeny and have not been exposed to immunologic challenge [23]. In contrast to adult bone marrow (BM) –derived HSCs, UCB progenitors have higher proliferative potential, reportedly up to eightfold greater than similar cells in BM, and four times as many CD34<sup>+</sup> cells [24–26]. Further, UCB contains a higher frequency of transplantable HSCs, a higher output of progenitor cells, formation of a greater number of colonies, a higher cell-cycle rate and a relatively long telomere length. Our laboratory found significant differences in gene expression of several functional groups of activated UCB monocyte genes, including those encoding for cytokines, immunoregulatory and signal transduction, and cell structure [27,28]. Because of the immunological immaturity of UCB, unrelated UCB transplantation tolerates greater human leukocyte antigen (HLA) disparity between the UCB donor and the recipient and may result in reduced severe acute graft-vs-host disease (GVHD) [1,29,30]. Rocha et al. [31] compared the records of recipients of UCB from HLA-matched siblings and recipients of HLA-matched bone marrow and found a lower risk of acute GVHD and chronic GVHD among the cord blood transplant patients [31].

UCB cells can be safely cryopreserved and stored for years without loss of viability. UCB offers substantial advantages over BM, including faster availability of banked cryopreserved UCB units, greater tolerance across 1 or 2 HLA-A, -B, and -DR mismatching, and lower risk of transmitting infections by latent viruses. Additionally, public banking of UCB has been established throughout the world, providing easy access to UCB units from worldwide registries. Further, the Cord Blood Transplantation study reported substantial numbers of CD34<sup>+</sup>/CD90<sup>+</sup> pluripotent progenitor cells in the cord blood units collected [3]. UCB-derived stem cells offer multiple advantages over adult stem cells and ES cells, including their immaturity, which may play a significant role in the rejection of generated tissue when transplanted into a mismatched host and their ability to produce larger quantities of homogenous tissue or cells for transplantation. While research with ES cells continues to generate considerable controversy, human umbilical stem cells provide an alternative cell source that has been more ethically acceptable and appears to have widespread public support. This review will summarize in

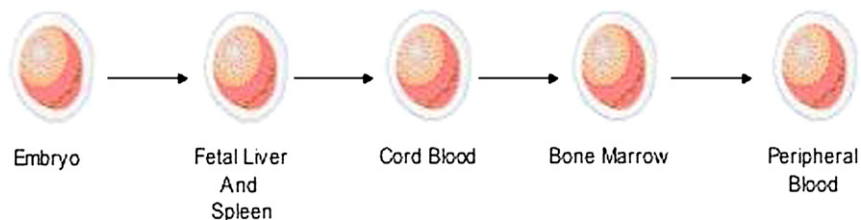


Figure 1. Ontogeny of developmental hematopoiesis of human pluripotent stem cells.

**Table 1.** Adult human stem cells and their primary direction of differentiation

| Cell type                                     | Tissue-specific location   | Cells or tissue produced   |
|---|--|--|
| Hematopoietic stem cells                      | Bone marrow, peripheral blood  | Bone marrow and blood lymphohematopoietic cells  |
| Mesenchymal stem cells                        | Bone marrow, peripheral blood  | Bone, cartilage, tendon, adipose tissue, muscle, marrow stroma, neural cells                               |
| Neural stem cells                             | Ependymal cells, astrocytes (subventricular zone) of the central nervous system                | Neurons, astrocytes, oligodendrocytes  |
| Hepatic stem cells                            | In or near the terminal bile ductules (canals of Hering)                                       | Oval cells that subsequently generate hepatocytes and ductular cells                                       |
| Pancreatic stem cells                         | Intra-islet, nestin-positive cells, oval cells, duct cells                                     | $\beta$ cells  |
| Skeletal-muscle stem cells or satellite cells | Muscle fibers  | Skeletal muscle fibers   |
| Stem cells of the skin (keratinocytes)        | Basal layer of the epidermis, bulge zone of the hair follicles                                 | Epidermis, hair follicles  |
| Epithelial stem cells of the lung             | Tracheal basal and mucus-secreting cells, bronchiolar Clara cells, alveolar type II pneumocyte | Mucous and ciliated cells, type I and II pneumocytes   |
| Stem cells of the intestinal epithelium       | Epithelial cells located around the base of each crypt   | Paneth's cells, brush-border enterocytes, mucus-secreting goblet cells, enteroendocrine cells of the villi |

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vitro and in vivo studies examining UCB stem cells and their potential use for therapeutic application for nonhematopoietic tissues and cell regeneration.

## Cell and tissue regeneration

### *Hematopoietic and immunologic reconstitution*

UCB transplantation has made allogeneic hematopoietic stem cell transplantation (HSCT) available to patients who would otherwise have no suitable donor. The clinical use of UCB HSCT has been well-documented by our group and others, with more than 9000 unrelated cord blood transplantations performed in children and adults [2,29,32–39]. The most important factor influencing the rate of engraftment of UCB is the total nucleated dose related to the weight of the patient. We and others have reported that patients transplanted with UCB units containing  $1.7$  to  $2.3 \times 10^5$  CD34<sup>+</sup> cells/kg have poorer survival [37]. Further, we and others have demonstrated, following both myeloablative and reduced-intensity conditioning and UCB transplantation, long-term full-donor chimerism, decreased severe acute GVHD, and comparable overall survival to other stem cell sources [2,34,37,39,40].

To address the need to increase the number of HSC for UCB transplantation in larger patients, several institutions have evaluated the infusion of multiple UCB units. Barker et al. [41] reported a higher engraftment rate associated with a low transplant-related mortality and no increase in severe GVHD in 23 high-risk patients transplanted with two partially HLA-matched UCB units. Other approaches to augment HSC for UCB transplantation include ex vivo

expansion of UCB hematopoietic or accessory cells that may enhance engraftment and graft-vs-tumor activity [42,43]. Our laboratory recently reported ex vivo expansion of lymphocyte subsets, enrichment of natural killer (NK) cell subsets and enhancement of NK and lymphokine-activated killer cell cytotoxicity from cryopreserved UCB [44]. Other investigators have demonstrated improved engraftment with the cotransplantation of MSCs with UCB [45–47].

While UCB transplantation has been clinically used most often for malignant disease, UCB has been successfully used to treat nonmalignant diseases, including lysosomal disorders (e.g., Krabbe's disease, Hurler's syndrome), hemoglobinopathies, aplastic anemia, and Fanconi's anemia [29,40,48–50].

### *Multilineage progenitor cells*

A multipotent cell (multilineage progenitor cells [MLPC]) potentially representing a new subset of stem cell was recently identified in UCB as a CD45<sup>+</sup>/CD34<sup>+</sup>/CD9<sup>+</sup>/nestin<sup>+</sup> plastic adherent population [51]. These cells have demonstrated extensive expansion capacity, while maintaining normal genetic stability [52], as well as the ability to be differentiated into cells representing all three germinal layers. These cells are thought to bridge the span between pluripotent ES cells and adult-source stem cells by demonstrating extensive plasticity without teratoma potential.

MLPC can be isolated from full-term UCB by a nonparticle-based negative cell selection methodology commercially marketed as PrepaCyte followed by plastic adherence. MLPC can be separated from MSC (also isolated by this methodology) by single-cell cloning [13,53,54]. Forty-five clonal cell lines of MLPC have been established from five

different cord blood samples by this methodology, as well as eight CB-derived MSC cell lines. MLPC distinguish themselves from MSC by having a more extensive expansion and differentiating capacity. MLPC have a doubling time ranging from 35 to 50 hours, depending upon culture conditions and are obligate adherent cells for both expansion and differentiation [55]. Molecular karyotyping by genomic DNA hybridization demonstrated karyotypically normal diploid cells that were able to maintain genomic integrity after 80 doublings [52]. These results contrasted with MSC that exhibited genetic instability after 20 doublings [52]. Standard in vitro culture in nondifferentiating media resulted in no instances of spontaneous differentiation and initial in vivo experiments have not resulted in teratoma formation [51].

MLPC exhibit two distinct morphologies and cell surface phenotypes, a leukocyte-like morphology associated with freshly isolated cells (CD45<sup>+</sup>, CD34<sup>+</sup>, CD133<sup>+</sup>, CD13<sup>+</sup>, CD29<sup>+</sup>, CD44<sup>+</sup>, CD73<sup>+</sup>, CD90<sup>+</sup>, CD105<sup>+</sup>, CD9<sup>+</sup>, SSEA-3<sup>+</sup>, SSEA-4<sup>+</sup>) and a fibroblastic morphology associated with cells after establishment of culture (CD45<sup>-</sup>, CD34<sup>-</sup>, CD133<sup>-</sup>, CD13<sup>+</sup>, CD29<sup>+</sup>, CD44<sup>+</sup>, CD73<sup>+</sup>, CD90 bimodal<sup>+</sup>, CD105<sup>+</sup>, CD9<sup>+</sup>, SSEA-3<sup>-</sup>, SSEA-4<sup>-</sup>, TNF-R<sup>+</sup>, nestin<sup>+</sup>,  $\beta$ -tubulin<sup>+</sup> (Fig. 2) [55]. The leukocyte morphology predominates during the first 7 days of culture. Shortly after the initial establishment of culture, MLPC will attach tightly to the plastic or glass substrate and will attain a more fibroblastic appearance. Figures 2E and 3H demonstrate the loss of CD45 coincidental with transition to the fibroblast morphology [55].

Clonally derived cell lines of MLPC have been compared to MSC obtained from BM and UCB and a number of similarities and differences were observed. Both cell types were observed to be positive for CD13, CD29, CD44, CD73, and CD105. Differences were observed in the expression of CD9 (bright for MLPC, dull for UCB-MSC, negative for BM-MSC), CD90 (bimodal for MLPC, a bright population for CD90 combined with a dull population for CD90; unimodal for MSC, one bright population for UCB- and BM-MSC), and CD106 (positive for MLPC and negative for UCB- and BM-MSC) (Fig. 3). Additionally, MLPC have been compared to MSC by microarray competitive hybridization mRNA analysis (Fig. 4). In a limited microarray analysis confined to 942 genes associated with stemness and commitment to lineage it was observed that 360 of 942 genes were significantly differently expressed. The analysis suggested that MLPC were a more quiescent and primitive cell, less committed to lineage, specifically connective tissue, when compared to MSC and that MLPC expressed more genes characteristic of HSCs than MSC [52].

MLPC have demonstrated a wide degree of plasticity in in vitro differentiation experiments (Fig. 5). Carefully controlled substrate and growth factor requirements enabled the differentiation of MLPC into cells representative of

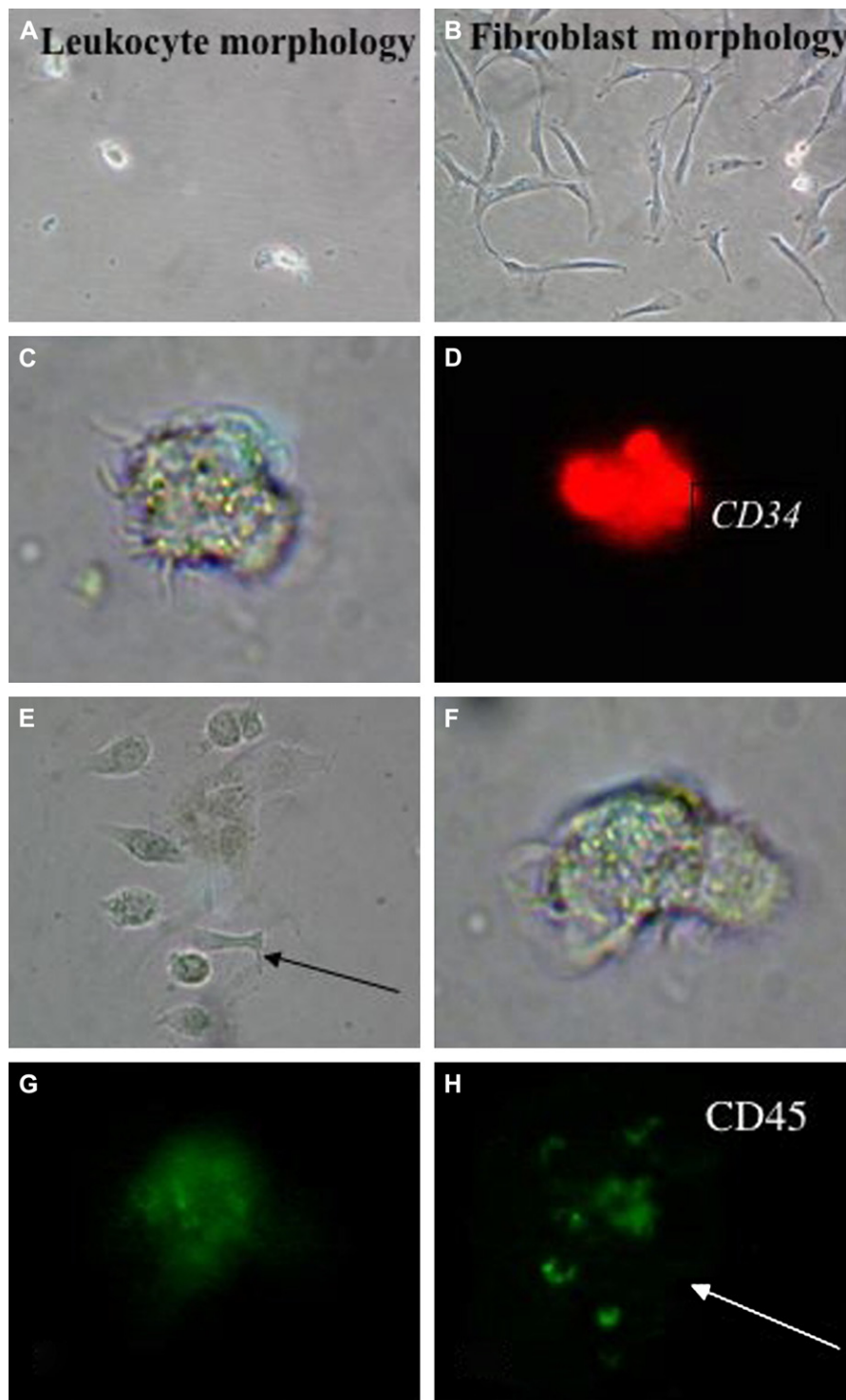
the three different embryonic germinal layers: endodermal (hepatopancreatic precursor cells, mature hepatocytes, type II alveolar pneumocytes), mesodermal (adipocytes, chondrocytes, osteoblasts, myocytes, endothelial cells), and ectodermal (neurons, astrocytes, oligodendrocytes) [55]. Interactions with three-dimensional biomatrices enhanced the differentiation of MLPC and could provide a working platform for tissue engineering. Distinct from ES cells, the MLPC have not demonstrated spontaneous differentiation in vitro or differentiation to mixed tissue types. The ability to carefully control the differentiation of these cells may provide an added safety feature not easily achieved with ES cells, while their extensive expansion; ability to develop pure clonal cell lines by limited dilution cloning; and expansive differentiating capacity could provide greater utility than currently described adult-sourced stem cells.

#### *Neuronal lineage*

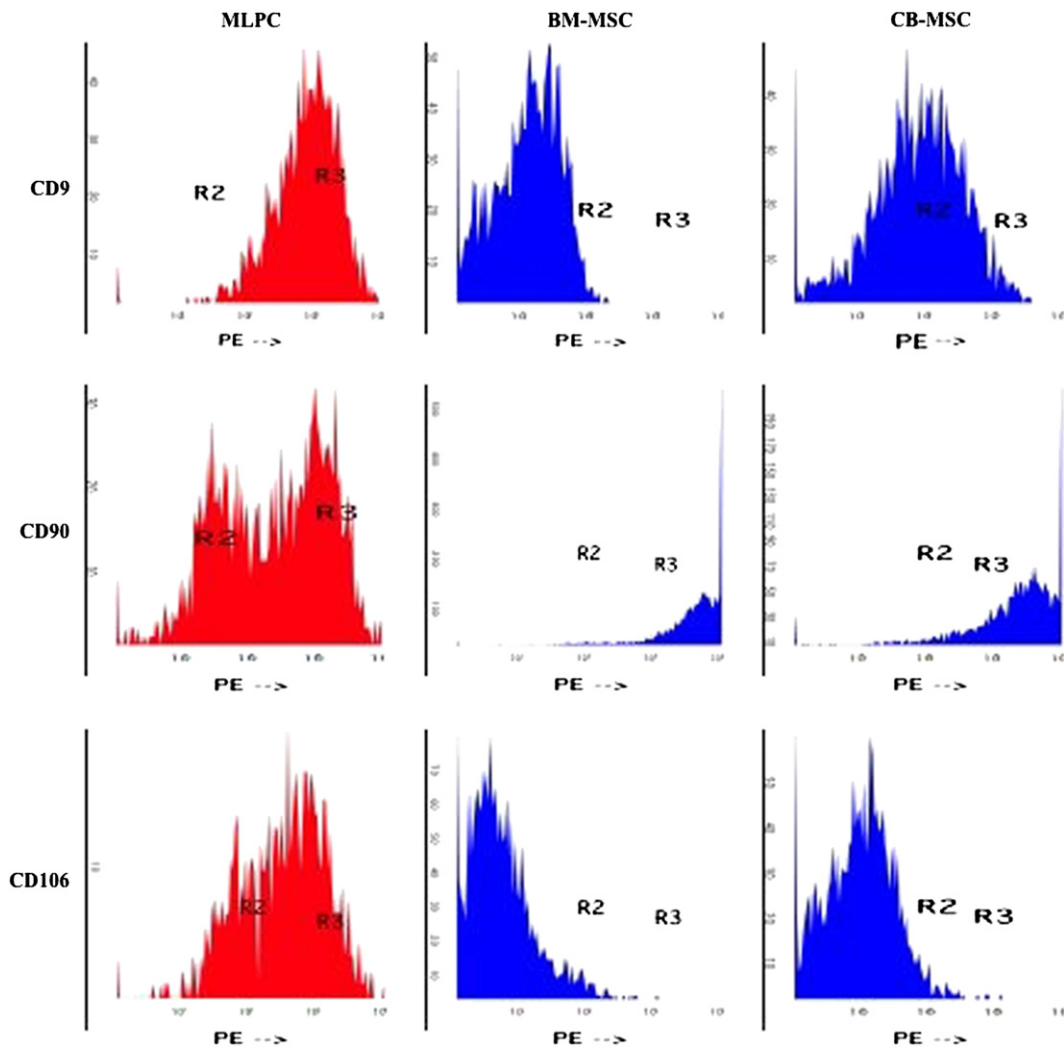
UCB transplantation has been shown to be a promising new therapy for neurodegenerative diseases, stroke, traumatic brain injury, spinal cord injury, and potential for neuronal replacement or gene delivery in neurodegenerative diseases, trauma, and genetic disorders. Numerous in vitro studies have demonstrated the generation of neuronal cells from UCB progenitors (Table 2).

Several investigators have reported that MLSC from UCB can be induced to transdifferentiate and give rise to glia and neuron-like cells during in vitro cell culture. UCB cells treated with retinoic acid and nerve growth factor exhibit a change in phenotype and express Musashi-1 and  $\beta$ -tubulin III, proteins found in early neuronal development [56]. Chen et al. [57] recently characterized a subpopulation of adherent cells in UCB cultures expressing vimentin, nestin, and A2B5, antigens typically found in brain tissue, and neurotrophic receptors, trk-A, trk-B, and trk-C receptors suggesting that the cells could be induced into becoming neural cells. McGuckin et al. [58] expanded primitive stem cells negative for hematopoietic lineage markers with thrombopoietin, flt-3, and c-kit ligand. A slow-dividing adherent cell population resulted with neuroglial progenitor morphology, upregulation of primitive neuroglial cell markers, and expression of glial fibrillary acidic protein, suggesting transdifferentiation into neuroglial progenitors [58]. A similar lineage switch was described when UCB were cultured on collagen-coated plates in conditioned media with added nerve growth factor. These 14-day cultures yielded the survival of progenitor cells with a neuronal morphology, expression of  $\alpha$ 1/2 integrin receptors, mRNA and protein markers specific to neuronal survival and increased gene expression related to neural differentiation [59].

Buzanska et al. suggested that UCB-derived progenitors could be effectively differentiated into functional neuron-like cells. Using the CD34<sup>-</sup> sub-fraction of UCB units, cells



**Figure 2.** Primary Isolates of multilineage progenitor cells (MLPC). (A) Initial isolates of MLPC. Freshly isolated MLPC and cells cultured less than 3 days exhibit a leukocyte-like morphology and a panel of key characteristic protein markers associated with these cells. (B) After morphologic conversion to fibroblastic phase (usually day 3 to day 7) MLPC attain their final culture-specific morphology and key characteristic protein phenotype. (C) and (F) Phase contrast of MLPC at culture day 3. (D) and (G), same cells seen in (C) and (F) CD34 (D) and CD45 (G) immunofluorescence. (E) and (H) Phase contrast (E) and CD45 immunofluorescence (H) of day 5 MLPC. The arrow in each photomicrograph points to a cell that has converted from the leukocyte phase to the fibroblast phase of morphology and has lost its expression of CD45 antigen. Cells that retain the leukocyte morphology and are positive for CD45 surround the fibroblastic stage cell. \*Arrows point to MLPC, which has attained fibroblastic morphology has lost expression of CD45, while the surrounding MLPCs in leukocyte morphology retain expression of CD45.

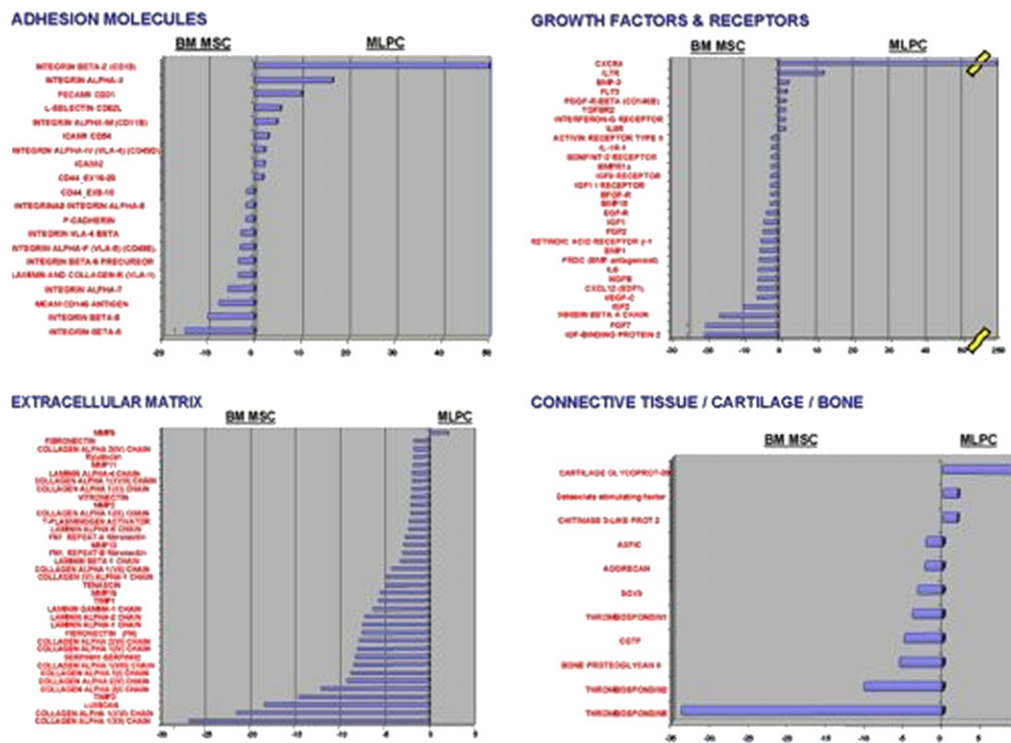


**Figure 3.** Comparison of multilineage progenitor cells (MLPC) to cord blood-derived mesenchymal stem cells (MSC) and bone marrow-derived MSC. MLPC and MSC cell lines were grown to 60% confluency in MSCGM medium (Cambrex). MLPC clone E8 was compared to BM-MSC (Cambrex) and cord blood-derived Clone A3 (BioE). While all cell types are typically positive for CD105, CD29, CD73, and CD44 the individual cell types could be clearly distinguished by examining CD9, CD90, and CD106. MLPC clones were positive for CD9 and CD106 while expressing a bright and dull population for CD90. The MSC lines were clearly negative for CD106, expressed a single bright population for CD90 and were either negative (BM-MSC) or dull (CB-MSC).

could be induced into functional neuron-like cells with neuronal, astrocytic and oligodendroglial features. They established a clonogenic non-immortalized UCB neural stem cell line that could be maintained in culture at different stages of neural progenitor development with trophic factors, mitogens and neuromorphogens [60]. Further, Habich and colleagues provided evidence that the UCB CD34<sup>-</sup> mononuclear fraction may contain a subpopulation of undifferentiated ES-like cells. They reported the spontaneous differentiation toward neural lineage after culturing Oct-3/4<sup>+</sup>, Sox-2<sup>+</sup>, Rex-1<sup>+</sup> UCB MLSCs at high cell density in DMEM/12 media. The cells acquired neural-like phenotypes and expressed genes and proteins known to be characteristic for neural stem/progenitors including nestin, glial fibrillary acidic protein, NF200,  $\beta$ -tubulin III, double Cortin, MAP2, s100 $\beta$ , O4, and GalC [61].

In vivo animal studies have been reported using UCB therapy to promote the resuscitation of rats with induced heatstroke by reducing circulatory shock, cerebral nitric oxide overload and ischemic injury [62]. Chen and colleagues compared the administration of human UCB vs peripheral blood stem cells before or after induced heatstroke. Pre-treatment with intravenous or intra-cerebroventricular doses of UCB significantly extended the resuscitation, latency and survival time for rats during heatstroke. Animals receiving UCB had significantly reduced arterial hypotension, cerebral hypoperfusion, cerebral ischemia and hypoxia [63].

Other studies suggest that UCB CD34<sup>+</sup> cells may promote either directly or indirectly an environment conducive to neovascularization of ischemic brain so that neuronal regeneration can proceed [64]. UCB HSC can also promote

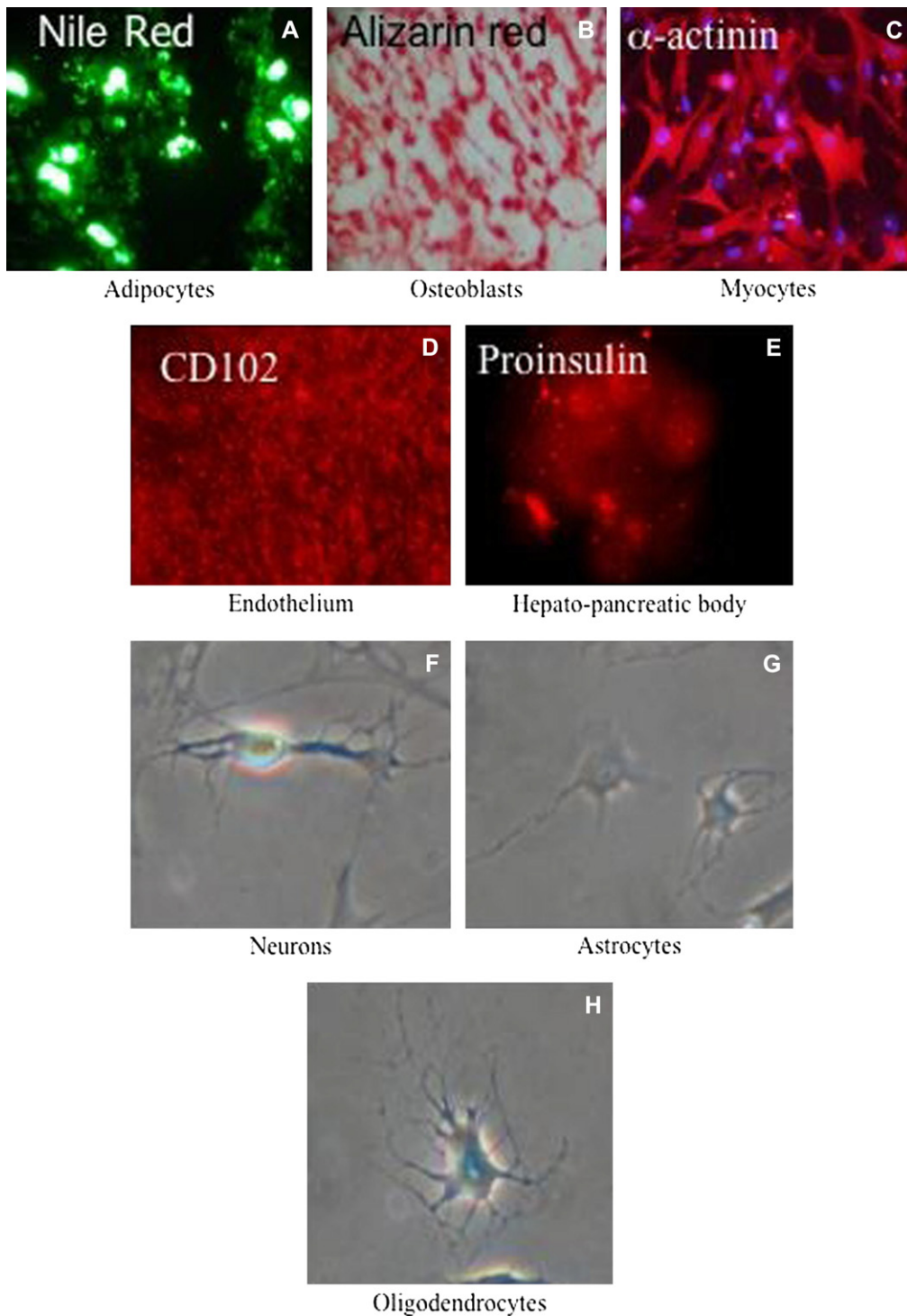


**Figure 4.** Comparative Expression of multilineage progenitor cells (MLPC) vs mesenchymal stem cells (MSC) mRNA by microarray. mRNA was obtained from logarithmically growing cultures of MLPC and bone marrow-derived MSC and subjected to competitive hybridization microarray utilizing a 942-gene array designed by Miltenyi to focus on stem cell-associated and lineage-commitment genes. Bars to the left of the zero axis are overexpressed by MSC, bars to the right of the zero axis are genes that are overexpressed by MLPC. Of the 942 genes analyzed by this array, 360 genes were expressed significantly different. MLPC were found to be relatively quiescent primitive cells that were capable of wide plasticity yet were not committed to lineage. MSC were found to be much more committed to stromal and connective tissue lineages overexpressing genes associated with extracellular matrix, growth factors, and proteins associated with bone and cartilage.

the neovascularization of ischemic brain. UCB derived CD34<sup>+</sup> cells were administered to immunocompromised mice 48 hours after induction of stroke. Results demonstrated an acceleration of neovascularization in the ischemic zone, induction of cortical expansion and endogenous neurogenesis as a result of enhanced migration of neuronal progenitor cells to the damaged area followed by their maturation and functional recovery.

Several *in vivo* studies have investigated transplantation of UCB for neurologic deficits associated with injury or disease. Weiss et al. used UCB matrix stem cells (UCBMSC) and assessed the behavioral effects in a rodent model of Parkinson's disease. UCBMSC are negative for CD34 and have genetic and surface markers of MSCs (positive for CD10, CD13, CD 29, CD44, CD90 and negative for hematopoietic progenitor markers). Undifferentiated UCBMSC were transplanted into the brains of hemi-parkinsonian rats and UCBMSC treated animals showed improvement in the apomorphine-induced rotations in the animals without inducing the formation of brain tumors or host immune rejection [65]. UCB therapy for neurologic deficits caused by intracerebral hemorrhage was also studied in a rodent model. Twenty-four hours after induction of intracerebral hemorrhage, rats received infusions of UCB mononuclear

cells and were evaluated at 1 and 2 weeks post transfusion. Animals receiving UCB exhibited significant improvement in neurologic testing compared to controls. These results suggest that UCB may ameliorate neurologic deficits associated with intracerebral hemorrhage [66]. Infusion of UCB after the induction of spinal cord injury was also evaluated in two separate studies. Saporta et al. injected UCB cells into rats 1 and 5 days after compression spinal cord injury. UCB cells reportedly localized around the site of injury, but not in the healthy spinal cord tissues and behavioral test scores improved in the group receiving UCB [67]. Another study administered UCB CD34<sup>+</sup> cells to rats one week after spinal cord injury. UCB treated animals had improved functional recovery, reduction of the area of cystic cavity at the site of injury, increased residual white matter and regeneration of axons in the injured spinal cord [68]. Finally, Garbuzova-Davis et al. studied the long-term effects of UCB on disease progression in a mouse model of amyotrophic lateral sclerosis and examined the distribution of transplanted UCB in the central nervous system. They reported UCB delayed disease progression, improved survival of diseased mice and transplanted UCB were observed to survive 10–12 weeks after infusion entering regions of motor neuron degeneration in the brain and spinal cord. Engrafted cells



**Figure 5.** Multipotential differentiating capacity of multilineage progenitor cells (MLPC). Mesodermal differentiation: (A–D), Endodermal differentiation: (E) Ectodermal differentiation: (F–H). (A) Adipocytic differentiation. MLPC were grown for 10 days in the presence of adipogenic induction medium (Cambrex). Triglyceride-containing liposomes were visualized with Nile Red stain. (B) Osteocytic differentiation. MLPC were grown in the presence of Osteogenic Induction Medium (Cambrex) for 21 days. Osteoblasts and mineralization were visualized by staining with Alizarin Red. (C) Myogenic differentiation. MLPC were grown on fibronectin-coated chamber slides in the presence of Skeletal Muscle Growth Medium (Cambrex) supplemented with fibroblast growth factor (FGF) basic (100 ng/mL), 5'-azacytidine (10  $\mu$ M, Sigma), and cardiogenol C (10  $\mu$ M, Sigma) for 30 days. Cells were stained for myocyte-specific  $\alpha$ -actinin. (D) Endothelial differentiation. MLPC are grown in the presence of Endothelial Growth Medium-Microvasculature (Cambrex) for 21 days. Cells were stained for endothelium-specific protein CD102. (E) Differentiation of hepatopancreatic precursor cells. MLPC were cultured for 30 days in the presence of Hepatocyte Complete Medium (Cambrex) supplemented with FGF basic (20 ng/mL), FGF-4 (20 ng/mL), and stem cell factor (SCF; 40 ng/mL). Three-dimensional bodies were stained for human proinsulin. (F,G,H) Neurogenic differentiation. MLPC were grown on laminin and poly-D-lysine-coated chamber slides in the presence of Neural Progenitor Maintenance Medium (Cambrex) supplemented with FGF-4 (20 ng/mL) for 30 days mixed cultures of neurons, astrocytes, and oligodendrocytes developed.

**Table 2.** Potential applications for umbilical cord blood–derived stem cells for tissue repair or regeneration

| Regenerative tissue lineage               | Model                       | Outcome  |
|---|-----------------------------|--|
| Hematopoietic/immunologic<br>[2,29,32–39] | Clinical in vivo (human)    | Engraftment of RBC, lymphocytes, natural killer cells, dendritic cells, myeloid cells, platelets   |
| Multiple [51]                             | In vitro                    | Development of 3 different germinal layers: endodermal, mesodermal, ectodermal (MLPC)  |
| Neuronal [57–61]                          | In vitro                    | Cells of neuroglial morphology expressed brain tissue markers, vimentin, nestin, A2B5; neurotrophic receptors, trk-A, trk-B trk-C; neural genes and proteins including nestin, GFAP, NF200, $\beta$ -tubulin III, double Cortin  |
| Neuronal [62–69]                          | Preclinical in vivo (mouse) | Reduced arterial hypotension, cerebral hypoperfusion, cerebral ischemia and hypoxia after induced heatstroke in mice<br>Accelerated neovascularization, induction of cortical expansion; enhanced migration of neuronal progenitor cells to damaged area after induced stroke in mice<br>Improved neurologic testing after induced intracerebral hemorrhage in mice<br>Improvement in apomorphine-induced rotations in hemi-parkinsonian rats<br>UCB delayed disease progression improved survival in murine ALS model<br>Localization of UCB to site of injury; improved functional scores, increased white matter and regeneration of axons after induced spinal cord injury |
| Neuronal [5,70]                           | Clinical in vivo (human)    | Improved survival in children with Krabbe's disease  |
| Cardiac [73,75–79]                        | Preclinical in vivo (mouse) | Improved cardiac function and reduction of infarction size in animals with induced acute MI  |
| Endothelial [81,82,89]                    | Preclinical in vivo (mouse) | Improved vascularization and regeneration of muscle fiber in induced ischemia in animals<br>Elimination of hyperglycemia after transplantation into diabetic mice  |
| Endothelial [84]                          | Clinical in vivo (human)    | Improved peripheral circulation in patients with Buerger's disease; relief of pain and healing of skin lesions   |
| Hepatic [85,86,88]                        | In vitro                    | Development of differentiated hepatic cells in culture   |
| Hepatic [87]                              | Preclinical in vivo (rat)   | Human AFP <sup>+</sup> cells found in liver of rats after induced hepatic injury   |
| Epithelial [90]                           | In vitro                    | Development of keratinocytes in culture  |

AFP =  $\alpha$ -fetoprotein; ALS = amyotrophic lateral sclerosis; GFAP = glial fibrillary acidic protein; MI = myocardial infarction; MLPC = multilineage progenitor cells; RBC = red blood cells; UCB = umbilical cord blood.

in those tissues expressed neural markers, nestin,  $\beta$ -tubulin III, and glial fibrillary acidic protein [69].

Clinical studies have evaluated UCB therapy for treatment of neurological disorders. Several institutions have utilized UCB to treat Krabbe's disease, an enzymatic disorder caused by deficiency of galactocerebrosidase that results in deficiency of myelin formation in the central and peripheral nervous system and progressive neurologic deterioration and death in early childhood [5,70].

#### Cardiac lineage

The permanent loss of cardiomyocytes and fibrous scar formation following myocardial infarction (MI) result in irreversible damage to cardiac function because adult cardiac myocytes do not replicate and cannot be replaced. Recent studies have demonstrated the rapid progress of stem cell therapy after MI using autologous BM stem cells for cardiac regeneration [71–73]. Because UCB is rich in MSC and EPC progenitors, it may also be a valuable alternative source of transplant stem cells for cardiac muscle regeneration and repair (Table 2).

Preclinical in vivo animal studies have reported encouraging results using UCB stem cells to rejuvenate infarcted

myocardium, enhance healing, and improve ventricular function. Henning et al. [74] investigated the use of human UCB mononuclear progenitor cells for the treatment of rats with induced acute MI. Their results demonstrated that UCB administered into the infarct border progressively increased the left ventricular (LV) function and reduced the infarction size in the animals [74]. Similarly, UCB CD34<sup>+</sup> cells were isolated and administered to conditioned male Wistar rats after induced MI. They reported that the intramyocardial transplanted UCB cells survived localized around the vessels of the ischemic myocardium and enhanced neovascularization in the injured myocardium and improved LV function [75]. UCB CD133<sup>+</sup> progenitor cells have also been used after MI [76]. UCB CD133<sup>+</sup> were infused into athymic nude rats, the coronary arteries of which had been ligated. One month after infusion, UCB cells were found to have migrated, colonized, and survived in the infarcted myocardium and human cells were identified near vessel walls and in the LV cavity. Functionally, LV fractional shortening improved and anterior wall thickness decreased. Ma et al. recently reported on the capacity of UCB mononuclear cells to migrate to the heart of nonobese diabetic/severe combined immune deficient mice with induced

MI [77]. Evidence of UCB migration was seen in 50% of the mice, as well as, smaller infarct size and increased capillary density. A large animal study by Kim et al. demonstrated similar results [78]. Using a porcine model with induced MI, UCB somatic stem cells were injected directly into the infarcted tissue. After 4 weeks grafted cells were detected in the tissue with improvement in perfusion and wall motion of the infarcted region, increased ejection fraction and decreased scar thickness.

Combined UCB stem cell and gene therapy have also been studied for cardiac repair after MI to deliver specific gene products that can restore physiological function [79]. UCB CD34<sup>+</sup> cells were transduced with a vector encoding human antiangiogenic-1cDNA and/or vascular endothelial growth factor cDNA and expanded in culture. After induction of MI in SCID mice, the expanded/transduced CD34<sup>+</sup> were injected into the left anterior free wall of the heart. After treatment, infarct size significantly decreased, capillary density increased and higher cardiac performance in echocardiography was seen.

#### *Endothelial lineage*

Novel approaches for the use of UCB for treatment of ischemia have also been reported. Vasculogenesis and angiogenesis from BM contribute to the development and repair of vascular tissue. Both processes involve EPCs that originate in the BM and migrate to the sites of neovascularization, thus, EPCs may potentially be used for angiogenic therapies. Numbers of EPCs in the peripheral circulation are very low, however, increase in their levels can be seen after trauma or ischemia. UCB contain higher numbers of EPCs than peripheral blood and show faster differentiation and greater proliferative activity [80]. Ingram et al. identified a unique population of high proliferative-potential endothelial colony forming cells in human UCB that can achieve at least 100 population doublings, replated into secondary and tertiary colonies and retain high levels of telomerase [18] (Table 2).

An early study described cells with an endothelial phenotype generated from UCB CD34<sup>+</sup> cells in an in vitro endothelial culture system [81]. When the cultured EPCs were transplanted into a nude rat model with induced hind limb ischemia, the cells were found to incorporate into endothelial capillaries among the skeletal myocytes of the ischemic limbs. Additionally, significantly improved ratios of ischemic/normal hind limb blood flow was observed in the treated animals [81]. A subsequent study demonstrated the differentiation of selected UCB CD34<sup>+</sup> cells into EPCs after in vitro expansion and differentiation [82]. Injection of the cultured cells into ischemic limbs of immunosuppressed mice confirmed the survival and colonization tissues of the affected adductor muscles and enhanced arteriole length density and regenerating muscle fiber density of the treated limbs.

A comparison of EPCs generated from mononuclear fractions of UCB and BM was recently reported. EPCs were immunophenotyped and transplanted into an ischemic nonobese diabetic/severe combined immune deficient mouse model. Surface CXCR4 expression and stromal markers were higher in UCB vs BM-derived EPCs, but no difference was seen in the in vivo experiments where injection of both sources of EPCs resulted in significantly improved perfusion after femoral artery ligation and increased capillary densities in muscle tissue samples [83].

A recent study reported results of UCB transplantation for ischemic limb disease in an animal model and the outcome of a clinical trial using UCB for the treatment of Buerger's disease [84]. UCB mononuclear cells were cultured and expanded for 4 weeks for the differentiation of mesenchymal stem cell lineage. Ischemia was induced in the hind limb of nude mice and UCB cultured MSCs were injected into the ischemic area. Up to 60% of the hind limbs were salvaged in the UCB-MSc treated animals and MSCs were detected in the arterial walls of the ischemic hind limbs. Further, the efficacy of UCB-derived MSCs for improvement of peripheral circulation and pain was tested in a clinical trial. HLA-matched UCB-derived MSCs were transplanted into men with Buerger's disease who had failed previous treatment and surgical therapy. After UCB transplantation, ischemic rest pain was ameliorated, necrotic skin lesions were healed, digital capillaries were increased in number and size, and peripheral circulation was improved [84].

#### *Hepatic, pancreatic, and epithelial lineages*

UCB stem cell therapy offers exciting promise for disease of other target organs including liver, pancreas, lung, and skin (Table 2). Several investigators have demonstrated that UCB-derived stem cells are capable of differentiating along a hepatic lineage. Hong et al. [85] reported that MSCs isolated from UCB can be stimulated in vitro to express a variety of hepatic lineage markers, Thy-1, c-kit, Flt-3,  $\alpha$ -fetoprotein, cytokeratin-18, albumin, and functionally incorporate DiI-acetylated low-density lipoprotein, a hallmark of differentiated hepatic cells [85]. McGuckin et al. [86] report the production of cells expressing ES cell markers (SSEA-4/-3, TRA-1-60, TRA-1-81, and Oct 4) from UCB after immunomagnetic removal of mononuclear cells, erythrocytes, and hematopoietic progenitors and culture with thrombopoietin, Flt-3 ligand, and c-kit ligand. The primitive cells could undergo further differentiation and express characteristic hepatic markers [86]. A similar study demonstrated the differentiation of human UCB into hepatocytes in vitro and in vivo [87]. After UCB mononuclear cells were cultured with fibroblast and hepatocyte growth factors, adherent cells stained positive for  $\alpha$ -fetoprotein and albumin hepatic markers. Further, hepatic injury was induced in adult Sprague-Dawley rats and treated with UCB mononuclear cells. After 1 month, cells positive for

human  $\alpha$ -fetoprotein and albumin were found in the sinus hepaticus, central veins of hepatic lobules, and portal area [87]. Recently, Peters et al. [88] reported the differentiation of nonadherent HSCs from UCB units into adherent hepatocytic lineages. MSCs were expanded from UCB units and the cells differentiated into hepatocyte cells expressing mRNA for albumin as well as hepatocyte-specific transport proteins.

UCB stem cell therapy could also provide a rational treatment for regenerative repair of chronic disease. Zhao and colleagues [89] identified stem cells from UCB with embryonic cell characteristics, including expression of ES cell-specific molecular marker and transcription factors Oct-4, Nanog, SSEA-3, and SSEA-4. These cells displayed low immunogenicity and could give rise to cells with endothelial-like characteristics when stimulated with vascular endothelial growth factor and neuronal-like characteristics when stimulated with neuronal growth factor. Interestingly, the UCB stem cells could be stimulated to differentiate into functional insulin-producing cells that eliminated hyperglycemia after transplantation into a diabetic mouse [89].

Another novel application for the use of UCB stem cells involves their differentiation to epithelial cells of the skin, keratinocytes. Kamolz et al. [90] isolated and cultured human keratinocytes from adult female skin donors on fibrin glue/fibroblast gels and male UCB cells were added for coculture. After 2 weeks, sheets of culture cells formed three to four layers of cells and using PCR and fluorescence in situ hybridization, the UCB cultures contained cells with Y-chromosome-specific sequences, thus indicating that UCB cells differentiated into epithelial cells that could serve as material for the isolation and expansion of cells for skin transplantation [90].

### Summary and future perspective

Stem cells from various sources, embryonic, BM, peripheral blood, or UCB have the potential to differentiate and contribute to the homeostasis and repair of multiple tissues. The utilization of ES cells present significant practical and moral/ethical problems that hamper their use at this time. However, other stem cell sources may provide comparable broad developmental potential without these obstacles. UCB offers many innate advantages as a stem cell source. It is readily available in a virtually unlimited supply, possesses a primitive ontogeny, has not been exposed to immunologic challenge, and has a high number of progenitor cells with intrinsic high proliferative potential. Further, these recent studies provide convincing evidence that UCB contain not only hematopoietic progenitors, but also several other types of stem cells from the very primitive ES cells to more differentiated MLPCs, MLSCs, EPCs, and MSCs. The high degree of plasticity of UCB stem cells has been demonstrated by the differentiation of UCB MLPC, HSC, EPC, and MSC, into cardiac, neuronal,

hepatic, and epithelial lineages in reports summarized in Table 2. Thus, UCB may be a suitable alternative to ES cells for tissue replacement and repair.

While the basic mechanisms of UCB stem cell differentiation are not yet completely understood, and methods to expand specific populations in sufficient numbers for transplantation must be established, the use of UCB stem cells shows promise for the treatment of both hematopoietic and nonhematopoietic diseases. Further, UCB stem cells still need to cross HLA-matching barriers upon transplantation, thus use of allogeneic UCB stem cells for tissue engraftment and repair may require transient (1–6 months) immunosuppressive treatment. The abovementioned studies and previous studies provide a solid foundation for future basic and translational research to determine the intrinsic potential of UCB pluripotent stem cells and their use for nonhematopoietic tissue and cell regeneration.

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