

Endothelial Colony Forming Cells and Mesenchymal Stem Cells are Enriched at Different Gestational Ages in Human Umbilical Cord Blood

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ABSTRACT: Endothelial progenitor cells (EPCs) are used for angiogenic therapies and as biomarkers of cardiovascular disease. Human umbilical cord blood (UCB) is a rich source of endothelial colony forming cells (ECFCs), which are EPCs with robust proliferative potential that may be useful for clinical vascular regeneration. Previous studies show that hematopoietic progenitor cells are increased in premature UCB compared with term controls. Based on this paradigm, we hypothesized that premature UCB would be an enriched source of ECFCs. Thirty-nine UCB samples were obtained from premature infants (24–37 wk gestational age (GA)) and term controls. ECFC colonies were enumerated, clonally isolated, and identified by expression of endothelial cell surface antigens and functional analysis. GA of 33–36 wk UCB yielded predominantly ECFC colonies at equivalent numbers to term infants. UCB from 24 to 28 wk GA infants had significantly fewer ECFCs. Surprisingly, 24–28 wk GA UCB yielded predominantly mesenchymal stem cell (MSC) colonies, capable of differentiating into adipocytes, chondrocytes, and osteocytes. MSCs were rarely identified in 37–40 wk GA UCB. These studies demonstrate that circulating MSCs and ECFCs appear at different GA in the human UCB, and that 24–28 wk GA UCB may be a novel source of MSCs for therapeutic use in human diseases. (*Pediatr Res* 64: 68–73, 2008)

Circulating endothelial progenitor cells (EPCs) in adult human peripheral blood were originally identified in 1997 by Asahara *et al.* (1). Since their original identification, EPCs have been extensively studied as biomarkers to assess the risk of cardiovascular disease in human subjects and as a potential cell therapeutic for vascular regeneration (2–5). We recently identified endothelial colony forming cells (ECFCs) from adult circulating and human umbilical cord blood (UCB) (6). ECFCs are characterized by robust proliferative potential and by their ability to form blood vessels *in vivo* when transplanted into immunodeficient mice (6,7). Based on these studies, ECFCs are currently being tested in preclinical models as a cell therapeutic for a variety of vascular disorders.

ECFCs circulate at a very low frequency in adult human blood and their function may be impaired secondary to complications from atherosclerosis, diabetes, and other vascular disorders (5,6,8). In contrast, ECFCs are enriched in term UCB compared with adult peripheral blood. Further, ECFCs from term UCB not only emerge earlier in culture, but proliferate faster and show enhanced vessel forming ability (6,7,9). The average number of cell progeny derived from a single plated cord blood ECFC is nearly 100-fold greater than the number of cells derived from an individual adult ECFC (6). In addition, cell progeny of single-cord blood-derived ECFCs (6) have the ability to be serially replated and expanded exponentially in long-term culture. For these reasons, UCB is a rich source of ECFCs, which may be readily banked for clinical use.

Although term UCB is a viable source of ECFCs, the frequency of circulating ECFCs in premature UCB is unknown. This is an important question since previous studies from our group demonstrated that primitive hematopoietic progenitor cells are greatly enriched in premature UCB compared with term controls (10). Given that previous genetic studies have defined the close embryonic origins of endothelial and hematopoietic cells in the developing fetus (11), we hypothesized that premature UCB would be an enriched source of ECFCs for potential clinical use.

MATERIALS AND METHODS

Umbilical cord blood samples. Human UCB samples (10–50 mL) were collected in heparin-coated syringes from the umbilical vein of 39 term and preterm infants (gestational age, 24–40 wk). The Institutional Review Board at the Indiana University School of Medicine reviewed and approved this study with exempt IRB status.

Preparation of mononuclear cells. UCB was diluted 1:1 with PBS (PBS; Invitrogen, Grand Island, NY) and overlaid onto Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ). Cells were centrifuged for 30 min at room temperature (RT) at 740g. Buffy coat mononuclear cells (MNCs) were isolated and washed with EBM-2 medium (Cambrex, Walkersville, MD)

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Abbreviations: DiI-AcLDL, DiI acetylated low-density lipoprotein; EC, endothelial cell; ECFC, endothelial colony forming cell; EPC, endothelial progenitor cell; MSC, mesenchymal stem cell; MNC, mononuclear cell; RT, room temperature; UCB, umbilical cord blood

supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 2% penicillin/streptomycin (Invitrogen), and 0.25 $\mu\text{g}/\text{mL}$ amphotericin B (Invitrogen).

Culture of umbilical cord blood mononuclear cells. For outgrowth of endothelial cell (EC) colonies, UCB MNCs were resuspended in complete EGM-2 medium (cEGM2) consisting of EBM-2 supplement with the entire EGM-2 bullet kit (Cambrex), 10% FBS, and 1% antibiotic supplement. A total of 30×10^6 MNCs/well were seeded onto 6-well tissue culture plates precoated with type 1 rat-tail collagen (BD Biosciences, Bedford, MA) and cultured as previously described (6).

For outgrowth of mesenchymal stem cell (MSC) colonies, UCB MNCs (30×10^6 MNCs/well) were cultured in 6-well tissue culture plates with DMEM (Invitrogen, Carlsbad, CA) and supplemented with 10% FBS as previously described with minor modifications (12,13).

EC colonies appeared between 5 and 15 d of culture and were identified as well-circumscribed monolayers of cobblestone-appearing cells (Fig. 1A). MSC colonies appeared between 3 and 10 d of culture and were identified as flattened and spindle-shaped cells (Fig. 1B). ECFC or MSC colonies were enumerated by visual inspection using an inverted microscope (Olympus, Lake Success, NY) under $40\times$ magnification.

Clonal isolation of ECFCs and MSCs. ECFC and MSC colonies were clonally isolated as previously described (6,14). ECFC and MSC colonies were isolated with cloning rings (Fisher Scientific, Hampton, NH), washed with PBS and detached with 0.25% trypsin-EDTA (Invitrogen). Cells from colonies were reseeded onto collagen-coated tissue culture plates in EGM-2 or DMEM + 10% FBS and were cultured at 37°C , 5% CO_2 , in a humidified incubator.

Immunophenotyping of ECFCs and MSCs. Early passage (1,2) ECFCs were detached from tissue culture flasks, washed with PBS, and stained with primary murine MAb directed against human CD31, CD45, and CD105 conjugated to FITC (all BD Pharmingen, San Diego, CA, unless otherwise indicated); against human CD73, CD105, and CD146 conjugated to phycoerythrin (PE); against human CD144 conjugated to Alexa Fluor 647; human CD54 and CD90 conjugated to allophycocyanin (APC) per manufacturer's recommendations in PBS + 2% FBS. Isotype controls used were mouse IgG $_{1\kappa}$ FITC, mouse IgG $_{1\kappa}$ PE, and mouse IgG $_{1\kappa}$ APC. Stained cells were analyzed using a FACSCalibur and Cell Quest software (Becton Dickinson, San Diego, CA).

Osteogenic differentiation. Passage 2 MSCs (50,000) were seeded onto a collagen-coated 6 well tissue culture plate and cultured in osteogenic differentiation media comprised of Mesencult mesenchymal basal cell media, human osteogenic stimulatory supplements (5% by volume), glycerophosphate (3.5 mM), dexamethasone (10^{-8} M), and ascorbic acid (50 $\mu\text{g}/\text{mL}$) (all Stem Cell Technologies, Vancouver, Canada) as per manufacturer's recommendations for 3 weeks. Nonadherent cells and debris were removed with twice weekly media changes.

Adipogenic differentiation. Early passage MSCs (50,000) were cultured in adipogenic media comprised of Mesencult mesenchymal basal cell media and adipogenic stimulatory supplement (Stem Cell Technologies) per the manufacturer's recommendations. Nonadherent cells and debris were removed with twice weekly media changes.

Chondrogenic differentiation. Early passage MSCs (10^6) were transferred to a 15 mL polypropylene conical tube and centrifuged for 5 min to form a cell pellet. Chondrogenic media, consisting of high-glucose DMEM (Invitrogen) supplemented with 10^{-4} M dexamethasone (Sigma Chemical Co.-Aldrich,

St. Louis, MO), 50 $\mu\text{g}/\text{mL}$ acetylsalicylic acid (Sigma Chemical Co.-Aldrich), 100 $\mu\text{g}/\text{mL}$ sodium pyruvate (Sigma Chemical Co.-Aldrich), 40 $\mu\text{g}/\text{mL}$ praline (Sigma Chemical Co.-Aldrich), 10 ng/mL TGF- β 1 (BD Pharmingen), and 50 mg/mL ITS $^+$ premix (BD Pharmingen), was added into the 15 mL conical tube. Media was refreshed twice weekly for 3 wk.

MSC and human umbilical vein endothelial cell (HUVEC) lines purchased from Lonza (Walkersville, MD) were used as a positive and negative control, respectively, for all differentiation experiments.

Cytochemical staining. To confirm adipogenic differentiation, differentiated MSCs and noninduced cells were cultured in 6-well plates, washed with Hank's Balanced Salt Solution (HBSS; Invitrogen), and fixed for 1 h in 10% formalin (Sigma Chemical Co.) at 37°C . Fixed cells were washed with HBSS, incubated with a 0.21% solution of oil red O (Sigma Chemical Co.) for 1.5 h (15). Plates were examined under $50\times$ magnification for positive red staining of lipid droplets.

To confirm osteogenic differentiation, differentiated MSCs and noninduced cells were cultured in 6-well plates, washed with PBS, and fixed in ice-cold 70% ethanol (Sigma Chemical Co.). Fixed cultures were washed with distilled water and stained for 30 min at RT with a 2% solution of alizarin red S (Sigma Chemical Co.) (15). Plates were examined under $50\times$ magnification for positive orange-red staining of calcium deposits.

To confirm chondrogenic differentiation, pellets were fixed with freshly prepared 4% paraformaldehyde overnight and embedded in paraffin. Tissue blocks were cut into 4–5 μm sections that were placed on slides and dried overnight at 37°C . Deparaffinization was carried out in xylene (2 times, 3 min each), and sections were hydrated in graded series of ethanol. Detection of proteoglycan was performed using Alcian blue staining (Sigma Chemical Co.). Sample slides were deparaffinized and hydrated to distilled water. One percent Alcian blue dye in glacial acetic acid (Sigma Chemical Co.) was added for 30 min. Counterstaining was done with nuclear fast red solution for 5 min. Slides were then washed and dehydrated using 95% alcohol. Slides were examined under $50\times$ magnification for positive blue staining of glycosaminoglycans (15).

Total RNA isolation and RT-PCR. RNA was extracted from MSCs, differentiating cells, or differentiated cells using RNeasy (Qiagen, Valencia, CA) per the manufacturer's instructions. The mRNA was reverse transcribed to cDNA using SuperScript III Reverse Transcriptase (Invitrogen) per the manufacturer's instructions. cDNA was amplified using a Platinum TaqDNA polymerase (Invitrogen) System at 94°C for 40 s, 56°C for 50 s, 72°C for 60 s for 35 cycles, after initial denaturation at 94°C for 5 min. The following specific primers were used for amplification: collagen type I (414 bp) S: 5'-ACGTCCTGGTGAAGTTGGTC-3', A: 5'-TCCAGCAATACCCTGAG-GTC-3' and osteopontin (330 bp) S: 5'-CTAGGCATCACCTGTGCCAT-ACC-3', A: 5'-CTACTTAGACTACTTGACCAGTGAC-3'. Primers were designed based on specific gene sequences retrieved from Ensemble human cDNA sequence library and queried against overlapping DNA sequences using BLAST. PCR products were imaged under UV fluorescence of ethidium bromide in 2% agarose gels.

Tube formation. Early passage cells (1,2) derived from ECFC and MSC colonies were seeded onto 96 well tissue culture plates precoated with Matrigel (BD Discovery Labware) as previously described (6) and observed by inverted microscopy over the course of 24 h for tube formation. MSCs and HUVEC cell lines were used as negative and positive controls, respectively.

Acetylated LDL uptake. To assess the ability to uptake acetylated LDL (AcLDL), early passage (1,2) cells derived from ECFC and MSC colonies were incubated with DiI-acetylated-LDL (DiI-AcLDL) (Invitrogen) as previously described (6). Cells were examined by fluorescence microscopy for uptake of DiI-AcLDL.

RESULTS

Two different colony-forming cells are present in premature UCB. ECFCs are enriched in term human UCB compared with adult peripheral blood (6). To determine whether ECFCs are further enriched in premature cord blood, UCB was obtained from 24 to 37 wk GA premature infants and term controls (37–40 wk GA). MNCs were isolated and seeded onto collagen-coated tissue culture plates in EGM-2 media for outgrowth of ECFCs. Presumed EC colonies with characteristic monolayer cobblestone morphology (Fig. 1A) arose between days 5 and 15 of culture in UCB from all GA infants except one 27-wk GA sample. Interestingly, in initial exper-

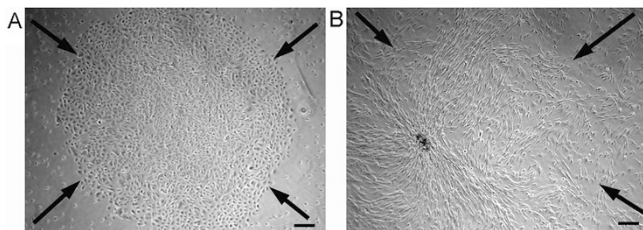


Figure 1. Isolation of two colony types from premature UCB. (A) Representative photomicrograph ($50\times$ magnification) of an EC colony (d11) cultured from UCB MNCs of a 32 wk GA infant. Colonies with similar cobblestone morphology were identified in cultures of UCB from 24–36 wk GA ($n = 24$) and full term infants. (B) Representative photomicrograph of a colony of spindle shaped cells arising from the UCB MNCs of a 24-wk GA infant. Colonies with similar cellular morphology were identified in cultures of UCB from 18 other 24–36 wk GA infants, but only one full term infant. Arrows indicate colony boundaries and scale bars represent $200 \mu\text{m}$.

iments, cell colonies comprised of flattened, fibroblast-like cells (Fig. 1B) appeared between day 3 and 10 in MNC cultures derived from 24 to 36 wk GA UCB. However, the fibroblast-like cell colonies rarely appeared in term UCB (1 of 13) under these defined ECFC culture conditions (data not shown).

Identification of two types of colony-forming cells in pre-term UCB. Given the unexpected appearance of two colony forming cells in premature cord blood, we performed detailed experiments to characterize the cell progeny derived from each distinct colony type before quantifying and comparing the distribution of colonies in UCB from premature and term infants. First, we characterized the presumed EC colonies (as shown in Fig. 1A), which were derived from UCB of various GAs (24 wk to term). Individual colonies were visually identified, clonally isolated, and expanded in cell culture to generate sufficient progeny for analysis. Immunophenotyping revealed that cell progeny derived from both preterm and term UCB expressed the EC surface antigens, CD31, CD105, CD146, and CD144 (Fig. 2A). Importantly, the cell progeny did not express the hematopoietic cell specific surface antigen CD45 (Fig. 2A). We next tested whether the EC-derived cells would incorporate DiI-Ac-LDL, and form capillary-like structures in Matrigel, which are functional characteristics of ECs (6). Both preterm and term cells subcultured from the adherent colonies uniformly incorporated DiI-Ac-LDL and formed capillary-like structures in Matrigel (Fig. 2B, C). Thus, these studies confirm that the cell progeny derived from preterm and term UCB colonies were endothelial in origin.

After identification of ECFCs, we next determined the phenotype of the second cell colony type (as shown in Fig.

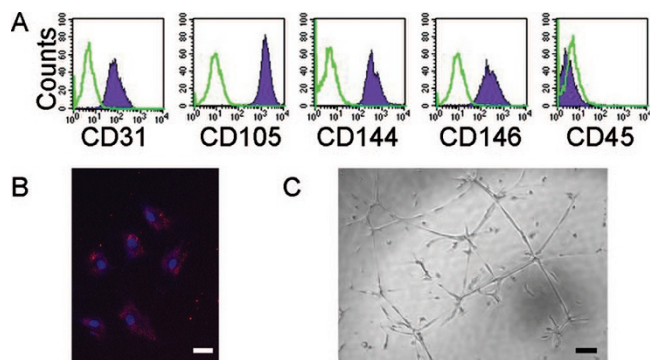


Figure 2. Phenotypic and functional analysis of ECFCs derived from premature UCB. (A) Representative FACS analysis of cells derived from a cobblestone appearing colony cultured from UCB MNCs of a 34 wk GA infant. Cells were uniformly positive for EC surface antigens, CD31, CD105, CD144, and CD146, but negative for hematopoietic specific surface antigen, CD45. Similar results were seen in 34 colonies from 25 premature donors and 26 colonies from 13 full term donors. (B) Representative photomicrograph of cells derived from a cobblestone appearing colony cultured from UCB MNCs of a 24 wk GA infant, following 4 h of incubation with DiI-Ac-LDL (red). Nuclei are counterstained with DAPI (blue). Similar AcLDL uptake was seen in cells derived from nine colonies from four premature donors and four colonies from two full term donors. Scale bar represents 50 μ m. (C) Representative photomicrograph of cells derived from a cobblestone appearing colony cultured from UCB MNCs of a 33 wk GA infant 24 h after seeding on Matrigel. Similar tube formation was seen for cells derived from eight colonies from six premature donors and four colonies from three full term donors. Scale bar represents 200 μ m.

1B), which was enriched in UCB derived from more premature infants. As noted above, the cells contained within the colony displayed a characteristic spindle-shaped and/or fibroblast morphology, reminiscent of MSC colonies derived from adult bone marrow (16–19). To determine whether these colonies were comprised of MSCs, individual cell colonies were visually identified, clonally isolated on day 11 of culture, and passaged for generation of sufficient cell numbers to characterize for a MSC phenotype. Immunophenotyping revealed that cell progeny derived from the colonies expressed numerous MSC cell surface antigens including CD54, CD73, and CD90 (Fig. 3A), but did not express EC surface antigens CD31, CD146, or CD144, or the hematopoietic cell surface antigen, CD45 (data not shown).

Previous studies demonstrate that adult-derived MSCs can be differentiated into chondrocytes, adipocytes, and osteocytes under defined culture conditions (20). Therefore, we next tested whether the cell progeny generated from the colonies yielded each cell type utilizing previously established methods (20). Well-characterized adult bone marrow-derived MSCs were used as a positive control and HUVECs as a negative control for all differentiation experiments. Consistent with a MSC phenotype, the cells derived from individual colonies could be differentiated into cells, which morphologically, histologically, and cytochemically appeared to be chondrocytes, adipocytes, and osteocytes (Fig. 3B–D). Adipogenic capability of cells derived from premature UCB colonies was investigated with serial passage of cells under defined adipogenic differentiation culture conditions. Morphologic cell changes and the presence of lipid vacuoles, which stained positive for oil red O, confirmed the adipocyte phenotype of the differentiated cells (Fig. 3B). Cells derived from the colonies were also induced in defined chondrogenic and osteogenic differentiation conditions. Following 3 wk of induction, presumed osteocytes and chondrocytes stained positive for alzirin red S and Alcian blue, respectively (Fig. 3C, D). To further confirm the chondrocyte and osteocyte phenotype of cells differentiated from the original colonies, we performed RT-PCR analysis for known chondrocyte and osteocyte genes. Following 3 weeks of induction, osteocyte appearing cells indeed expressed osteopontin, and chondrocyte appearing cells expressed type I collagen (Fig. 3E). Thus, multiple lines of evidence indicate the cell colonies enriched in premature UCB are MSCs.

ECFCs and MSCs are enriched at different gestational ages in human UCB. After identification of the two colony types derived from UCB, we next quantitated and compared the frequency of ECFC and MSC colonies circulating at different GA in UCB. MNCs were isolated from 39 human UCB samples and seeded into tissue culture plates in either EGM-2 or defined MSC media for outgrowth of MSC and ECFC colonies. Similar to other reports (12,13,21,22), MSCs were rarely identified in UCB from infants between 32 and 40 wk GA (0.6 ± 0.4 colonies/ 10^8 MNCs in 32–36 wk GA, $n = 12$ and 0.02 ± 0.02 colonies/ 10^8 MNCs in 37–40 wk GA, $n = 13$, Fig. 4A). However, there was a 12-fold enrichment of MSCs in 24–31 wk GA UCB (Fig. 4A). Conversely, there was a 3-fold enrichment of ECFCs in 37–40 wk GA UCB com-

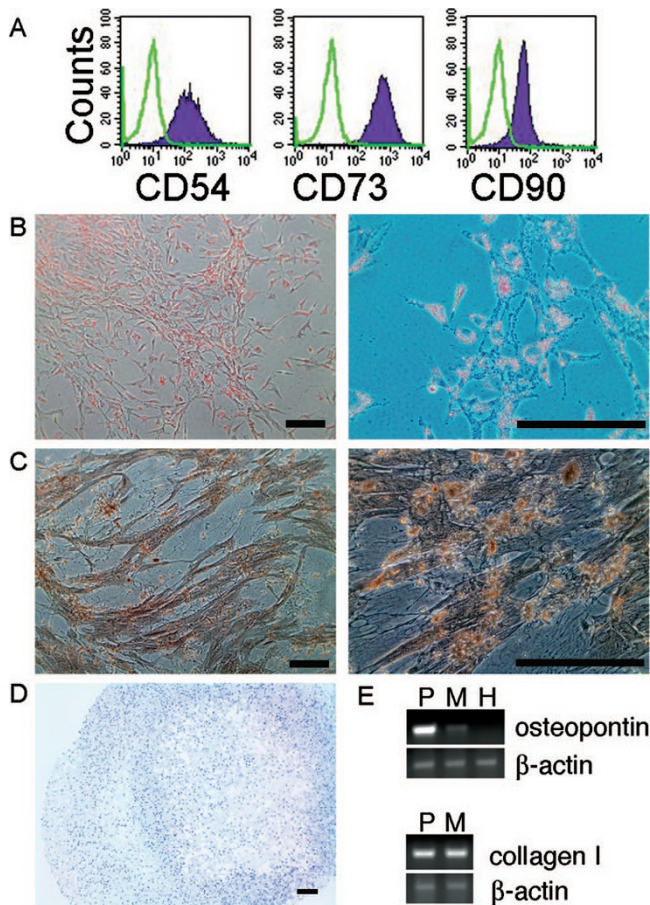


Figure 3. Phenotypic analysis and lineage differentiation of MSCs derived from premature UCB. (A) Representative FACS analysis of cells derived from a colony consisting of spindle shaped cells cultured from UCB MNCs of a 24 wk GA infant. Cells were uniformly positive for MSC surface antigens, CD54, CD73, and CD90. Similar results were seen in 28 colonies from 19 premature donors. (B) Representative photomicrograph of spindle cells from a 24 wk GA infant stained with oil red O following 3 wk of induction in adipogenic-specific cell culture conditions. Similar results were seen in 10 colonies from four premature donors. Scale bars represent 200 μ m. (C) Representative photomicrograph (left panel 10 \times magnification, right panel 32 \times magnification) of spindle cells from a 24 wk GA infant stained with Alzirin red S following 3 wk of induction in osteogenic-specific cell culture conditions. Similar results were seen in 10 colonies from 4 premature donors. Scale bars represent 200 μ m. (D) Representative photomicrograph of spindle cells from a 24 wk GA infant stained with Alcian blue following 3 wk of induction in chondrogenic-specific cell culture conditions. Similar results were seen in five colonies from four premature donors. Scale bars represent 200 μ m. (E) RT-PCR analysis of spindle cells from a 24 wk GA infant (P), commercially available MSCs (M), and HUVECs (H) following 3 wk of induction in osteogenic-specific (top panel) or chondrogenic-specific (bottom panel) cell culture conditions, for gene expression of osteopontin, type I collagen, and β -actin. RT-PCR results represent three independent experiments using cells from different donors.

pared with 24–31 wk GA UCB (Fig. 4B). Upon closer examination of the extremely premature group, there was no difference in ECFC frequency between 24–28 and 29–31 wk GA infants (data not shown). Strikingly, MSCs were 10 times more frequent in the UCB of 24–28 wk GA infants than 29–32 wk GA infants (Fig. 4C). Importantly, culture of MNCs in either EGM-2 or defined MSC media did not significantly alter the colony numbers in different GA UCB (data not

shown). Thus, these studies demonstrate that ECFCs and MSCs circulate at higher frequencies at different GA in the human fetus.

DISCUSSION

Human UCB is a viable source of somatic stem/progenitor cells for potential therapeutic applications in a variety of diseases (14). However, there is little information about which types of progenitor cells circulate during human embryogenesis. This is an important question since somatic stem/progenitor cells may emerge and circulate at different times during gestation. Therefore, we conducted a comparative cell culture analysis of premature and term infant UCB to determine the appearance and frequency of MSCs and ECFCs at different gestational ages.

Employing lineage specific cell surface-marker phenotyping and *in vitro* differentiation assays to identify colony outgrowth cell types, we successfully isolated both MSCs and ECFCs from individual cord blood cultures and quantitated the frequency of each cell type in UCB at different gestational ages. Surprisingly, MSCs were enriched in premature infant cord blood, whereas ECFCs were routinely isolated during later gestational ages as previously described by our group and others (6,23). Specifically, UCB of 24–28 wk gestational age infants had a significantly higher concentration of MSCs compared with term gestation UCB. Further, the concentration of MSCs decreased as gestation proceeded to term, leading to a near absence of MSCs in 37–40 wk UCB. Specifically, we found that 24–31 wk gestation UCB had a mean MSC concentration of 8.0 MSCs/100 million MNCs, which decreased to 0.6 MSCs/100 million MNCs at 32 wk gestation.

In contrast to the emergence pattern of MSCs in UCB, we determined that the concentration of ECFCs was lower in premature gestation infants (24–28 wk) compared with term gestation infants. We cultured approximately 3.0 ECFCs/100 million MNCs in 24–28 wk GA UCB, which increased to 9.0 ECFCs/100 million MNCs in term gestation UCB. This experimental observation suggests that premature infants may have lower numbers of circulating ECFCs, which then increase in frequency as the fetus matures toward term gestation. These data contrast the observation that premature infant blood has a higher concentration of hematopoietic stem and progenitor cells (10) thereby contradicting our original hypothesis that premature infant UCB comprises an abundant source of stem and progenitor cells of all lineages.

MSCs have been isolated from a plurality of tissue extracts, illustrating the ubiquitous nature of MSCs (24), yet controversy exists as to whether UCB contains MSCs (13,21,22,25). Our data suggests that this controversy may have arisen due to the inherent temporal concentration changes of progenitor cells in UCB. Erices *et al.* were able to cultivate MSCs from term and preterm UCB, noting that the relative frequency of MSCs was increased in preterm (30 wk gestation) UCB (13). Additionally, Goodwin *et al.* identified cells capable of multiple lineage differentiation in UCB cultures (21). In 2001, Wexler *et al.* reported that MSCs could only be isolated from bone marrow but not term infant UCB (22). Since then, MSCs

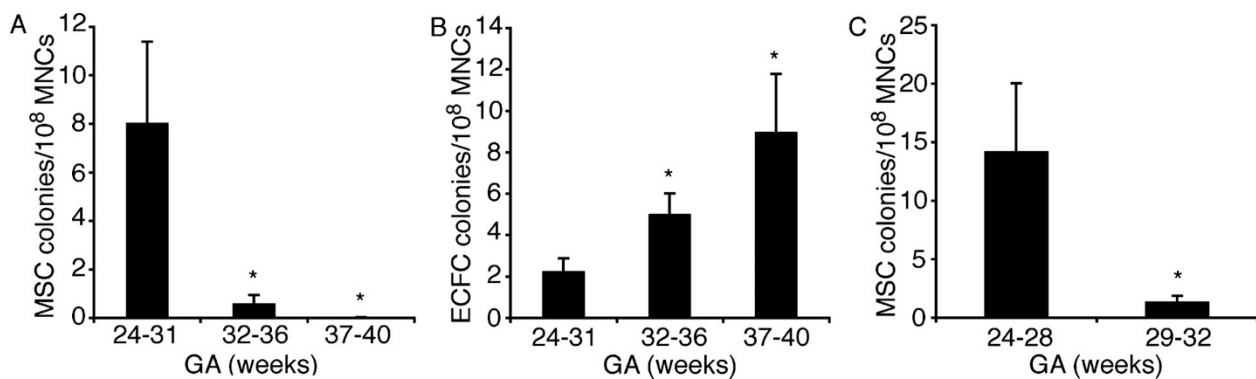


Figure 4. Quantitation of MSC and ECFC colonies in UCB of premature and full term infants. (A) Quantitation of MSC colonies per 100 million MNCs plated. * $p < 0.04$ for 24–31 ($n = 12$) weeks GA vs. 32–36 ($n = 12$) and 37–40 ($n = 13$) weeks GA by t test. (B) Quantitation of ECFC colonies per 100 million MNCs plated. * $p < 0.04$ for 24–31 ($n = 12$) weeks GA vs. 32–36 ($n = 12$) and 37–40 ($n = 13$) weeks GA by t test. (C) Quantitation of MSC colonies per 100 million MNCs plated. * $p < 0.03$ for 24–28 ($n = 6$) weeks GA vs. 29–32 ($n = 8$) weeks GA by t test.

have apparently been described in very low concentrations in term infant UCB requiring significant expansion *ex vivo* to generate sufficient cell numbers for therapeutic applications (12). Specifically, Kogler *et al.* reported the ability to obtain differentiating MSCs from only 35% of 573 term UCB samples (25). It is interesting to note that Igura *et al.* found chorionic villi MSCs (26), while others have found placental mesenchymal cells (27), which indicate possible additional sources of MSCs in afterbirth waste. Thus, it appears that UCB retains MSCs capable of *in vitro* multilineage differentiation throughout gestation, but our comparative study clearly indicates that the circulating concentration of MSCs is significantly increased in preterm UCB.

MSCs may hold therapeutic potential for several human diseases, which is under investigation in a number of ongoing clinical trials (28). Specifically, MSCs are pluripotent, self-renewing cells with the potential for tissue regeneration, including repair of bone, cartilage, tendon, ligament, skeletal muscle, and cardiac muscle. The MSCs we describe herein share the differentiation capacity of previously reported MSCs, and thus may represent a new, and renewable, source of MSCs. Similarly, EPCs are currently used as biomarkers and as potential cell therapeutics for a variety of cardiovascular diseases (2–5,29). Although the physiologic role of the temporal change in circulating ECFCs and MSCs in the developing fetus is currently not understood, it is clear from our comparative analysis that MSCs and ECFCs can be routinely isolated with maximum efficiencies at discrete gestational ages. We speculate that the MSCs may circulate at higher frequency in preterm infants since this is a period of rapid bone, cartilage, and fat deposition, which are tissues that are in part derived from MSCs. As the fetus approaches 33 wk GA, it is possible that ongoing vascular patterning and growth is more critical in later stages of gestation as the infant nears term. Nevertheless, given the growing interest in the potential use of cell therapy in clinical medicine, banking and cryopreservation of both preterm and term UCB may be an important development to the field of cell-based therapeutics.

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