Endothelial Fate and Angiogenic Properties of Human CD34$^+$ Progenitor Cells in Zebrafish

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Objective—The vascular competence of human-derived hematopoietic progenitors for postnatal vascularization is still poorly characterized. It is unclear whether, in the absence of ischemia, hematopoietic progenitors participate in neovascularization and whether they play a role in new blood vessel formation by incorporating into developing vessels or by a paracrine action.

Methods and Results—In the present study, human cord blood–derived CD34$^+$ (hCD34$^+$) cells were transplanted into pre- and postgastrulation zebrafish embryos and in an adult vascular regeneration model induced by caudal fin amputation. When injected before gastrulation, hCD34$^+$ cells cosegregated with the presumptive zebrafish hemangioblasts, characterized by *Scl* and *Gata2* expression, in the anterior and posterior lateral mesoderm and were involved in early development of the embryonic vasculature. These morphogenetic events occurred without apparent lineage reprogramming, as shown by CD45 expression. When transplanted postgastrulation, hCD34$^+$ cells were recruited into developing vessels, where they exhibited a potent paracrine proangiogenic action. Finally, hCD34$^+$ cells rescued vascular defects induced by *Vegf-c* in vivo targeting and enhanced vascular repair in the zebrafish fin amputation model.

Conclusion—These results indicate an unexpected developmental ability of human-derived hematopoietic progenitors and support the hypothesis of an evolutionary conservation of molecular pathways involved in endothelial progenitor differentiation in vivo. (***Arterioscler Thromb Vasc Biol.* 2011;31:1589-1597.)**

Key Words: endothelium ■ vascular biology ■ angiogenesis ■ embryology ■ stem cells

It has long been supposed that cells present in the bone marrow, peripheral blood (PB), and cord blood (CB), which copurify with hematopoietic stem cells (HSCs), also give rise to endothelial cells. This speculation was supported by the notion that CD34$^+$ and CD133$^+$ cells differentiate, in culture and in vivo, into cells that express mature endothelial cell markers. These cells, called endothelial progenitor cells (EPCs),[1–3] have been the subject of numerous basic and translational studies showing their participation in neovascularization. Recent studies have revealed possible separation between hematopoietic- and nonhematopoietic-derived EPCs.[4–8] These 2 cell types have been called early and late EPCs,[9] or colony-forming unit–endothelial cells (CFU-ECs) and endothelial colony-forming cells (ECFCs).[7] They are fundamentally distinguished on the basis of hematopoietic marker expression (eg, CD45) and the ability to proliferate or to differentiate into endothelial cells.[10] Separation between hematopoietic and the endothelial lineages during early mouse[11] and zebrafish[12] development is established during by asymmetrical division of primitive cells located in the dorsal aorta endothelium, through a novel differentiation event called endothelial-hematopoietic transition (EHT).[13–16] More uncertain is whether cells with a similar potency of generating HSCs or endothelial cells are present at postnatal stages[17]; recently, however, derivation of endothelial cells from CD34$^+$/CD38$^+$/CD45$^+$/CD133$^+$ CB progenitors was demonstrated,[18] suggesting the existence of similar progenitors at least during fetal life.

The zebrafish (*Danio rerio*) embryo provides several advantages for in vivo study of vascular morphogenesis, based on the opportunity to observe in real time the development of embryonic blood vessels[19,20] and also on the

Received on: June 21, 2010; final version accepted on: March 31, 2011.

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Arterioscler Thromb Vasc Biol **is available at http://atvb.ahajournals.org** **DOI: 10.1161/ATVBAHA.111.226969**

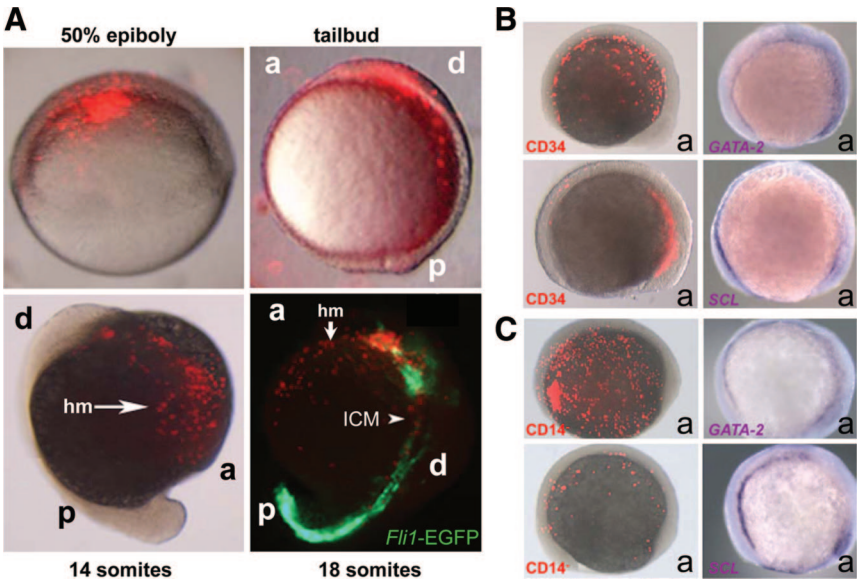

Figure 1. Early segregation of hCD34$^+$ cells after injection into the zebrafish blastula. *Tg(fli1*:enhanced green fluorescent protein [EGFP])^{y1} embryos were injected with Orange Cell Tracker–labeled hCD34$^+$ cells at the blastula stage. A, Injected gastrula at 50% epiboly (top left), tailbud (top right), 14-somite-stage (bottom left) and 18-somite-stage (bottom right) injected embryo. Images result from merging of bright-field and rhodamine fluorescence pictures indicating injected human cells (arrows). The 18-somite injected embryo image results from merging dark-field green and red fluorescence pictures, representing the embryonic vasculature and human cells, respectively. In all pictures, a, d, and p indicate the anterior, dorsal, and posterior sides of the embryos, respectively; ICM, intermediate cell mass. B and C, Different distribution of blastula-injected hCD34$^+$ (B) and hCD14$^-$ (C) cells in 3- to 5-somite-stage developing embryos (left) and expression of *Gata2* and *Scl* transcripts (right). Pictures showing human cells were obtained by merging rhodamine fluorescence with bright-field images of the injected embryos. The same transplanted embryos are shown on the right after in situ detection of *Gata2* and *Scl* transcripts. In all pictures, embryos are oriented with the anterior (a) on the right side of each picture. hm indicates head mesenchyme.

remarkable conservation of molecular pathways regulating vascular development in mammals, such as those regulated by endothelial (eg, *Flk-1*) or hematopoietic (eg, *Scl, Runx1*) master genes.[21–23]

The objective of the present work was to explore the developmental potency and the vascular competence of human CB CD34$^+$ cells, exploiting the flexibility offered by zebrafish system.

Materials and Methods

An expanded supplemental version of the Methods section is available online at http://atvb.ahajournals.org.

Ethical Statement

Collection of CB samples was performed with written consent on a voluntary basis and after approval by institutional review boards. Authorization numbers are provided in the supplemental material.

Fish Maintenance

Breeding zebrafish, wild-type AB strain, transgenic (Tg) strain Tg(*KDRI*:enhanced green fluorescent protein [EGFP])^{S843} (ZIRC, University of Oregon), and the Tg(*fli1*:EGFP)^{y1} line (from the laboratory of Nathan Lawson, University of Massachusetts Medical School) were maintained at 28°C on a 14-hour light/10-hour dark cycle, according to established procedures.

hCD34$^+$ and hCD14$^-$ Cell Selection and Characterization

Isolation of human CB–derived CD34$^+$ (hCD34$^+$) and CD14$^-$ cells was performed by using magnetic selection with a MINI-MACS system after separation of CB and PB mononuclear cells onto density gradients. hCD34$^+$ cell purity (87.4±2.9%; n=9) was assessed by flow cytometry in preliminary experiments. Before injection, cells were labeled using Orange Cell Tracker (Molecular Probes, Invitrogen) according to the manufacturer's instruction.

Cell Transplantation Assays and Morpholino RNA Injection

Blastula stage or tricaine (0.04 mg/mL, Sigma) anesthetized 48 hours post fertilization (hpf) stage AB or Tg(*fli1*:EGFP)^{y1} zebrafish strains embryos were injected with Orange Cell Tracker–labeled CD34$^+$, CD14$^-$ cells (4 nL injection volume, 500 to 1000 cells/embryo), or *Vegf-c* morpholino (MO) RNA.

Embryo Sectioning and Confocal Microscopy

For tissue sectioning, CD34$^+$ cell-injected embryos were fixed with 4% paraformaldehyde, embedded in cryostat mounting medium (Killik, Bioptica), and transversally cut (8 μm) at different sectioning planes in the regions of the head and the dorsal artery/dorsal aorta locations for immunofluorescence.

Whole-Mount Alkaline Phosphatase Staining, Immunodetection Assays, and In Situ Hybridization

Zebrafish control and cell-injected embryos were fixed at the indicated time points using 4% paraformaldehyde for 2 hours at room temperature, after which they were processed for in situ hybridization, alkaline phosphatase staining, terminal deoxynucleotidyl transferase dUTP nick-end labeling, or phosphohistone H3 whole-mount immunofluorescence.

Statistical Analysis

Statistical analysis was performed by unpaired *t* test or 1-way ANOVA with Newman-Keuls post hoc analysis using Prism GraphPad statistical software; each experiment was carried out in at least in triplicate. Data are indicated as mean±SE.

Results

Fate of hCD34$^+$ Progenitors Transplanted Into the Developing Zebrafish Blastula

Orange Cell Tracker–labeled hCD34$^+$ cells were transplanted in Tg(*fli1*:EGFP)^{y1} embryos before the onset of gastrulation, at midblastula stage. The distribution of hCD34$^+$ cells in the developing embryos was analyzed immediately after injection and at tailbud and somite stages. Initially, cells remained mostly localized at the injection site at the embryo animal pole (Figure 1A). From tailbud stage to somite stage, a number of human cells were found in the midline, the head

mesenchyme, and the intermediate cell mass regions (Figure 1A). In situ hybridization using riboprobes to detect *Scl* and *Gata2* mRNAs were then performed. The results revealed a strict association of human cells with multipotent *Scl*$^+$ and *Gata2*$^+$ mesoderm cells (Figure 1B), suggesting cosegregation of human cells with zebrafish hemangioblasts. This behavior was specific; in fact, PB cells devoid of the CD14$^+$ monocyte cell population, having proangiogenic activity,[24] were not associated with *Scl*$^+$ and *Gata2*$^+$ regions (Figure 1C and Supplemental Figure I).

Proliferation and survival of hCD34$^+$ cells transplanted into Tg(*fli1*:EGFP)^{y1} blastulas were evaluated at the 28 hpf stage by whole-mount staining for phosphohistone H3 and terminal deoxynucleotidyl transferase dUTP nick-end labeling. Both tests revealed little proliferation and only occasional apoptosis (Supplemental Figure II). At this stage, human cells were found in primary and secondary blood vessels of the head, in the heart, in the dorsal aorta, in the posterior cardinal vein, in the caudal vein plexus, and in intersegmental and longitudinal vessels (Figure 2A to 2C). At the blastula stage, injection of CB-derived CD34$^+$/CD45$^-$ cells, considered to be containing putative ECFC progenitors,[10] produced analogous results (Supplemental Figure III), whereas injection of melanoma cancer cells gave rise to formation of micrometastases in the head and the caudal vein plexus regions (Supplemental Figure IVA). Finally, observation of transversal sections revealed the presence of hCD34$^+$ cells in the lumen, as well as in the wall of the dorsal aorta and in other vessels in strict association with EGFP$^+$ host endothelial cells (Figure 2D).

To further investigate the phenotype of human cells, blastula-injected embryos were dissociated in single-cell suspensions at 28 and 48 hpf, followed by staining with antibodies recognizing human hematopoietic and endothelial markers and flow cytometry (Figure 3). Human cells were easily recognized as a red-labeled population in dot plots representing red versus green fluorescence. In these cells (Figure 3 and Supplemental Figures V and VI), CD3, CD4, CD8, and CD144 (VE-cadherin) were not expressed before or after injection; CD38, CD48, and CD105 exhibited a significant downregulation; endothelial marker CD146 was increased; and kinase insert domain receptor (KDR) was transiently upregulated at 28 hpf. Interestingly, the monocyte marker CD14 was expressed in a very small subset of hCD34$^+$ cells before injection, to be progressively upregulated at the 28 and 48 hpf stages; finally, endothelial marker CD31 and panhenatopoietic marker CD45 were expressed at high levels at all stages. To reveal coexpression of endothelial and hematopoietic markers in CD34$^+$ cells in the embryos, immunofluorescence in whole-mount embryos and on transversal frozen sections was performed. This revealed that vessel-associated human cells expressed the hemangioblast/endothelial marker KDR (Figure 4A) and the endothelial marker endothelin (Figure 4B). Strikingly, irrespective of their location in developing embryonic vessels, human-derived cells expressed the hematopoietic lineage marker CD45 (Figure 4C). Coexpression of CD45 with KDR was, finally, detected in vessel-associated human cells (Figure 4D).

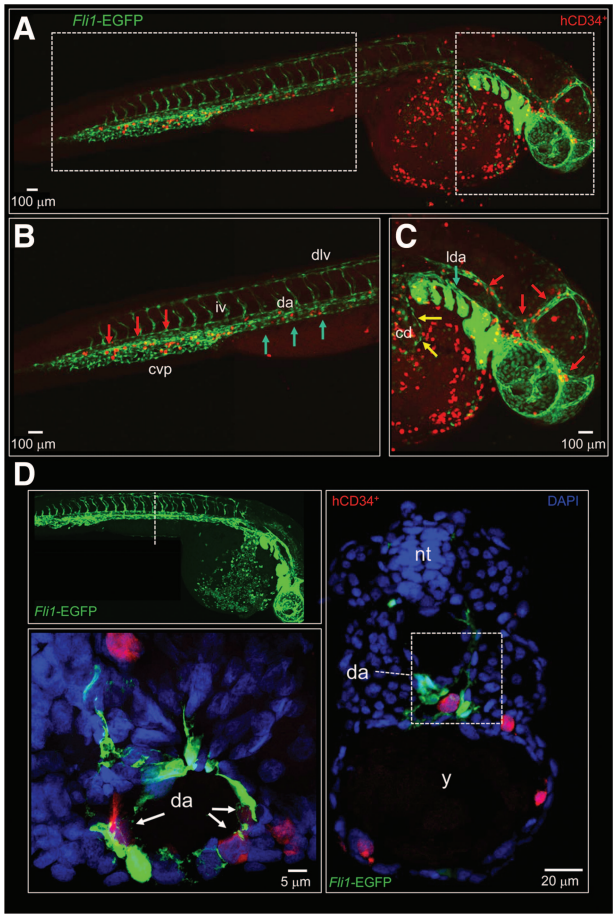

Figure 2. Fate of blastula-injected hCD34$^+$ cells at postgastrulation stages. *Tg*(*fli1*:enhanced green fluorescent protein [EGFP])^{y1} embryos were injected at the blastula stage with Orange Cell Tracker–labeled hCD34$^+$ cells and fixed with paraformaldehyde at the 28 hours post fertilization (hpf) stage of development. A, Confocal microscope low-power view of a hCD34$^+$ injected embryo. hCD34$^+$ cells are represented by red dots. Dashed boxes represent magnified regions in B and C. B and C, Magnification of the tail and head regions, respectively, of the embryo shown in A. Note in B the preferential association of hCD34$^+$ cells to the caudal vein plexus (cvp; red arrows) and the dorsal artery (da; green arrows) and in C the presence of hCD34$^+$ cells (red arrows) lining the head vessels, 1 of the 2 branches the dorsal aorta (lda; green arrows) and the Cuvier duct (cd; yellow arrows). dlv indicates dorsal lateral vein; iv, intersomitic vessels. D, Low-power view of an uninjected Tg(*fli1*:EGFP)^{y1} embryo at the 28 hpf stage (top left) and transversal section at the midtrunk level of 2 hCD34$^+$ injected Tg(*fli1*:EGFP)^{y1} embryos at different magnification (bottom right and left). In the top left panel, the dashed line indicates the approximate sectioning plane represented in the 2 other panels; dashed box in the right panel indicates the region of the dorsal aorta (da) shown at a higher magnification in the bottom right panel, representing human cells (arrows) in close association with EGFP$^+$ zebrafish endothelial cells of the dorsal aorta. nt indicates neural tube.

Transition from endothelial to hematopoietic phenotype of cells localized in the wall of the dorsal aorta has been described as the major event associated with emergence of definitive HSCs in the zebrafish embryo.[13,16] To assess whether EHT is affected by the presence of human cells, a 16-hour time-lapse video recording of Tg(*KDRI*:EGFP)^{S843} embryos injected at the blastula stage with CD34$^+$ cells was

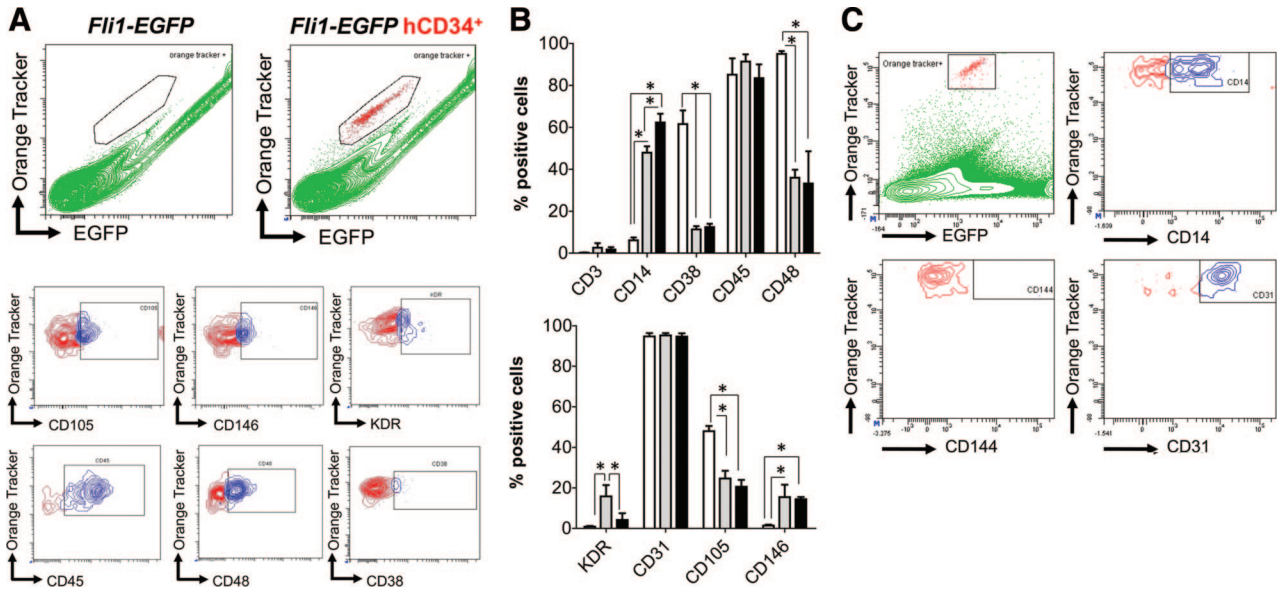

Figure 3. Flow cytometry analysis of blastula-injected hCD34$^+$ cells phenotype at 28 and 48 hours post fertilization (hpf). *Tg*(*fli1*:enhanced green fluorescent protein [EGFP])^{y1} embryos were injected with hCD34$^+$ cells and then dissociated and labeled with human antibodies recognizing hematopoietic and endothelial cell markers for analysis by flow cytometry. A to C, Human cells were recognized in green vs red fluorescence dot plots by comparison with uninjected embryos. These cells were logically gated for their red fluorescence by use of 488 nm (A) and 561 nm (C) lasers and analyzed for the expression of each of the human markers. B, Quantification of results shown in A and C for hematopoietic (top) and endothelial (bottom) markers in hCD34$^+$ cells before injection (open bars) and at 28 (gray bars) 48 hpf (black bars). *$P<0.05$ by 1-way ANOVA with Newman-Keuls post hoc analysis; n≥3. Orange Tracker indicates Orange Cell Tracker.

performed. As shown in Supplemental Figure VII and Supplemental Movie I, budding of KDR$^+$ HSCs was not perturbed by transplanted cells.

Postgastrula Stage Transplantation of hCD34$^+$ Cells Modulates Embryonic Vascular Development

The behavior of hCD34$^+$ cells injected into the circulatory system at the 48 hpf stage Tg(*fli1*:EGFP)^{y1} embryos was assessed by confocal time-lapse analysis. Early homing events in the blood vessels were observed as early as 30 minutes after injection (Supplemental Figure VIIA). hCD34$^+$ cell rolling and adhesion to zebrafish intersegmental vessels were also observed (Supplemental Movie II).

Transgenic embryos injected with hCD34$^+$ cells at the 48 hpf stage were analyzed at the 72 hpf stage, ie, 24 hours after injection. Altered blood vessel sprouting in the growing tail vasculature (85% of 72 transplanted embryos; Supplemental Figure VIIIB) and at the level of the subintestinal vein (80% of 17 injected embryos; Supplemental Figure VIIIC) were observed. Angiogenesis abnormalities were never found after injection of PB-derived hCD14$^-$ cells (Supplemental Figure VIIID) but were detected in embryos transplanted with the intestinal cancer cell line CACO2 (Supplemental Figure IVB). To further examine whether blood vessel development was perturbed by transplanted hCD34$^+$ cells, the vascular development of 48 hpf stage Tg(*fli1*:EGFP)^{y1} injected embryos was monitored from 2 to 12 hours postinjection by confocal videomicroscopy. A strict association was found between red-labeled cells and the regions of ectopic vascular sprouting (Supplemental Figure IX and Supplemental Movie III).

Transplanted hCD34$^+$ Cells Rescue Defective Angiogenesis in the Zebrafish Embryo by Multifactorial Paracrine Effect

The previous data suggested that transplanted hCD34$^+$ cells have paracrine effects in the developing embryos. To assess this hypothesis, cells were injected at the blastula stage in Tg(*fli1*:EGFP)^{y1} embryos, where *Vegf-c* was knocked down by MO RNA injection at the 1- to 4-cell stage. As shown in Figure 5A, embryos depleted of *Vegf-c* exhibited defects in the development of the vascular system (30% of 50 MO RNA-injected embryos). *Vegf-c* depletion mostly affected the formation of the dorsal aorta, the posterior cardinal vein, the intersegmental vessels, and specific head arteries[25] (Figure 5B). These defects were observed in only 9% (n=32) of *Vegf-c*-depleted/hCD34$^+$ cell-injected embryos (Figure 5C). Interestingly, phenotype rescue was complete even in vascular regions where CD34$^+$ cells failed to localize, suggesting complementation of *Vegf-c* knockdown defects by soluble factors secreted by human cells. Finally, hCD14$^-$ cell injection in *Vegf-c*-depleted embryos did not rescue defective blood vessel formation (100% of vascular abnormalities, n=5 embryos injected with *Vegf-c* MO RNA and hCD14$^-$ cells; data not shown). To identify paracrine factors participating in the rescue effect by injected cells, the secretion of interleukins, chemokines, and angiogenic growth factors was assessed by Luminex assay (Table). The results showed production of several factors, including chemokines, proinflammatory, and proangiogenic cytokines, suggesting a potent paracrine effect by hCD34$^+$ cells.

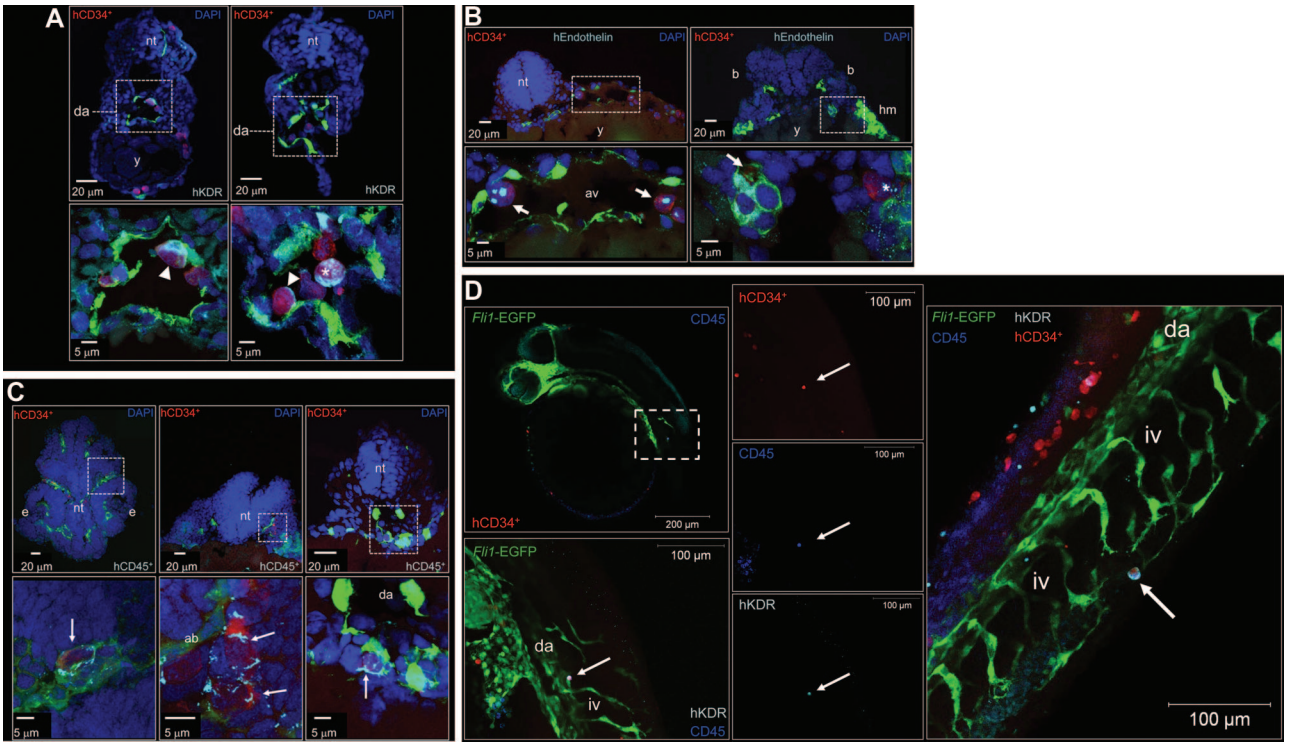

Figure 4. Marker analysis of vessel-associated hCD34$^+$-derived cells after blastula injection by immunofluorescence on embryo sections. Transversal frozen sections (8 μm) of hCD34$^+$ injected Tg(*fli1*:enhanced green fluorescent protein [EGFP])^{y1} embryos at the 28 hours post fertilization (hpf) stage. A, hKDR staining in 2 embryos cut at different levels in the trunk region. nt indicates neural tube; y, yolk; da, dorsal aorta/dorsal artery. Dashed boxes in the 2 top panels indicate the regions shown at higher power view in the bottom panels, where endothelium-associated hCD34$^+$ cells (arrowheads) and a round human kinase insert domain receptor (KDR)$^+$ cells in the lumen of the dorsal aorta/dorsal artery (*) are shown. B, Human endothelin staining of 2 transversally cut embryos at the anterior trunk (left panel) and brain (b; right) levels. Note the presence of Orange$^+$/endothelin$^+$ cells in the abdominal vessels (av; arrows, left) and associated with the head vessels endothelium (arrow, right panel). Orange$^+$/endothelin$^+$ cells (*, right panel) were also found close to head mesenchyme (hm), which also expresses *Fli1* at this stage. C, hCD45 expression in human cells at different locations in 28 hpf stage embryos. Panels on the left show developing blood vessels in a transversal section of the head, where the eye vasculature is present. The higher magnification on the bottom shows a CD45$^+$ human cell (arrow) associated with EGFP$^+$ endothelial cells. e indicates eye. Panels in the center show a developing vessel located in the anterior part of the trunk. A cluster of CD45$^+$ human cells (arrows) is recognizable within EGFP$^+$ endothelial cells forming abdominal vessels (ab). Panels on the right show CD45 expression in a human cell (arrow) in the developing dorsal aorta (da). D, Whole-mount immunofluorescence for detection of KDR and CD45 in human cells transplanted at the blastula stage in Tg(*fli1*:EGFP)^{y1} embryos. The 2 panels on the left show different magnifications of an embryo containing a hCD34$^+$ cells double positive for CD45 and KDR (arrows), located in the region of the dorsal aorta (da). The panel on the right depicts a CD45$^+$/KDR$^+$ human cell in the region of the dorsal lateral vein and close to intersomitic vessels (iv).

hCD34$^+$ Cells Accelerate Vascular Repair in Adult Zebrafish

The ability of hCD34$^+$ cells to enhance vascular repair in mammalian models of myocardial infarction and limb ischemia has been shown by us[26,27] and by others.[28–33] In the present study, we examined whether hCD34$^+$ cells contributed to vascular repair in adult zebrafish by injecting these cells in immunosuppressed Tg(*fli1*:EGFP)^{y1} adult animals after caudal fin amputation (Figure 6A), an established model of vascular regeneration in zebrafish.[34–36] A significant increase in angiogenesis in hCD34$^+$ cell–transplanted versus vehicle-injected animals was found (Figure 6B).

Discussion

Bone marrow, PB, and CB contain EPCs that contribute to neovascularization in ischemic tissues.[1] EPCs have long been thought to derive from alternative differentiation pathways of hematopoietic progenitors because of the expression of markers (CD34, CD133, KDR) that they have in common with primitive HSCs. Embryology has suggested a different scenario. In fact, during vertebrate embryogenesis, HSCs derive from primitive cells located in a morphologically conserved region, called the aorta-gonad-mesonephros region,[37] whence definitive HSCs emerge by a novel modality of asymmetrical division of pluripotent nonhematopoietic cells, the so-called hemogenic endothelium.[13,14] This division modality has been characterized in the zebrafish dorsal aorta and has been called EHT.[13] In the adulthood, the existence of 2 EPCs types, namely CFU-ECs (early EPCs) and ECFCs (late EPCs),[7] has been proposed. These cells likely have a different origin, although they are, at least in part, functionally overlapping. The most important proofs in favor of lineage separation between these 2 EPC types are (1) the expression of the panhematopietic marker CD45 in CFU-ECs but not in ECFCs,[4] and (2) the finding that ECFC clones from patients with myeloproliferative disorders do not harbor HSCs specific mutations, therefore identifying ECFCs as nonhematopoietic in origin.[7,8,10]

According to this definition of EPCs,[5] progenitors obtained by bone marrow, PB, or CB cell sorting with CD34, CD133,

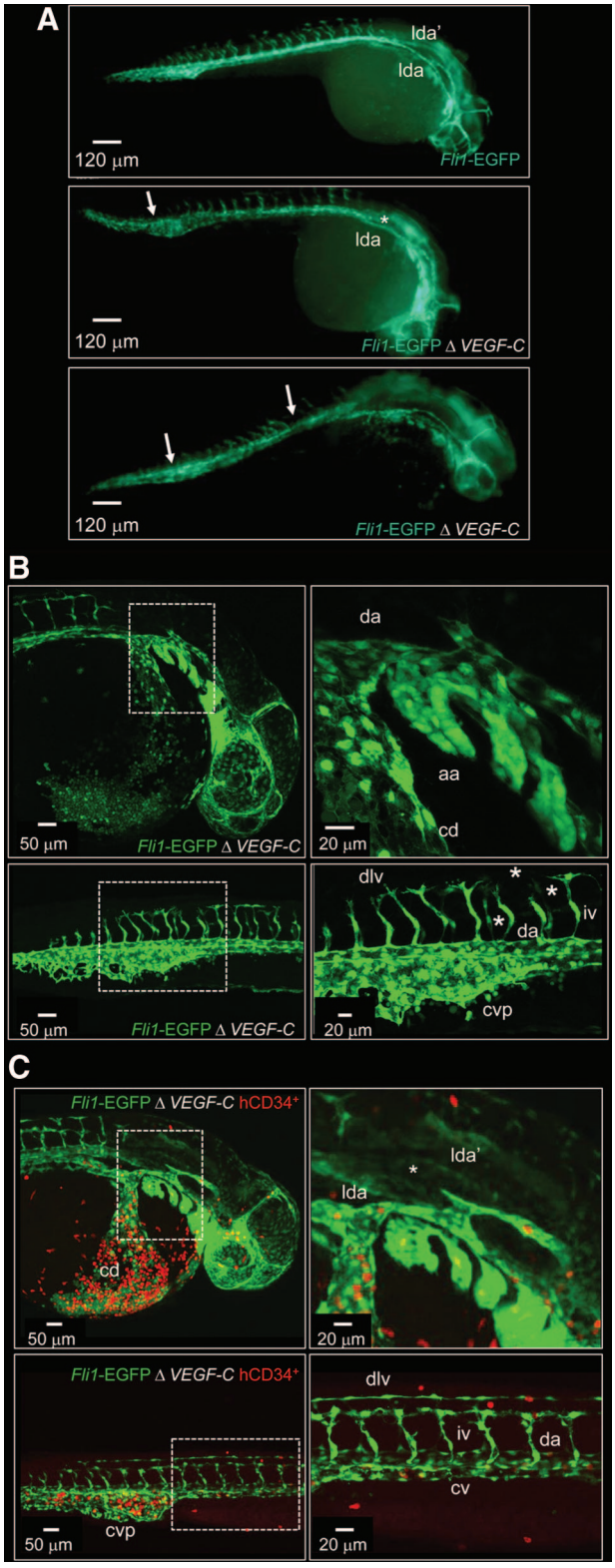

Figure 5. Rescue of angiogenic defects in *Vegf-c*-depleted embryos by blastula transplantation of hCD34$^+$ cells. Shown is in vivo knockdown of *Vegf-c* by injection of antisense MO RNA at 1 to 8 cell stage into *Tg*(*fli1*:enhanced green fluorescent protein [EGFP])^{y1} embryos. A, Low-power views of a control (Fli-GFP) and 2 *Vegf-c* MO RNA–injected embryos (Fli-GFPΔ*VEGF-C*). Note the absence of the bifurcation between the 2 lateral dorsal aorta branches in midpanel (*) and the irregular spacing of the intersomitic vessels (arrows), caused by absence of *Vegf-c* expression. B, Defective vascular phenotype caused by *Vegf-c* in vivo knockdown. Panels on the left show low-power views of the head/yolk regions (top) and the trunk/tail regions (bottom). Dashed box in the top left panel (magnified in the top right) shows the absence of the dorsal aorta (da). aa: aortic arches; cd: Cuvier duct. Dashed box in the bottom left panel (magnified in the bottom right) shows the irregular spacing of the intersomitic vessels (iv) of a Fli-EGFP Δ*VEGF-C* embryo. *Regions of the trunk where defects in the organization of these vessels and the dorsal lateral vein (dlv) were observed. da indicates dorsal artery; cvp, caudal vein plexus. C, Injection of red-labeled hCD34$^+$ cells into *Vegf-c* knockdown embryos rescued the vascular phenotype. Low-power (left) and high-power (right) views of regions similar to those represented in B show the presence of hCD34$^+$ cells associated with normal formation of the 2 lateral branches of the dorsal aorta (*, lda and lda′) and to regular spacing and branching of the intersomitic vessels (iv) in the trunk/tail regions. Note the presence of several red-labeled cells in the Cuvier duct (cd), and in the caudal vein plexus (cvp). dlv indicates dorsal lateral vein; cv, cardinal vein; da, dorsal artery.

and KDR markers without counterselection for CD45 should give rise only to CFU-ECs[38] and support neovascularization by paracrine interactions with resident endothelial cells. This latter hypothesis, however, is contradicted by several experimental findings showing that blood-borne stem cells give rise to endothelial cells in vitro and in ischemic tissues irrespective of their hematopoietic origin[2,3,26,29,33,39–42] and by a very recent report showing that CD34$^+$/CD38$^+$/CD45$^+$/CD133$^+$ CB progenitors differentiate in culture into clonally expanding endothelial cells, which, similarly to ECFCs, participate in neovascularization by incorporating into new blood vessels.[18]

Transplantation of hCD34$^+$ Cells at Early Stages of Embryonic Zebrafish Development Unravels an Unexpected Hemangioblast-Like Behavior

Transplantation into midblastulas was performed to reveal the fate of hCD34$^+$ cells throughout the earliest stages of vascular development. These experiments showed that blastula-transplanted hCD34$^+$cells were mostly cosegregated with the presumptive zebrafish *Scl*$^+$ and *Gata2*$^+$ progenitors in the anterior and posterior lateral mesoderm. Thus, hCD34$^+$ cells migrated in concert with zebrafish mesoderm progenitors and colonized the anterior region, ie, the site where embryonic uncommitted progenitors are known to establish primitive hematopoiesis.[43] During somitogenesis, hCD34$^+$ cells migrated medially and posteriorly along the developing intermediate cell mass, to finally colonize the entire cardiovascular system at the 28 hpf stage. As shown by flow cytometry and immunofluorescence (Figures 3 and 4), these morphogenetic events were associated with transient upregulation of KDR and overexpression of CD146 at all stages; importantly, this also coincided with downregulation of hematopoietic progenitor–specific markers CD38 and CD48. It was also remarkable that hCD34$^+$ cells showed increasing amounts of monocyte marker CD14 expression; that CD45 was not downregulated in KDR$^+$ human cells (Figures 3 and 4); and that injection of hCD34$^+$/CD45$^-$ cells, the putative ECFC progenitors,[10] produced the same segregation pattern into various vascular districts (Supplemental Figure III). Taken together, these results suggest that blastula-injected hCD34$^+$ cells were committed to multiple differentiation into

Table. Quantification of Human Cytokines in Culture Medium Conditioned by hCD34$^+$ Cells

Factor (pg/ml per 10^5 Cells)	Mean	SE (n=4)
Interleukins		
Hu IL-1ra	25.5	14
Hu IL-5	13	1.8
Hu IL-9	2.9	0.8
Hu IL-8	7220	2468
Hu IL-10	3.0	1.8
Hu IL-12	1.1	0.76
Hu IL-13	11.2	3.0
Hu IL-15	0.13	0.09
Hu IL-17	1.3	0.54
Chemokines		
Hu IP-10	196	103
Hu MCP-1	162	101
Hu MIP-1a	119	54
Hu MIP-1b	419	271
Hu RANTES	72.5	45
Proangiogenic factors		
Hu PDGF-bb	2.0	1.3
Hu FGF basic	0.43	0.2
Hu G-CSF	14.8	5.7
Hu GM-CSF	12.3	5.9
Hu VEGF	19.1	18

Hu indicates human; IL, interleukin; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor.

monocytes or primary endothelial cells, irrespective of their hematopoietic lineage programming, detected by CD45 expression.

Interestingly, a number of blastula-transplanted human cells were enclosed in the endothelial layer of the dorsal aorta at 28 hpf (Figures 2D and 4). This is consistent with the timing of EHT occurrence in the dorsal aorta of the developing zebrafish embryos.[13,16] To check whether the presence of human cells alters the normal behavior of multipotent endothelial progenitors lining the dorsal aorta, we performed EHT imaging in *KDR*-EGFP embryos, which showed no abnormalities in the budding process of definitive HSCs (Supplemental Figure VII, Supplemental Movie I). The lack of substantial perturbation of the EHT process is an indication of a physiological integration of human cells into zebrafish developing vascular network, even if future work will be necessary to establish whether human cells actively participate in EHT in this system.

Paracrine Activity of hCD34$^+$ Cells in the Zebrafish Embryo

The angiogenic activity of human bone marrow–derived,[2] PB-derived,[44] or CB-derived[40] progenitors has been demonstrated in several studies based on injection of these cells in preclinical models of ischemic disease. Except for a few reports describing results of intravital microscopy analyses of stem cells homing to mouse vessels,[45] so far only indirect

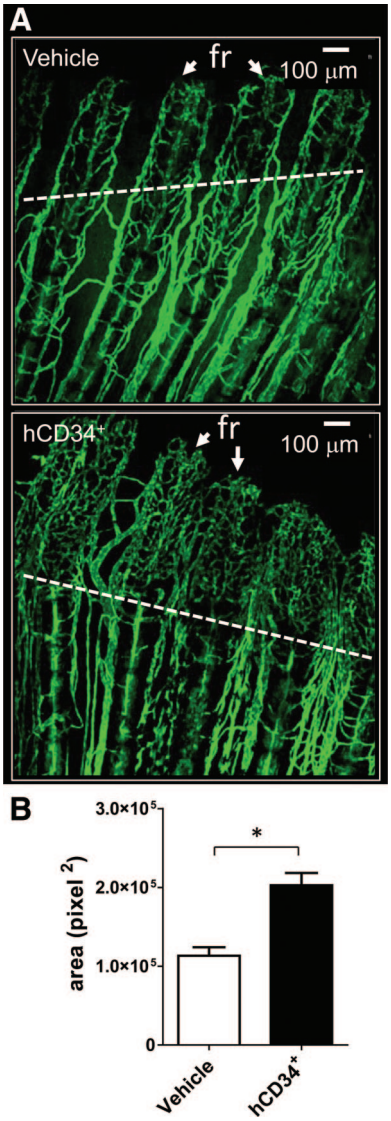

Figure 6. hCD34$^+$ cells accelerate vascular repair in immunosuppressed adult zebrafish. An adult zebrafish fin amputation model was used to quantify vascular regeneration in regenerating fin rays. A, Regenerating caudal fin of an adult fish at 7 days after vehicle (top) or unlabeled hCD34$^+$ cells (bottom) intraperitoneal injection. The newly formed vascular network is more developed in hCD34$^+$ cell–injected zebrafish than in controls. Dashed lines indicate the fin amputation level. B, Histogram shows the quantification of the enhanced green fluorescent protein (EGFP)$^+$ area (pixel^2), corresponding to the vascular network in confocal images of regenerating caudal fins in vehicle and hCD34$^+$ cell–injected adult fishes. fr indicates fin rays. *$P<0.05$ by unpaired Student t test; n=3.

evidence of homing events in ischemic tissues, mostly based on tissue sectioning, has been provided.[26]

The results of our hCD34$^+$ cell injection in postgastrulation-stage (48 hpf) embryos, showed that these cells circulated throughout the organism and produced early and late homing events, associated with abnormal or ectopic embryonic vasculature formation (Supplemental Figure VIII). Interestingly, homing of hCD34$^+$ cells was observed in several districts of the vasculature; this was different from the behavior of the human PB-derived CD14$^-$ cells, which were used as a negative control (Supplemental Figure VIII), or that of human melanoma and

CACO-2 stem cells, whose presence was mostly associated with the head; the caudal vein plexus, where they formed micrometastases; or the yolk region, where they caused ectopic vessel sprouting (Supplemental Figure IV).[46]

Direct participation in primary angiogenesis was not the only effect of $hCD34^+$ cell injection into developing zebrafish embryos. In fact, $hCD34^+$ cells rescued vascular defects caused by *Vegf-c* knockdown (Figure 6), and when transplanted into immunosuppressed adults, they accelerated regeneration of the vascular network in the amputated caudal fins. This activity was due to secretion of several cytokines and chemokines implicated in (lympho)angiogenesis and inflammatory response (Supplemental Table I). Taken together, and in line with the large existing literature on EPCs, these results suggest that transplanted $hCD34^+$ cells exert a potent paracrine effect[47] that rescues vascular developmental defects due to knockdown of Vegf-C, deregulates formation of normal vascular network in postgastrulation zebrafish embryos, and accelerates wound repair in adults.

Concluding Remarks

The identity of human-derived endothelial progenitors is a still open issue. Although the distinction between the 2 EPC types[10] has allowed reconciliation of the embryo with the adult stem cell worlds, it is still not clear whether, under pathological conditions or in developmental biology contexts, these cells may cross their lineage barriers and noncanonically differentiate into alternative or mixed phenotypes. Our findings suggest that this might be the case and call for further investigation aimed at clarifying the molecular nature of these possible events.

To our knowledge, the present study represents the first example of successful human stem cell transplantation into developing zebrafish embryos. In fact, apart from previous investigations showing the fate of human cancer cells in the developing zebrafish[48] and the recent demonstration of angiogenic activity of mouse and human tumor cell lines,[46] no other evidence exists that human stem cells colonize the developing zebrafish embryos and respond to specific developmental cues. In this context, our results may also have important translational implication for in utero correction of vascular defects in human embryos by injected stem cells.

It has been discussed that the developing zebrafish is an interesting microenvironment in which modulatory effects on the fate of injected cells are likely due to the presence of developmental mechanisms that are conserved among vertebrates.[48] In addition, given the relative ease of zebrafish genetic manipulation systems, xenotransplantation of human cells in zebrafish has been proposed as a reference technique to define the properties of human cancer (stem) cells.[49] Our study is in line with these emerging concepts and shows the versatility of this system to also address fundamental human developmental and cell biology questions.

Acknowledgments

We thank Dr Mammoliti and the obstetrics group at Melzo Hospital for CB collection, and we thank Dr Nathan Lawson for the Tg(*fli1*:EGFP)^{y1} line. We also thank Drs Serena Balasso and Laura Masala for fish breeding and transgenic line screenings and Drs Gianluca Deflorian and Federica Pezzimenti of the Zebrafish Facility care at Istituto FIRC di Oncologia Molecolare Institute, Milan, Italy.

Sources of Funding

This work was supported by European Union–funded grants Ulcer Therapy (contract no: LSHB-CT-2005-512102) and Thercord (contract no: LSHB-CT-2005-018817) and by Fondazione Cariplo NOBEL Project "Tumour Stem Cells" 2006 (to F.C.).

Disclosures

None.

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