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).10 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 mouse11 and zebrafish12 development is established during an 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 stages11; 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 vessels19,20 and also on the

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The 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.

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(KDR:enhanced green fluorescent protein [EGFP])$^{ZIRC}$ (ZIRC, University of Oregon), and the Tg(fl/1: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 tricane (0.04 mg/mL, Sigma) anesthetized 48 hours post fertilization (hpf) stage AB or Tg(fl/1: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, Biopita), and transversally cut (8 μm) at different sectioning planes in the regions of the head and the dorsoventral aorta to 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(fl/1: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, 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, 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 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 panhematopoietic 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 (da; green arrows) and the Cuvier duct (cd; yellow arrows). div indicates dorsal lateral vein; iv, intersomitic vessels. D, Low-power view of an un.injected 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. To assess whether EHT is affected by the presence of human cells, a 16-hour time-lapse video recording of Tg(KDR1:EGFP)S843 embryos injected at the blastula stage with CD34+ cells was...
performed. As shown in Supplemental Figure VII and Supple-
mental Movie I, budding of KDR

/H11001

HSCs was not per-
turbed by transplanted cells.

Postgastrula Stage Transplantation of hCD34

/H11001

cells Modulates Embryonic Vascular Development

The behavior of hCD34

/H11001

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

/H11001

cell rolling and adhesion to zebrafish intersegmental vessels

were also observed (Supplemental Movie II).

Transgenic embryos injected with hCD34

/H11001

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 ob-
served. Angiogenesis abnormalities were never found after

injection of PB-derived hCD14

/H11002

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

/H11001

cells, the vascular develop-

ment of 48 hpf stage Tg(

fli1

):EGFP)y1 injected embryos was

monitored from 2 to 12 hours postinjection by confocal video-

microscopy. A strict association was found between red-labeled

cells and the regions of ectopic vascular sprouting (Supplemen-
tal Figure IX and Supplemental Movie III).

Transplanted hCD34

/H11001

cells Rescue Defective

Angiogenesis in the Zebrafish Embryo by

Multifactorial Paracrine Effect

The previous data suggested that transplanted hCD34

/H11001

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 arteries25 (Figure 5B). These defects were

observed in only 9% (n=32) of Vegf-c-depleted/hCD34

/H11001

cell-injected embryos (Figure 5C). Interestingly, pheno-
type rescue was complete even in vascular regions where

CD34

/H11001

cells failed to localize, suggesting complementa-
tion of Vegf-c knockdown defects by soluble factors

secreted by human cells. Finally, hCD14

/H11001

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

/H11001

cells; data not shown). To identify paracrine factors participat-
ing 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 chemo-
kines, proinflammatory, and proangiogenic cytokines, sug-
gesting a potent paracrine effect by hCD34

/H11001

cells.
The ability of hCD34⁺ cells to enhance vascular repair in mammalian models of myocardial infarction and limb ischemia has been shown by us²⁶,²⁷ 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, 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.¹²,¹³ This division modality has been characterized in the zebrafish dorsal aorta and has been called EHT.¹²,¹³ 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 panhematopoietic 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 HSC-specific mutations, therefore identifying ECFCs as nonhematopoietic in origin.⁷,⁸,¹⁰

According to this definition of EPCs,⁷ progenitors obtained by bone marrow, PB, or CB cell sorting with CD34, CD133,
and KDR markers without counterselection for CD45 should give rise only to CFU-ECs 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 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.

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. 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, 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 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 ΔVEGF-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 (\(\ast\), Ida and Ida') 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.
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. 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, PB-derived, or CB-derived 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, so far only indirect evidence of homing events in ischemic tissues, mostly based on tissue sectioning, has been provided.

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 micrometa-tases; 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 effect47 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 types10 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 zebrafish48 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.

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Disclosures
None.

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SUPPLEMENT MATERIAL

Expanded ethical statement

An Institutional Review Board formal approval for cord blood collection at Melzo Hospital was obtained to this aim (December 12, 2008; authorization no 843). Collection of peripheral blood was performed at Centro Cardiologico Monzino on a voluntary basis from healthy subjects. The experimentation involving the use of animals conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the Helsinki Declaration. Specific authorization to proceed with animal experimentation in the present study was obtained by Italian Ministry of Health (authorization no 08/2009) from the zebrafish Facility at IEO/IFOM campus.

Cells isolation and culture; flow cytometry

CD34+ cells were isolated using a magnetic isolation method (MINI-MACS, Miltenyi Biotech) already described by us 1. After diluting 1:3 freshly collected cord blood with PBS, whole blood was layered over Lymphoprep (1,077g/mL density) followed by centrifugation at 800xg for 30min at room temperature. Cord blood mononuclear cells (CBMNCs) were then collected, washed twice with PBS containing 2mM EDTA and 5% FBS and finally processed for CD34+ cells isolation and culture. MACS-isolated hCD34+ cells were maintained in culture for one day before injection into a serum-free medium (Stem Span, Stem Cell Technologies) supplemented with IL-3/6 (20 ng/ml), SCF and Flt3-L (both at 100 ng/ml).

For the isolation of CD14- cells, peripheral blood samples from healthy volunteers were collected. After dilution 1:3 with PBS, peripheral blood was loaded onto Lymphoprep for the peripheral blood mononuclear cell (PBMCs) separation. CD14- cells were isolated by negative selection using the CD14 Isolation MINI-MACS Kit (Miltenyi Biotech). After isolation and before culture, CD14- cells
were maintained in DMEM supplemented with 10% FCS for one day before injection. The immunophenotype of CD34⁺ and CD14⁺ cells was assessed by flow cytometry as follows.

**Immunophenotype of CD34⁺ cells at **T₀

Immunophenotype of CD34⁺ cells prior injection was assessed by incubating cells with 1-10µg specific Pacific Blue-CD3, FITC-CD34, APC-Cy7-CD45, Alexa-700-CD48, APC-KDR, APC-CD144, APC-CD14 and APC-CD31 antibodies. All antibodies were purchased from BD-Pharmingen except for CD48 and KDR that were purchased from Ex-Bio and R&D Systems, respectively. Mouse IgG conjugated with the same fluorochromes were used as isotype controls. Cells (1-5x10⁵) were incubated at RT for 15’ after, which they were washed and immediately analyzed by FACSAria high throughput cell sorter system (Beckton-Dickinson). 5x10⁴ events were scored for each sample.

**Immunophenotype of CD34⁺-derived cells at 28 and 48 hpf**

To assess the phenotype of human cells after blastula injection, Tg(*fli1*:EGFP)*y₁* embryos injected with Orange⁺hCD34⁺ cells were dissociated at 28 and 48 hpf by using a dissociation solution containing 0.1 mg/ml Collagenase IV and 1mM EDTA in Ca²⁺-free Ringer’s Solution, until reaching a homogeneous unicellular suspension. Cells were then filtered through a 70µm mesh filter and then labeled with the indicated antibodies under conditions as described above. The following gating strategy was adopted to recognize human-derived cells. In a first dot plot, the Orange tracker fluorescence was plotted against the EGFP fluorescence, to clearly discriminate human cells from embryos’ endothelial cells (Figure 3). The Orange⁺ cells were recognized by comparing the plots obtained from cells uninjected and cells-injected embryos (Figure 3). These cells were finally gated to analyze the expression of hematopoietic and endothelial markers in multicolor analysis. Given the small percentage (0.2%-0.5%) of human cells compared to cells of the host embryos, this analysis was performed by analyzing a minimum of 10⁵ total events.
Purity checking of CD14 cells

Flow cytometry was used to assess negative depletion of CD14\(^+\) cells from PBMNCs before labeling with Orange Tracker and injection into developing zebrafish embryos. To this purpose, a PE-labeled anti-CD14 antibody (Beckton Dickinson) was used (Figure I). hCD14\(^-\) cells were used as negative control as they are similar in morphology to hCD34\(^+\) cells but, unlike CD34\(^+\) cells and CD14\(^+\) monocytes, they do not behave as endothelial progenitor cells and exhibit no angiogenic activity\(^2\).

Isolation of CD34\(^+\)/CD45\(^-\) cells

Isolation of CD34\(^+\)/CD45\(^-\) cells was performed by a high throughput cell sorting method using FACS\textregistered Aria system. Briefly, hCD34\(^+\) cells were first isolated using MINI-MACS (see above) and then cultured for 2 days before sorting. On the day of sorting, CD45\(^-\) cells were separated from CD45\(^+\) by flow cytometry, after staining with an anti CD45-APC antibody. After sorting, cells were cultured for 5 additional days to let them grow in sufficient numbers to perform embryo injection. Before injection, these cells were labeled using Orange Tracker dye as described below.

Human cells transplantation; Vegf-C morpholino RNA injection

Embryos were collected by natural spawning and staged. Tg(\textit{fli1}:EGFP);\(^1\) blastula-stage embryos or tricaine (0.04 mg/mL, Sigma)-anesthetized 48 hpf stage embryos were injected with 4 nL PBS containing 100-500 Orange Tracker (Invitrogen) labeled CD34\(^+\) or CD14\(^-\) cells per embryo by using a FemtoJet Express Microinjector (Eppendorf), as described by Traver et al. (2003)\(^3\). Blastula-stage embryos were also injected with a 4 nL drop/embryo of PBS and 100-500 Orange Tracker labeled CD34\(^+\)/CD45\(^-\) cells, sorted from a freshly isolated CD34\(^+\) cell population as described above. Tg(\textit{fli1}:EGFP);\(^1\) CD34\(^+\)/CD45\(^-\) injected embryos were imaged by confocal microscopy in order to visualize human cells homing and embryonic vascular phenotype.
Tg(fli1:EGFP)$^{y1}$ blastula-stage embryos or tricaine (0.04 mg/mL, Sigma)-anesthetized 28 hpf stage embryos were injected with about 100 Orange Tracker labeled melanoma cancer cells (from primary culture) and CACO-2 cells respectively, then imaged by confocal microscopy (LSM710, Zeiss) 24h after injection. Furthermore, CACO-2 injected embryos were cultured until 7dpf and imaged in order to visualize ectopic vessels induced by cancer cells.

Zebrafish Vegf-c antisense morpholino (MO) RNA was injected into 1-4 cell stage transgenic Tg(fli1:EGFP)$^{y1}$ embryos, as described$^4$.

**Embryo sectioning, immunofluorescence and confocal microscopy**

**Embryo sectioning**

Zebrafish control and injected embryos at 72 hpf stage were fixed using 4% PFA for 2h at room temperature. 8 µm transversal frozen sections of OCT-embedded 4% PFA fixed embryos, were cut, immunostained and mounted. Images were acquired by confocal microscopy.

**Immunofluorescence**

Immunofluorescence on whole-mount embryos and frozen sections was performed by using polyclonal mouse and rabbit anti-human antibodies, diluted from 1:50 to 1:1000: CD45 (Abcam), KDR (Abcam), Endothelin (Abcam) and Ph-H3 (Bethyl Antibodies, TEMA). Goat anti-mouse and anti-rabbit Alexa Fluor 633 and 350 (Molecular Probes, Invitrogen), diluted 1:200, were used for the secondary staining. DAPI staining (Sigma) was performed for nuclear labeling where present. TUNEL assay was performed according to manufacturer’s instructions (Roche Applied Science).

**Confocal imaging**

Live injected Tg(fli1:EGFP)$^{y1}$ embryos were imaged either by a Leica stereo-microscope equipped with a digital camera and the Leica IM100™ software, or by a Leica TCSSP2AOBS confocal microscopy and a Leica software for time-lapse in vivo imaging, equipped with a Ar/Kr and a UV
laser (GFP: 488 nm filter for excitation and 500-600 nm AOBS filter for emission; Orange Cell Tracker dye: 594 nm filter for excitation and 600-700 nm AOBS filter for emission).

Injected and/or immunostained whole-mount embryos and embryo sections were imaged by the LSM710 Zeiss confocal inverted microscope and the Zeiss software Zen 2008, equipped with a Ar/Kr and a UV laser (GFP: 488 nm filter for excitation and 500-600 nm filter for emission; Orange Cell Tracker dye: 594 nm filter for excitation and 600-700 nm filter for emission; Alexa 633: 612 nm filter for excitation and 700 nm filter for emission).

To document EHT events during vascular development of hCD34+ injected embryos, we performed a 16h time-lapse imaging of live injected Tg(KDRI:EGFP)S843, 24h after blastula injection with a high magnification (20X) of the dorsal aorta region (1-12 somites), by using the LSM710 Zeiss confocal inverted microscope and the Zeiss software Zen 2008, equipped with the laser as described above.

Whole-mount alkaline phosphatase staining and in situ hybridization methods

**Alkaline phosphatase staining**

After fixation and dehydration using a methanol series, embryos were stained with a solution containing NBT/BCIP (Roche Applied Science).

**In situ hybridization**

In situ hybridization to detect Scl and Gata2 genes was performed as described in 5. Riboprobes were synthesized by using the T3/T7 MaxiScript kit (Ambion). Hybridization was performed in 50% formamide at 65°C for an overnight. After washing, color reaction was developed by BM purple (Roche Applied Science).

**TUNEL**
TUNEL staining was performed using the Fluorescein *In Situ* Cell Death Detection kit (TUNEL, Roche Applied Science).

**Fin regeneration model**

Regenerative angiogenesis experiments were performed on caudal fins of adult Tg(*fltl*:EGFP)*y1* zebrafish that were amputated at approximately 50% proximal/distal level. Amputated fish were kept individually, deprived of food, and were immunosuppressed using 0.02 µg/µl Cyclosporine A in fish water starting 1 day before the amputation throughout the experiment. The third day post-amputation, adults were injected intraperitoneally with vehicle or hCD34+ cells, and kept at 25°C for 7 additional days. Ten days post-amputation injected adults were sacrificed and whole regenerating caudal fins were cut and fixed in 4% PFA. Images of the regenerating fin rays were obtained by confocal microscopy, and angiogenesis was quantified by using AxioMeasure software (Zeiss). Quantification of the blood vessels regeneration in the fin rays of amputated fins was performed using the Axio Measure plugin in the AxioVision software (Zeiss). The areas of three fin rays occupied by green fluorescence was measured (in pixel²) over a fixed selected area in the LSM710 Zeiss confocal microscope field showing the amputation (see also figure 6A).
SUPPLEMENTARY REFERENCES


SUPPLEMENTARY FIGURES LEGENDS

Figure I. Purity checking of negatively selected human peripheral blood-derived CD14$^-$ cells. After negative selection by MINI-MACS, CD14$^-$ and CD14$^+$ cells were cultured for one day. They then were labelled with anti CD14-PE antibody and analyzed by flow cytometry. Panel on the top shows that the majority of CD14$^+$ cells (blue gate in physical dot plot) were retained in the MINI-MACS column, while CD14$^-$ cells (red gate in physical dot plots in panels on the bottom) were fairly pure and were not contaminated by cells expressing CD14.

Figure II. Proliferation and cell death of hCD34$^+$ labeled cells injected into the zebrafish blastula. Tg(fli1:EGFP)$^{y1}$ and wild-type (WT) embryos were injected with Orange Cell-Tracker labeled hCD34$^+$ cells, 24 hours after isolation from human cord blood. Transplanted embryos were cultured and PFA-fixed at 28 hpf stage of development. Whole-mount embryos are oriented with anterior to the right and dorsal to the top.

A) Whole-mount Ph-H3 immunofluorescence detected a low hCD34$^+$ cells proliferation rate (1.25±1.01% of injected cells). Pictures in the head and the trunk regions were taken at low (left panels) and high (right panels) magnification. Dashed boxes in the left panels indicate magnified regions in right panels. Green fluorescence represents the embryonic vessels in the Tg(fli1:EGFP)$^{y1}$ embryos; h: heart; e: eye; cvp: caudal vein plexus; da: dorsal artery; iv: intersegmental vessels. Arrows indicate Ph-H3$^+$ human cells.

B) TUNEL staining revealed only occasional hCD34$^+$ cells apoptosis (0.54±0.60% of injected cells). Pictures in the yolk sac and trunk regions were taken at low (left panels) and high (right panels) magnification. Dashed boxes in the left panels indicate magnified regions in right panels. Arrows indicate TUNEL$^+$ human cells in the magnified regions.
Figure III. Localization of blastulae-injected CD34⁺/CD45⁻ cells in the developing embryos at 28 hpf. High throughput sorted CD34⁺/CD45⁻ cells were injected at blastula stage. At this stage, Orange Tracker⁺ cells were observed in several vascular districts such as the head, the dorsal aorta (da), the dorsal lateral vein (dlv) and the caudal vein plexus (cvp) in proximity of intersegmental vessels (iv).

Figure IV. Injection of human cancer stem cells (CSCs) from melanoma primary culture and CACO-2 cell line.

(A) Cancer stem cells were injected at the blastula stage (top panel) or in the Cuvier’s duct after gastrulation (bottom panel). Note the presence in both cases of red-labelled CSCs in the yolk and the caudal vein plexus regions, where these cells formed micro-metastases.

(B). Angiogenic sprouting in the same embryo depicted in bottom panel in A at 7 days post fertilization (7 dpf). The CSCs were not present at this stage likely due to rejection by the host immune system. However, the presence of CACO-2 cells in the yolk region caused an evident ectopic vessel sprouting (arrows), as evidenced by the presence of an expanded vascular network localized ventrally to the yolk in this embryo (right panels). Left panels indicate the vascularisation of the yolk in an uninjected embryo at a comparable developmental stage.

Figure V. Immunophenotype of hCD34⁺ cells before injection into developing blastulae. Cells were analyzed for the expression of the indicated hematopoietic or endothelial markers by flow cytometry one day after isolation. The dot plots at the top of each panel indicate the physical parameters of cells that were gated to analyze the expression of hematopoietic and endothelial cells markers. The histogram plots show the marker positive and negative for each marker. Gate was established by staining with isotype control antibodies.
Figure VI. Flow cytometry analysis of CD4 and CD8 markers expression in hCD34+ cells before (T0 CD34+) and after injection into developing blastulae. None of these markers was expressed at all time points. Logical gating strategy applied to recognize human cells in suspensions obtained from enzymatically dissociating the embryos is the same as that used in Figure 3.

Figure VII. EHT imaging in a Tg(KDR:EGFP)S843 transgenic embryo transplanted with hCD34+ cells at blastula stage. The four panels are four subsequent time frames of the same 16 hrs time lapse experiment, started at 24hpf (see also Movie 1). The dashed square indicates a region of the dorsal aorta where budding of a KDR+ HSCs (*) appears to emerge from the hemogenic endothelium (arrow).

Figure VIII. Homing of hCD34+ cells in the vasculature of post-gastrulation stages zebrafish embryos. Tg(fli1:EGFP)y1 and wild-type (WT) embryos received hCD34+ cells into the Cuvier duct at 48 hpf stage. Transplanted embryos were cultured until 72 hpf stage.

A) Two-hour confocal microscopy tracking of circulating red-labeled hCD34+ cells, immediately after transplantation into a Tg(fli1:EGFP)y1 embryo. The four images represent the 12, 24, 48 and 110 minute time frames, respectively. hCD34+ cells produced early and late homing events as shown by the presence of red cells associated to the vasculature as early as 12 minutes after injection (dashed squares) and at later time points (dashed circles). For further details about circulation, rolling and homing of hCD34+ cells in the vasculature at post gastrulation stages see also Movie 1 in the supplementary material. Embryos are oriented with the anterior on the top left and posterior to the lower right. Da: dorsal aorta/dorsal artery; iv: intersegmental vessels.

B) Injection of unlabeled hCD34+ cells into the zebrafish embryo peripheral circulation caused vascular abnormalities in the tail vasculature. These defects were never observed in vehicle-injected embryos. Upper panels show the tail region of an embryo injected with vehicle only (low magnification in the left and high magnification in the right upper panels). The lower panel shows
the tail region of a hCD34⁺ cells injected embryo; ectopic vessels sprouting is apparent between the dorsal artery and the intersegmental vessels. Embryos are oriented with the anterior to the right and posterior to the left. cvp: caudal vein plexus; da: dorsal artery; dlv: dorsal longitudinal vessels; iv: intersegmental vessels; pcv: posterior cardinal vein.

C) Stereoscope imaging of whole-mount alkaline phosphatase staining at the trunk-yolk region in one vehicle-injected post-gastrulation WT embryo (upper panel) and one injected with unlabeled hCD34⁺ cells (lower panel). Note the abnormal development of the subintestinal vein (siv) in the embryo transplanted with human cells, compared to that injected with vehicle only. Embryos are oriented with the anterior to the right and posterior to the left.

D) Confocal microscope imaging of the tail region in a post-gastrulation embryo injected with vehicle only (upper panel) or orange tracker labeled CD14⁻ cells (lower panel). hCD14⁻ cells did not preferentially home into the vasculature and were never associated to ectopic vessels sprouting. Embryos are oriented with the anterior to the right. iv: intersomitic vessels; da: dorsal aorta.

**Figure IX. Imaging of ectopic vessel sprouting in embryos injected at post-gastrulation stages.**

High magnification fields from (2-12h) confocal *in vivo* videomicroscopy (see also Movie 2 in supplementary material) of tail region in a Tg(*fli1:*EGFP)y1 embryo, injected with hCD34⁺ cells at 48 hpf, showing an altered secondary blood vessel associated to human cells localization. Arrows indicate a hCD34⁺ cells cluster located in the forming dorsal longitudinal vessel (dlv); dashed squares indicate a single hCD34⁺ cell in an intersegmental vessel (iv). Note the presence of an abnormally developed branching between the dorsal lateral vein (dlv) and two intersegmental vessels (iv). The embryo is oriented with anterior to the right and dorsal to the top.

**Movie 1. Time lapse experiment in a hCD34+ cells blastula-injected Tg(KDRI:EGFP)S843 started at 24hpf.** A budding of a KDR⁺ HSCs appears to emerge from the hemogenic endothelium located in the wall of the dorsal aorta.
**Movie 2.** Short time (0-2 hrs) confocal videomicroscopy recording showing in real time circulation, rolling and homing events of red-labeled hCD34<sup>+</sup> cells into the embryonic vasculature, recognized by green fluorescence. Representative time frames from this video are also shown in Figure 5A.

**Movie 3.** Long time (2-12 hrs) confocal videomicroscopy recording showing abnormal growth of a branch between dorsal lateral vein and intersegmental vessels in proximity with a hCD34<sup>+</sup> cells cluster. Representative time frames from this video are also shown in Figure V.
FIGURE I
FIGURE II
FIGURE III
FIGURE IV

A

28 hpf  
Blastula injection  
Fl1-EGFP  

Melanoma CSCs

48 hpf  
Cuvier duct injection  
Fl1-EGFP  

CACO2 CSCs

B

7 dpf  
Uninjected  

7 dpf  
CACO2 CSCs

7 dpf  
Uninjected  

7 dpf  
CACO2 CSCs
FIGURE V
**FIGURE VI**

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**T0 CD34⁺**

**Fli1-EGFP**

**Fli1-EGFP hCD34⁺**

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FIGURE VII
FIGURE VIII
FIGURE IX
Summary

그 동안의 내피전구세포 등을 비롯한 심혈관계 전구세포에 대한 집중적인 연구에도 불구하고 인간 유래 조혈전구세포(hematopoietic progenitor)가 태생기후 혈관조성(postnatal vascularization)에 어떠한 역할을 갖는지는 아직도 불분명한 상태이다. 더구나, 허혈 손상이 없는 태생기의 정상 심혈관계 발생에 조혈전구세포가 신생혈관조성(neovascularization)에 참여하는지 그리고 태아기에 발생하는 혈관망에 조혈전구세포가 직접적으로 포함되지는 않으면 측분비 효과(paracrine action)를 나타내는지도 불분명하다. 이번 연구에서는 인간 제대혈 유래 CD34\(^{+}\) 세포를 zebrafish의 장배형성기(gastrulation) 전과 후, 그리고, 성체 혈관재생의 형태인 꼬리지느러미 절단 모델에서 현관 재생 효과를 갖는 것을 밝혔다. 이 결과들은 인간 유래 조혈전구세포가 심혈관계 발생에 상당한 역할을 하며 내피전구세포로의 분화에 분자경로의 진화적 보존을 뒷받침하는 단서가 된다.
골수, 말초혈액, 그리고 제대혈에 존재하는 일부의 세포가 내피세포로 분화되며, 이는 HSC(hematopoietic stem cell)와 발생학적 연관이 있음이 밝혀져 있다. 그리고, CD34와 CD133를 보이는 세포들이 체내, 외에서 성숙한 내피세포 표지자를 갖는 세포로 분화되어 이러한 세포들을 EPC(endothelial progenitor cell)로 부르게 되었다. 하지만, 최근의 결과에서 EPC가 조혈전구세포 유래와 그렇지 않은 세포로 나눌 수 있다는 것이 밝혀졌고, 이는 전, 후기 EPC로 불릴게 되었으며, 또한, CFU-EC(colony-forming unit-endothelial cell)와 ECFC(endothelial colony-forming cell)로 불러지기도 한다. 이들은 기본적으로 조혈세포 표지자인 CD45의 발현여부와 내피세포로의 분화력에 의하여 분류된다. 조혈세포와 내피세포계로의 분리는 생쥐와 zebrafish의 발생에서 배측 대동맥(dorsal aorta endothelium)에 위치한 원시세포가 비대칭 분리를 보이는 EHT(endothelial-hematopoietic transition) 동안에 나타나게 된다. 더구나, 조혈절기세포에서 유래된 세포와 내피조직세포가 같은 혈관조성능력을 베어가와 출생 후에 갖는지 그리고 제대유래 CD34와 CD38의 진정성에서 얻어진 내피세포가 베어에게 어떠한 역할을 하는지도 불분명한 상태이다.

이번 연구자들은 hCD34+ 세포를 zebrafish의 장배행성기 전, 후와 성체 신생혈관조성 모델인 꼬리지느라미 절단 모델에 주입하여 신생혈관에 미치는 영향을 검증하였다. Zebrafish의 중기포배의 신생혈관의 생기로 hCD34+ 세포를 포배기에 주입하면 IL-8, IL-1ra, MIP-1, G-CSF, GM-CSF, VEGF 등의 다양한 interleukin, cytokine, chemokine를 생성하여 강력한 혈관신생 효과를 측정한다(Figure. 1). 이러한 다양한 혈관신생 효과는 바로 생후 7일령의 zebrafish 성체의 꼬리지느라미 절단 모델에 만든 이후 hCD34+ 세포를 주입하면 신생혈관이 대조군에 비하여 현격하게 증가하는 것을 알 수 있다. 결론적으로, 인간 제대 유래 세포 중 조혈전구세포 표지자인 CD34+ 세포는 내피세포로의 분화력을 보이며 이는 zebrafish의 HSC의 연관된 직접적인 분
화력과 다양한 조혈인자와의 물리학적 상호작용으로 배아의 심혈관계 신생에 영향을 미치고 성체에서도 심혈관계 조절능을 보여주는 이는 조혈세포로서의 reprogramming과의 무관하게 일어난다. 이는 임신 용기에 발생하는 심혈관계의 발생 이상을 줄기세포의 주입으로 교정할 수 있다는 가능성을 보여준다.

REFERENCES
Endothelial Fate and Angiogenic Properties of Human CD34\(^+\) Progenitor Cells in Zebrafish

Ombretta Pozzoli, Pietro Vella, Grazia Iaffaldano, Valeria Parente, Paolo Devanna, Marta Lacovich, Carla Lora Lamia, Umberto Fascio, Daniela Longoni, Franco Cotelli, Maurizio C. Capogrossi, Maurizio Pesce

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 ScI 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
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. The objective of the present work was to explore the developmental potential 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(KDR:enhanced green fluorescent protein [EGFP])S843 (ZIRC, University of Oregon), and the Tg(fli1:EGFP)K41 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
Blotnia stage or tricaine (0.04 mg/mL, Sigma) anesthetized 48 hours post fertilization (hpf) stage AB or Tg(fli1:EGFP)K41 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 (Kliik, Biopica), 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 Blotnia
Orange Cell Tracker–labeled hCD34+ cells were transplanted in Tg(fli1:EGFP)K41 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

![Image of Figure 1](http://atvb.ahajournals.org/Downloaded from)
mesenchyme, and the intermediate cell mass regions (Figure 1A). In situ hybridization using riboprobes to detect Sc1 and Gata2 mRNAs were then performed. The results revealed a strict association of human cells with multipotent Sc1" 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 Sc1" 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 IV). 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 panhematopoietic 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 postgasturalation 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). 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)y5843 embryos injected at the blastula stage with CD34+ cells was
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 VIII A). 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, i.e., 24 hours after injection. Altered blood vessel sprouting in the growing tail vasculature (85% of 72 transplanted embryos; Supplemental Figure VIII B) and at the level of the subintestinal vein (80% of 17 injected embryos; Supplemental Figure VIII C) were observed. Angiogenesis abnormalities were never found after injection of PB-derived hCD14+ cells (Supplemental Figure VIII D) but were detected in embryos transplanted with the intestinal cancer cell line CACO2 (Supplemental Figure IV B).

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 arteries25 (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 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) and 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.
hCD34<sup>+</sup> Cells Accelerate Vascular Repair in Adult Zebrafish

The ability of hCD34<sup>+</sup> cells to enhance vascular repair in mammalian models of myocardial infarction and limb ischemia has been shown by us<sup>26,27</sup> and by others.<sup>28–33</sup> In the present study, we examined whether hCD34<sup>+</sup> cells contributed to vascular repair in adult zebrafish by injecting these cells in immunosuppressed Tg(fli1:EGFP)<sup>Y1</sup> adult animals after caudal fin amputation (Figure 6A), an established model of vascular regeneration in zebrafish.<sup>34–36</sup> A significant increase in angiogenesis in hCD34<sup>+</sup> 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.<sup>1</sup> 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,<sup>37</sup> whence definitive HSCs emerge by a novel modality of asymmetrical division of pluripotent nonhematopoietic cells, the so-called hemogenic endothelium.<sup>13,14</sup> This division modality has been characterized in the zebrafish dorsal aorta and has been called EHT.<sup>13</sup> In the adulthood, the existence of 2 EPC types, namely CFU-ECs (early EPCs) and ECFCs (late EPCs),<sup>7</sup> 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 panhematopoietic marker CD45 in CFU-ECs but not in ECFCs,<sup>4</sup> 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.<sup>7,8,10</sup>

According to this definition of EPCs,<sup>4</sup> progenitors obtained by bone marrow, PB, or CB cell sorting with CD34, CD133,
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 Vegf-c expression.

**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::EGFP)\(^{11}\) embryos. A, Low-power views of a control (Fli-EGFP) and 2 Vegf-c MO RNA-injected embryos (Fli-EGFP\(\Delta\)VEGF-C). Note the absence of the bifurcation between the 2 lateral dorsal aorta branches in midpanel (\(\ast\)) and the irregular spacing of the intersomitic vessels (arrows), caused by absence of.
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.\textsuperscript{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\textsuperscript{+} Cells in the Zebrafish Embryo**

The angiogenic activity of human bone marrow-derived,\textsuperscript{2} PB-derived,\textsuperscript{44} or CB-derived\textsuperscript{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,\textsuperscript{45} so far only indirect evidence of homing events in ischemic tissues, mostly based on tissue sectioning, has been provided.\textsuperscript{26}

The results of our hCD34\textsuperscript{+} 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\textsuperscript{+} cells was observed in several districts of the vasculature; this was different from the behavior of the human PB-derived CD14\textsuperscript{+} cells, which were used as a negative control (Supplemental Figure VIII), or that of human melanoma and

### Table. Quantification of Human Cytokines in Culture Medium Conditioned by hCD34\textsuperscript{+} Cells

<table>
<thead>
<tr>
<th>Factor (pg/ml per 10\textsuperscript{6} Cells)</th>
<th>Mean</th>
<th>SE (n=4)</th>
</tr>
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<tbody>
<tr>
<td><strong>Interleukins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hu IL-1ra</td>
<td>25.5</td>
<td>14</td>
</tr>
<tr>
<td>Hu IL-5</td>
<td>13</td>
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<tr>
<td>Hu IL-9</td>
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<tr>
<td>Hu IL-8</td>
<td>7220</td>
<td>2468</td>
</tr>
<tr>
<td>Hu IL-10</td>
<td>3.0</td>
<td>1.8</td>
</tr>
<tr>
<td>Hu IL-12</td>
<td>1.1</td>
<td>0.76</td>
</tr>
<tr>
<td>Hu IL-13</td>
<td>11.2</td>
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</tr>
<tr>
<td>Hu IL-15</td>
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</tr>
<tr>
<td>Hu IL-17</td>
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<tr>
<td><strong>Chemokines</strong></td>
<td></td>
<td></td>
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<tr>
<td>Hu IP-10</td>
<td>196</td>
<td>103</td>
</tr>
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<tr>
<td>Hu MIP-1b</td>
<td>419</td>
<td>271</td>
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<tr>
<td>Hu RANTES</td>
<td>72.5</td>
<td>45</td>
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<tr>
<td><strong>Proangiogenic factors</strong></td>
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<tr>
<td>Hu PDGF-bb</td>
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<td>Hu G-CSF</td>
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</tr>
<tr>
<td>Hu VEGF</td>
<td>19.1</td>
<td>18</td>
</tr>
</tbody>
</table>

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.
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). 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 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 an open issue. Although the distinction between the 2 EPC types 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 and the recent demonstration of angiogenic activity of mouse and human tumor cell lines, 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. 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. 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.

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