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Generation of Hematopoietic Stem Cells from Purified Embryonic Endothelial Cells by a Simple and Efficient Strategy

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ABSTRACT

Recent progress by versatile approaches supports the new hypothesis that multi-potent hematopoietic stem cells (HSCs) are directly formed from a rare population of endothelial cells in mid-gestation mouse embryos. This process is therefore known as the endothelial-to-hematopoietic transition (EHT). Nevertheless, there is no functional evidence that documents the HSC transition from purified endothelial cells. In this study, we developed an OP9-DL1-based co-culture system that was able to facilitate the HSC specification and/or expansion *in vitro* of mouse embryonic day 10.5 (E10.5) Tie2^{+} cells remarkably. Then, the immunophenotypically defined endothelial cells were harvested by a combination of surface markers (Flk1^{+}CD31^{+}CD41^{-}CD45^{-}Ter119^{-}) from the caudal half of E10.0−E11.0 mouse embryos. The transplantation of the endothelia/OP9-DL1 co-cultures led to long-term, high-level, multi-lineage, and multi-organ hematopoietic reconstitution in the irradiated adult recipients. The induced HSC activity was initially observed at E10.5, and a significant increase was detected at E11.0, which suggests a temporally specific regulation. Taken together, for the first time, we provide functional evidence showing the HSC potential of purified embryonic endothelial cells, which is indispensable for the emerging EHT concept. Moreover, the newly defined co-culture system will aid the exploration of the key molecules governing the HSC transition from embryonic and even postnatal endothelial cells, which has enormous significance in basic and translational research.

KEYWORDS: Hematopoietic stem cells; Endothelial-to-hematopoietic transition; Endothelial cells; Aorta−gonad−mesonephros region

INTRODUCTION

Hematopoietic stem cells (HSCs) are the foundation of the blood system throughout the lifetime of an individual. Bona fide HSCs are able to provide all types of functional blood cells through multi-lineage differentiation and to simultaneously maintain the stem cell pool homeostasis through asymmetric self-renewing. In recent years, research examining HSC development has become increasingly important because a clear understanding of its physiological ontogeny will significantly facilitate the advancement of blood regeneration and clinical applications. Using a standard HSC transplantation assay, the canonical HSCs, as defined by their long-term, high-level, and multi-lineage ability to reconstitute the hematopoietic system of irradiated immunocompetent adult recipients, have been found to develop in the embryonic day 10.5−11.0 (E10.5−E11.0, 36−45 somite pairs, sp) aorta−gonad−mesonephros (AGM) region, head, yolk sac, and placenta, although the total number of HSCs is very low (Muller et al., 1994; Kumaravelu et al., 2002; Gekas et al., 2005; Medvinsky et al., 2011; Li et al., 2012). In comparison to other sites at E10.5, the HSCs that reside in the AGM region can repopulate the hematopoietic system of recipients

Abbreviations: AGM, aorta−gonad−mesonephros; EHT, endothelial-to-hematopoietic transition; FL, Flt3 ligand; HSCs, hematopoietic stem cells; IL-3, interleukin-3; sp, somite pairs; SCF, stem cell factor.

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more efficiently (Muller et al., 1994; Kumaravelu et al., 2002; Gekas et al., 2005). Interestingly, the E10.5 AGM region can autonomously generate a large number of HSCs by organ culture at the air/liquid interface, which has not been observed in the yolk sac and placenta (Medvinsky and Dzierzak, 1996; Robin et al., 2006). Furthermore, the addition of interleukin-3 (IL-3) to the organ culture medium allows the detection of HSC activity earlier in the E10.0 AGM region (30−34 sp), at which time HSCs cannot be found through direct transplantation (Robin et al., 2006).

For many years, the identity of the HSC precursor in developing mammalian embryos has been controversial (Dzierzak and Speck, 2008). New technologies have been continuously used to investigate the morphological, functional, and molecular aspects at the key times of initial HSC emergence in rapidly growing embryos (Eilken et al., 2009; Bertrand et al., 2010; Boisset et al., 2010; Kissa and Herbomel, 2010; Medvinsky et al., 2011). A significant body of evidence obtained through multifaceted approaches strongly suggests that HSCs may emerge directly from the endothelial cells in specific vascular beds through a dynamic process called endothelial-to-hematopoietic transition (EHT) (Medvinsky et al., 2011). First, the unique cellular structure of intra-aortic hematopoietic clusters may reflect the stepwise budding of blood cells from endothelial layers of the dorsal aorta (North et al., 1999; de Bruijn et al., 2002; North et al., 2002; Yokomizo and Dzierzak, 2010). Importantly, this speculated EHT *in vivo* has been largely recapitulated through live imaging of non-fixed AGM tissue slices *in vitro* (Boisset et al., 2010). Second, the endothelial cells purified using a surface marker combination from different hematopoietic locations can generate erythromyeloid cells and lymphoid cells (B and T cells) *in vitro* through their co-culture with stromal cells (Matsubara et al., 2005; Yoshimoto et al., 2011, 2012; Li et al., 2012). Third, as determined through an inducible Cre recombinase-based fate mapping assay, the embryonic cells with vascular endothelial (VE)-cadherin promoter activity (mainly endothelial cells) were documented to contribute to the adult hematopoietic system, including HSCs (Zovein et al., 2008). Fourth, in principle, the course of EHT may have some specific regulatory mechanisms that differ greatly from those of hematopoiesis. This paradigm was first established by Speck and colleagues, who showed that Runx1 is transiently required in the EHT (using VE-cadherin-Cre to mark the earlier endothelial stage) but not thereafter in hematopoiesis (using Vav-Cre to mark the later hematopoietic stage) (Chen et al., 2009; Tober et al., 2013). Conceivably, regarding the new concept of EHT, the most important outcome of the transition should be HSCs rather than any other type of hematopoietic cells. Success has been achieved in the induction of $CD41^+CD45^-$ or $CD45^+$ pre-HSCs into HSCs through a re-aggregation or co-aggregation system (Taoudi et al., 2008; Rybtsov et al., 2011). However, there is no functional evidence proving that the functional HSCs can be generated from accurately defined endothelial cells *in vitro*.

In this study, we first established a simple and reliable system, namely, the OP9-DL1 stromal cell co-culture, to allow efficient HSC maturation and/or expansion *in vitro*. More importantly, using this system, a successful endothelial-to-HSC transition was realized *in vitro*, which will be notably useful for the dissection of the genetic control of this process and the exploration of new strategies to reprogram HSCs from endothelial cells more efficiently.

RESULTS

OP9-DL1 stromal cell co-culture significantly increased HSC activity of $Tie2^+$ cells in E10.5−E11.0 embryos

Previous reports have indicated that the HSC potential in the E10.5−E11.5 AGM region can only be maintained or amplified *in vitro* through an organ, re-aggregation, or co-aggregation culture strategy (Medvinsky and Dzierzak, 1996; Taoudi et al., 2008; Rybtsov et al., 2011). A common feature of these methods is the placement of a three-dimensional structure at an air/liquid interface. In the present study, we determined whether a routine OP9-DL1 stromal cell co-culture can significantly support pre-HSC maturation and/or HSC self-renewal *in vitro*. As shown in Fig. 1A, the E10.5 caudal half-derived $Tie2^+$ cells, which predominantly include hematopoietic precursors and endothelial cells, were sorted by magnetic-activated cell sorting (MACS) from GFP^+ embryos and then co-cultured with OP9-DL1 in media supplemented with stem cell factor (SCF), Flt3 ligand (FL), and IL-3, similar to the re-aggregation system described previously (Taoudi et al., 2008). After 7 days, the semi-adherent cells were collected by manual pipetting and transplanted into irradiated adult mice. To analyze the short- and long-term hematopoietic repopulation, the peripheral blood of the recipients was bled 1 month and 4 months post transplantation, respectively. According to the formula $(GFP^+CD45^+)/(CD45^+GFP^- + CD45^+GFP^+)$, the hematopoietic chimerism of the donor-derived cells was calculated, and those with a chimerism of at least 10% were considered a successful repopulation. Expectedly, as a control, the transplantation of cultured OP9-DL1 cells never resulted in any reconstitution signals in recipients (data not shown). As shown in Fig. 1B, after infusion with the culture-expanded cells from the E10.5 caudal half $Tie2^+$ population of two embryo equivalents (ee), all of the adult recipients (7/7) were reconstituted after 1 month with an average chimerism of 89.7% ± 2.6%. After 4 months, the average chimerism was 77.6% ± 18.0%. Similarly, for E11.0 embryos, after the infusion of culture-expanded cells of one ee, all of the recipients (7/7) were repopulated successfully with an average short-term chimerism of 89.7% ± 1.6% (1 month) and an average long-term chimerism of 85.2% ± 8.0% (4 months) (Table 1 and Fig. 1B). Moreover, the multi-lineage potential of the donor-derived cells in the peripheral blood of the recipients was analyzed by flow cytometer. The transplantation assay with E10.5 embryonic cells revealed GFP^+ cells in the myeloid ($Gr\text{-}1^+/Mac\text{-}1^+$), B lymphoid ($B220^+$), and T lymphoid ($CD3^+$) lineages (Fig. 1C). Compared with the results from the direct transplantation of E10.5 (36−40 sp) AGM cells in

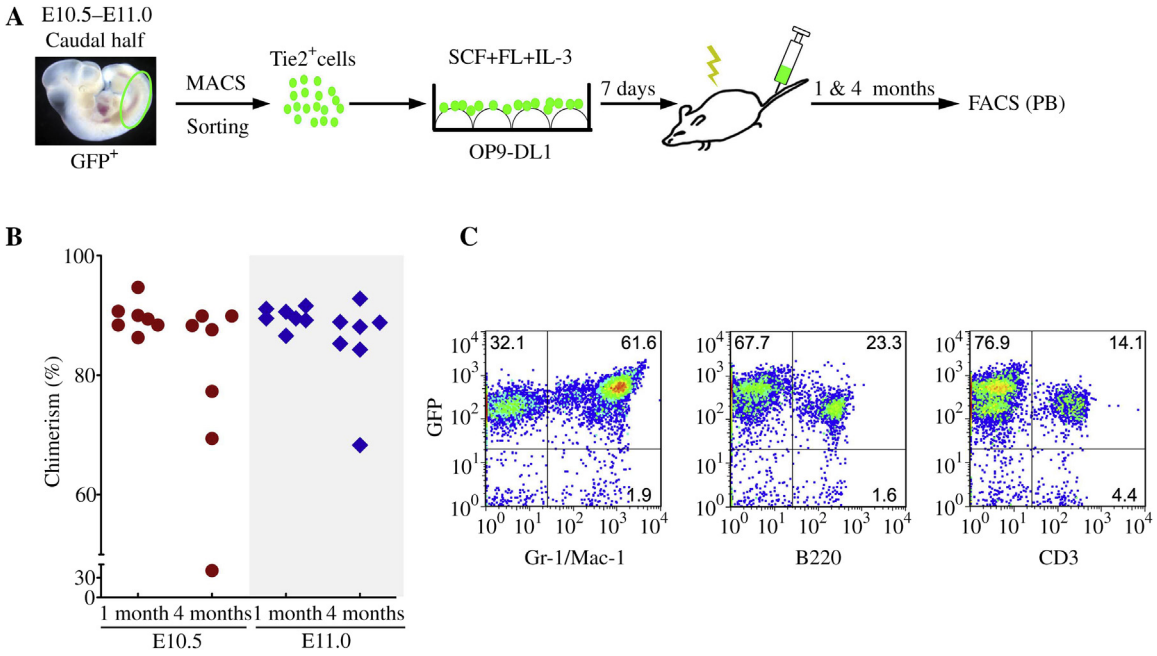

Fig. 1. Increased HSC activity of embryonic Tie2$^+$ cells after co-culture with OP9-DL1 stromal cells.

A: experimental design. **B:** donor-derived chimerism in recipients transplanted with the co-cultures of OP9-DL1 and Tie2$^+$ cells from the E10.5 (36−40 sp) or E11.0 (41−44 sp) caudal half. The symbols represent the donor chimerism in the peripheral blood of individual recipients 1 month or 4 months post transplantation. **C:** representative data of the multi-lineage reconstitution of the peripheral blood of the recipients repopulated by E10.5 co-cultured cells. MACS, magnetic-activated cell sorting; FACS, fluorescence-activated cell sorting; PB, peripheral blood.

our previous work (Li et al., 2012), the repopulation rate (100% *vs.* 29.4%) and the average chimerism (77.6% *vs.* 40.7%) after incubation with OP9-DL1 were significantly enhanced, which indicates increased HSC activity through the amplification of the existing HSCs, the maturation of pre-HSCs, or the endothelial to HSC transition.

Generation of adult-repopulating HSCs from stringently defined endothelial cells *in vitro*

To further test the HSC potential of precisely defined endothelial cells, a set of surface markers was used for flow cytometric purification. According to the experimental schematic diagram (Fig. 2A), the immunophenotypically defined endothelial cells from the E10.5−E11.0 caudal half, which are negative for standard hematopoietic markers and positive for an endothelial marker (CD31$^+$CD41$^-$CD45$^-$Ter119$^-$), were fractionated and co-cultured with OP9-DL1. The derivatives could give rise to standard HSCs with robust multi-lineage differentiation and self-renewal capacity in irradiated recipients, like those from Tie2$^+$ cells (data not shown). Interestingly, an incubation period of 4 to 6 days was sufficient to induce HSC-like potential in the endothelial cells. Of note, the pre-HSCs, which were purified by CD31$^+$CD41$^+$CD45$^-$Ter119$^-$ in parallel, were capable of maturing into functional HSCs efficiently (data not shown). Unlike CD31, which is expressed in both endothelial and intra-aortic blood cells, the expression of Flk1 is more restricted to the endothelial lining of the dorsal aorta, as revealed by three-dimensional confocal imaging (Yokomizo and Dzierzak, 2010). Therefore, the Flk1 antibody was further included in the combination to purify the endothelial cells more stringently. In accordance with the strategy shown in Fig. 2A, we harvested the endothelial cells by fluorescence-activated cell sorting (FACS) using a cocktail of antibodies (Flk1$^+$CD31$^+$CD41$^-$CD45$^-$Ter119$^-$) from the E10.0 (33−35 sp), E10.5 (36−40 sp), and E11.0 (41−44 sp) caudal half of CD45.1/2 embryos (Fig. 2B). The frequencies of the endothelial population in the E10.0, E10.5, and E11.0 caudal half were 0.8%−1.1%, 1.1%−1.3%, and 1.2%−1.7%, respectively. After a 4-day co-culture with OP9-DL1, the round semi-adherent cells were harvested and injected into irradiated CD45.2/2 mice. The short- and long-term chimerism was analyzed 1 month and 6 months after transplantation, respectively. As shown in Table 1 and Fig. 2C, the transplantation of E10.0 caudal half-derived cells failed to give rise to short- or long-term hematopoietic repopulation (0/8, three independent experiments). However, the injection of 2−2.3 ee of E10.5 caudal half-derived cells led to a somewhat hematopoietic repopulation. One of a total of 13 recipients (five independent experiments) exhibited a chimerism of 11.8% 1 month after transplantation, but this chimerism decreased to 4.9% after 6 months. Interestingly, the transplantation of co-cultured endothelial cells from 2 ee of the E11.0 (41−44 sp) caudal half resulted in a repopulation of 50% of the recipients (2/4) with efficient short- (46.0% and 27.7%) and long-term chimerism (48.2% and 51.8%). A similar repopulation occurred in myeloid (Gr-1$^+$/Mac-1$^+$), B lymphoid (B220$^+$), and T lymphoid (CD3$^+$) lineages in the peripheral blood of both recipients (Fig. 2D). Similarly, efficient engraftment was

Table 1
Adult-repopulating HSC activity of cells from E10.0−E11.0 mouse embryos co-cultured with OP9-DL1

Cell type	Stage[a]	ee[b]	Experiment (*n*)	>1 month		>4 months	
				Re[c]/total chimerism (%)		Re/total chimerism (%)	
Tie2$^+$ cells	E10.5	2.0	2	7/7	89.7 ± 2.6	7/7	77.6 ± 18.0
	E11.0	1.0	1	7/7	89.7 ± 1.6	7/7	85.2 ± 8.0
Endothelial cells[d]	E10.0	1.3−2.0	3	0/8	NA[e]	0/8	NA
	E10.5	2.0−2.3	5	1/13	11.8	0/10	NA
	E11.0	1.5−2.0	2	2/4	46.0, 27.7	2/4	48.2, 51.8

[a] Stage, E10.0, 33−35 sp; E10.5, 36−40 sp; E11.0, 41−44 sp; [b] ee, embryo equivalents; [c] Re, repopulated; [d] Endothelial cells, purified as Flk1$^+$CD31$^+$CD41$^-$CD45$^-$Ter119$^-$ cells in this study; [e] NA, not available.

observed in the bone marrow, spleen, and thymus (Fig. 2D). Furthermore, the transplantation of bone marrow cells from the repopulated recipients into secondary recipients led to marked chimerism that ranged from 31.8% to 43.2%, which suggests the self-renewal capacity of endothelia-derived HSCs (data not shown). Therefore, the results suggest that the culture conditions facilitated the formation of adult-repopulating HSCs from embryonic endothelial cells *in vitro*.

DISCUSSION

The validation of functional HSCs transitioned from purified endothelial cells is pivotal for the understanding of the EHT concept. In the absence of appropriate inductive signals, the putative hemogenic endothelial cells appear to be non-hematopoietic, as determined by their inability to form hematopoietic colonies in a CFU-C assay (our unpublished data) and their inability to repopulate recipients' hematopoietic system by direct transplantation (Matsubara et al., 2005). These features further indicate that these cells are not within the specified hematopoietic hierarchy, and also unlike the specifying CD41$^+$CD45$^-$ population. In contrast, in the presence of stromal cells that mostly belong to the mesenchymal lineages, these endothelial populations demonstrate robust growth and differentiation toward various lympho-myeloid cells *in vitro*, despite their lack of HSC-like potential *in vivo*. In the current long-term transplantation setting, the putative HSCs, particularly those that were transitioned immediately from non-hematopoietic endothelial cells *in vitro*, should exhibit superior abilities, such as homing, survival, proliferation, and differentiation, to become bona fide HSCs *in vivo*. Fortunately, our current strategy using OP9-DL1 plus a cytokine cocktail may largely resemble the *in vivo* niche that confers HSC-like potential to hemogenic endothelial cells, which constitute a very small population of the endothelial compartment. The close interactions within the co-cultures *in vitro* may mimic, to some extent, those interactions between the endothelial layer and the surrounding perivascular cells of the dorsal aorta *in vivo*. Our group is currently intensively investigating whether the purified endothelial cells from other hemogenic sites, such as yolk sac, head, and placenta, have similar HSC potential.

Of note, HSC maturation in mid-gestation embryos is stepwise, and the endothelial cells may be the starting population. To precisely define the heterogeneous HSC-related populations in different maturity states, Medvinsky and colleagues established a novel and efficient co-aggregation method to promote HSC maturation (Taoudi et al., 2008; Rybtsov et al., 2011). As a result, two types of pre-HSCs have been unambiguously recognized: type I (VE-cadherin$^+$CD41$^+$CD45$^-$) and type II (VE-cadherin$^+$CD45$^+$). The expression of hematopoietic markers in both types may indicate that these pre-HSCs are specifying or specified hematopoietic precursors and thus distinct from endothelial cells in nature. Interestingly, type II pre-HSCs are intermediates of the maturation of type I pre-HSCs into functional HSCs. In fact, the OP9-DL1 co-culture system also efficiently promotes the maturation of pre-HSCs, such as the CD31$^+$CD41$^+$CD45$^-$ population in E10.5 embryos (Rybtsov et al., 2011). However, the efficiency of inducing HSCs from endothelial cells appears to be lower than that achieved from CD41$^+$ pre-HSCs (data not shown), which implies that more signals are required for the endothelial-HSC transition. Future efforts are still needed to delineate the hierarchical relationship between these populations, which will be the prerequisite for the dissection of the critical mechanisms of HSC specification.

Together with previous knowledge regarding the parallel development of intermediate pre-HSCs (Taoudi et al., 2008; Rybtsov et al., 2011) and mature HSCs (Muller et al., 1994; Kumaravelu et al., 2002; Gekas et al., 2005; Li et al., 2012), in this study, we demonstrate similar developmental kinetics of HSC-competent endothelial cells in E10.5−E11.0 embryos, which become detectable in the presence of appropriate supportive signals (Fig. 3). Within the adult hematopoietic hierarchy, all of the progenitor and mature cells are differentiated stepwise from the multi-potent HSCs. In contrast, during mouse embryogenesis, hematopoietic progenitors emerge at E8.5 initially, followed by HSC generation 2 days later, suggesting a separated or independent developmental kinetics. Interestingly, the time-course of hemogenic potential of endothelial cells, based on previous literatures and this study, appears very consistent with that of hematopoietic capacity. It remains very interesting to investigate the distinct identities of hemogenic endothelial cells with or without HSC potential in

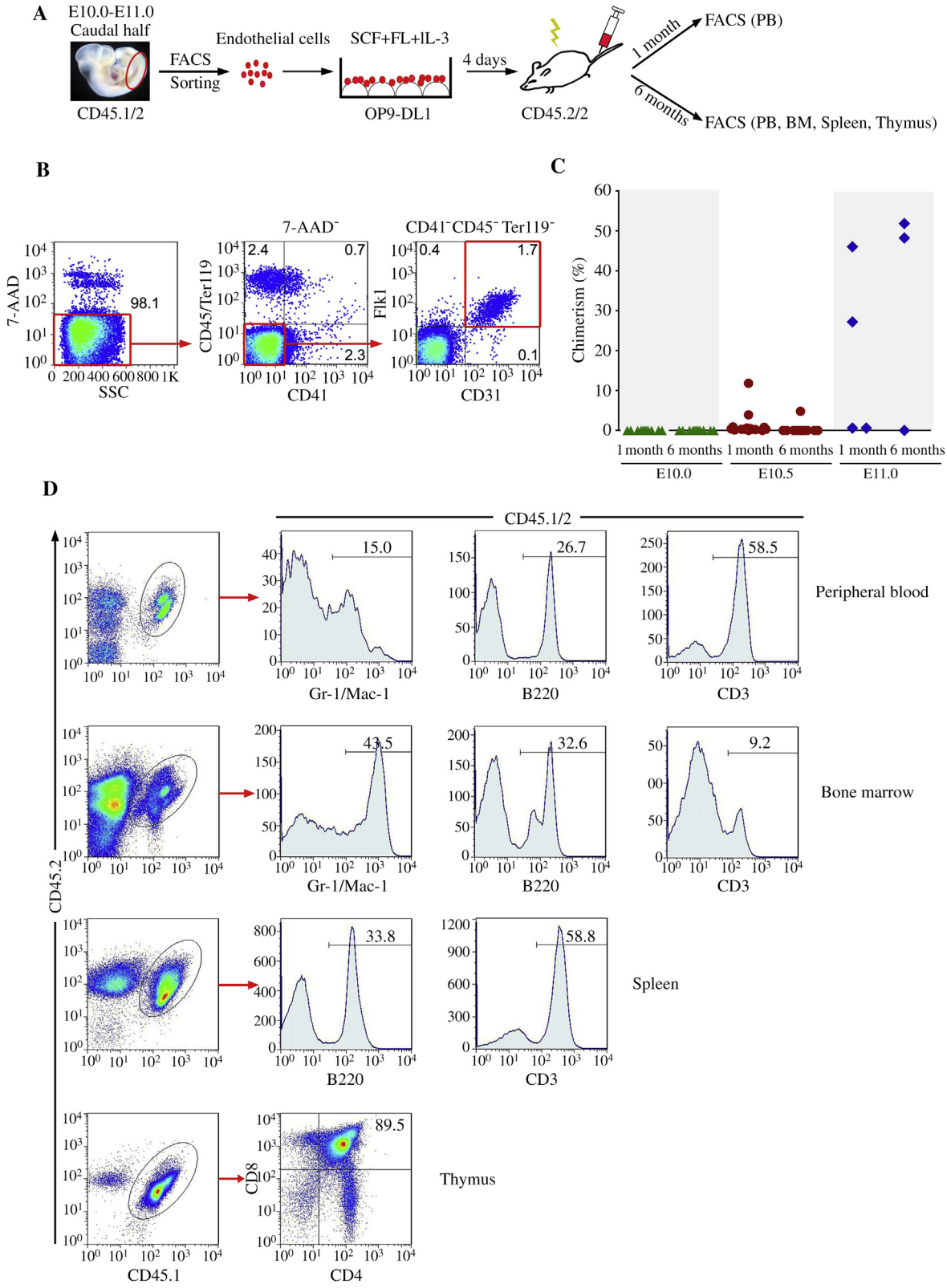

Fig. 2. Generation of adult-repopulating HSCs from E10.0−E11.0 endothelial cells by OP9-DL1 co-culture.

A: experimental design. **B:** putative endothelial cells (Flk1$^+$CD31$^+$CD41$^-$CD45$^-$Ter119$^-$) sorted by flow cytometry. **C:** donor-derived chimerism in recipients transplanted with the co-cultures of OP9-DL1 and endothelial cells from the E10.0 (33−35 sp), E10.5 (36−40 sp), and E11.0 (41−44 sp) caudal half, respectively. The symbols represent the donor chimerism in the peripheral blood of each recipient 1 month or 6 months post transplantation. **D:** representative data of the multi-lineage reconstitution of the peripheral blood in the recipients repopulated by E11.0 co-cultured endothelial cells. The donor-derived multi-lineage chimerism is shown by the presence of CD45.1/2 cells in the myeloid (Gr-1$^+$/Mac-1$^+$), B lymphoid (B220$^+$), and T lymphoid (CD3$^+$, or CD4$^+$/CD8$^+$) populations of the peripheral blood (PB), bone marrow (BM), spleen, and thymus of a representative recipient 6 months post transplantation. FACS, fluorescence-activated cell sorting.

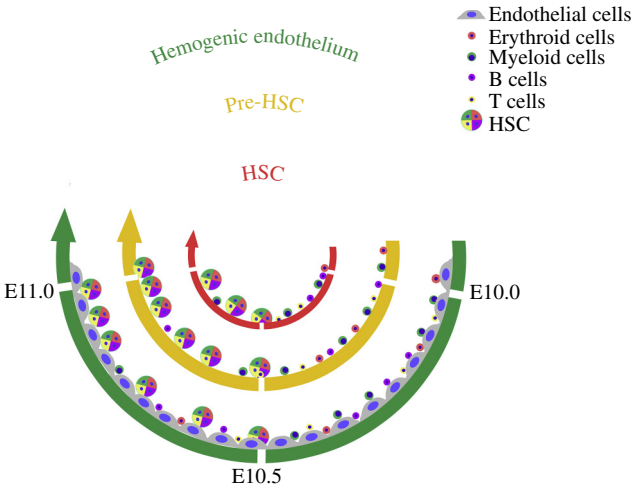

Fig. 3. Hierarchical continuity of the endothelial-to-HSC transition in E10.0−E11.0 mouse embryos.

The green ring indicates the hemogenic endothelial stage, which remains negative for the known hematopoietic surface markers CD41 and CD45, and can initiate transition to HSCs upon induction *in vitro*. The yellow ring indicates the pre-HSC stage (positive for CD41 or CD45), which may be specified from the hemogenic endothelium and can proceed further to the mature HSC stage (red ring) upon induction *in vitro*. In comparison, only mature HSCs can be determined by direct transplantation.

mouse embryos in the future. This scenario indicates multiple differentiation stages or HSC precursors during the course of EHT, which may involve dynamic and fine-tuned regulatory mechanisms.

MATERIALS AND METHODS

Mice

The animals were maintained under specific pathogen-free conditions at the Animal Center of the Academy of Military Medical Sciences and handled according to institutional guidelines. The use of EGFP-transgenic (CD45.2/2) mice on the C57BL/6 background has been described previously (Li et al., 2012). Male EGFP-transgenic mice were crossed with female wild-type mice (CD45.2/2) to obtain GFP^+ embryos. Alternatively, male (CD45.1/1) and female (CD45.2/2) mice were paired to generate CD45.1/2 embryos.

Antibodies

With the exception of CD41-FITC (MWReg30), which was obtained from BD Biosciences (USA), the other antibodies, including biotinylated anti-Tie2 (TEK4), biotinylated anti-Flk1 (Avas12a1), CD31-PE (MEC 13.3), CD45-PE-Cyanine7 (30-F11), Ter119-PE-Cyanine7 (TER-119), CD45.1-APC (A20), CD45.2-PE (104), CD11b-PE-Cyanine7 (M1/70), Gr-1-PE-Cyanine7 (RB6-8C5), B220-PE/FITC (RA3-6B2), CD3-APC/APC-eflour780 (145-2C11), CD4-FITC (GK1.5), CD8-PE-Cy7 (53-6.7) and Streptavidin APC-eFluor® 780, were purchased from eBioscience (USA).

OP9-DL1 co-culture

To collect the embryonic endothelial cells, the caudal half, including the AGM region, was dissected between the forelimbs and hindlimbs, and the ventral tissues (mainly including the liver and gastrointestinal tract) were removed. The tissues were dissociated into single-cell suspensions as described elsewhere (Lan et al., 2007; Li et al., 2012). The $Tie2^+$ cells were sorted from the GFP^+ caudal half according to the MACS manufacturer's instructions. The endothelial cells ($CD31^+CD41^-CD45^-Ter119^-$ or $Flk1^+CD31^+CD41^-CD45^-Ter119^-$) were sorted from the CD45.1/2 caudal half using a flow cytometer (Aria 2, BD Biosciences). The live cells were gated as 7-amino-actinomycin D (7-AAD)-negative cells. The $Tie2^+$ cells or endothelial cells were cultured on mouse OP9-DL1 stromal cells with media composed of IMDM (Invitrogen, USA), hematopoietic cytokines (50 ng/mL SCF, 20 ng/mL FL, and 50 ng/mL IL-3; all from PeproTech, USA), and 10% fetal bovine serum (Hyclone, USA). After 4 or 7 days, the semi-adherent cells were carefully harvested by mechanical pipetting for a subsequent transplantation assay.

HSC transplantation assay

Eight- to ten-week-old female C57BL/6 (CD45.2/2) mice were exposed to a split dose of 9 Gy γ-irradiation (^{60}Co). The culture-expanded cells (GFP^+ or CD45.1/2) and 20,000 CD45.2/2 bone marrow carrier cells were injected into irradiated adult recipients *via* the tail vein. The peripheral blood of the recipients was collected by bleeding the lateral tail vein at the indicated time points. The mice demonstrating ≥10% donor-derived chimerism after 4 months were considered reconstituted.

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