学位論文
Doctoral Thesis

Regulation of bipotential state of hemogenic endothelial cells by CXCR4 signaling
（造血性内皮細胞における二分化能の CXCR4 シグナルによる調節）

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1. Abstract

Background and objective: The origin of hematopoietic stem cells (HSCs) is considered to be hemogenic endothelial cells (HECs) located in the aorta-gonad-mesonephros (AGM) region. HECs have been identified in differentiating embryonic stem cells (ESCs) as VE-cadherin^{+} cells with both hematopoietic and endothelial potential in single cells (bipotential). Although the bipotential state of HECs is critical for fate determination toward HSCs, the molecular basis of the regulation of the bipotential state has not been well understood. In this study, I sought to identify an enriched ESC derived HEC population and investigated their regulatory mechanism.

Methods: KTPU8 ESCs were co-cultured with OP9 stromal cells to induce differentiation of CD45^{-} CD31^{+} VE-cadherin^{+} endothelial cells (ECs). ECs are then separated based on CD41 surface expression and tested for their hematopoietic and endothelial differentiation potential and transcription factor expressions. Single cell analysis was done to check the bipotential progenitors. CXCL12 was added during the ESC culture period to evaluate the effect of CXCR4 signaling. Embryonic equivalent populations were also investigated from single cell suspension of lower trunk of embryo proper and yolk sacs of E8.5-E10.5 murine embryos.

Results: I found that CD41^{+} fraction of CD45^{-} CD31^{+} VE-cadherin^{+} ECs from ESCs encompasses an enriched HEC population. The CD41^{+} ECs contained progenitors for both ECs and hematopoietic cells (HCs) at a high frequency. Expression of hematopoietic transcription factors, Runx1 and Tal1, and endothelial transcription factors, Etv2 and Sox17, in the CD41^{+} ECs indicated the hemogenic endothelial nature. Clonal analyses of cell differentiation confirmed that one out of five HC progenitors in the CD41^{+} ECs possessed the bipotential state that led also to EC colony formation. A phenotypically identical cell population was found in mouse embryos, although the potential was more biased to hematopoietic fate with rare bipotential progenitors. ESC-derived bipotential HECs were further enriched in the CD41^{+} CXCR4^{+} subpopulation. Stimulation with CXCL12 during the generation of CD41^{+} CXCR4^{+} cells reduced the frequency of bipotential progenitors by suppressing the EC potential.

Conclusion: The results in the current study suggest that CXCL12/CXCR4 signaling negatively regulates the bipotential state of HECs independently of the hematopoietic fate. Identification of signaling molecules controlling the bipotential state is crucial to modulate the HEC differentiation and to induce HSCs from ESCs.

Key Words: Mouse embryonic stem cells, Hemogenic endothelial cells, CXCR4 receptor, CXCL12 chemokine, CD41 antigen, VE-cadherin.

2. List of publications

1. **Tanzir Ahmed, Kiyomi Tsuji-Tamura, and Minetaro Ogawa.** "CXCR4 Signaling Negatively Modulates the Bipotential State of Hemogenic Endothelial Cells Derived from Embryonic Stem Cells by Attenuating the Endothelial Potential." ***Stem Cells*****, 2016.** doi:10.1002/stem.2441.

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4. Abbreviations

HSC	Hematopoietic stem cell
HEC	Hemogenic endothelial cell
ESC	Embryonic stem cell
EC	Endothelial cell
HC	Hematopoietic cell
E	Embryonic day
AGM	Aorta-gonad-mesonephros
EMP	Erythroid and Myeloid progenitor
pSP	Para-aortic-splanchnopleura
EHT	Endothelial-to-hematopoietic transition
EB	Embryoid body
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
PCR	Polymerase chain reaction
mAb	Monoclonal antibody
Etv2	ets variant 2
Sox17	SRY (sex determining region Y)-box 17
Foxc2	Forkhead box protein C2
Coup TFII	COUP transcription factor 2 / NR2F2 (nuclear receptor subfamily 2, group F 2
Runx1/AML1	Runt-related transcription factor 1 / acute myeloid leukemia 1 protein (AML1) / core-binding factor subunit alpha-2 (CBFA2)
Tal1	T-cell acute lymphocytic leukemia protein 1
HoxA3	Homeobox protein Hox-A3
GATA	GATA-binding factor
Myb	Transcriptional activator Myb
LIF	Leukemia inhibitory factor
IL	Interleukin
G-CSF	Granulocyte colony-stimulating factor
Flt3	FMS-like tyrosine kinase 3
SCF	Stem cell factor
CXCR4	CXC chemokine receptor 4

CXCL12	CXC chemokine ligand 12
Raldh	Retinaldehyde dehydrogenase
Bry	Brachyury
Sca1	Stem cell antigen 1
cKit	tyrosine-protein kinase Kit/CD117
CD	Cluster of differentiation
CD45	CD45 / Protein tyrosine phosphatase, receptor type, C
CD31	CD31 / Platelet endothelial cell adhesion molecule (PECAM-1)
CD41	CD41 / Integrin alpha-IIb
CD34	Hematopoietic progenitor cell antigen CD34
CD19	B-lymphocyte antigen CD19
CD150	CD150 / Signaling lymphocytic activation molecule 1
CD48/BLAST1	CD48 / B-lymphocyte activation marker / signaling lymphocytic activation molecule 2 (SLAMF2)
CD11b	CD11b / Integrin alpha M
VE-cadherin	Cadherin 5, type 2 or (vascular endothelial cadherin)
Flk1/KDR/VEGFR-2	Fetal Liver Kinase 1 / Kinase insert domain receptor / vascular endothelial growth factor receptor 2
Gr1	GR1 / Myeloid Differentiation Antigen, Ly-6G
TER119	TER119 / Erythroid cell marker, Ly-7
B220	CD45R (CD45 isoform B220)
AA4.1	CD93 / Early B cell antigen AA4.1
SEM	Mean standard error

5. Background and objective

5.1. Hematopoiesis

Hematopoiesis is the process of production of all types of blood cells. The hematopoietic system is one of the earliest tissues to develop during vertebrate development. Hematopoiesis occurs in two sequential programs: the primitive (pre-circulation) hematopoiesis and the definitive hematopoiesis (Galloway and Zon, 2003).

5.1.1. Primitive hematopoiesis

In murine embryos, the first blood cells are originated from the extra-embryonic mesoderm at around embryonic day (E) 7.0 (Figure 1). Primitive hematopoiesis spans from E7 to E9 and is marked by appearance of nucleated erythroid progenitors in blood islands of extra-embryonic yolk sac (Yoder, 2014). These erythroid progenitors are known as primitive erythoblasts and are functionally and morphologically distinct from mature erythroid progenitors. Primitive erythoblasts are large, nucleated and express embryonic globins. They are exclusively detected in the extra-embryonic yolk sac for a short time period (~48 hours) before disappearing. Primitive hematopoietic program also involves the emergence of some primitive or embryonic macrophages and megakaryocytes. Some of these embryonic macrophages can persist as tissue-resident macrophage populations in the later developmental stages (Palis et al., 1999; Yoder, 2014; Schulz et al., 2012). Primitive hematopoiesis does not produce lymphoid cells or HSCs and thus serves as a source of primary red blood cells that facilitates tissue oxygenation during the early rapid development of the embryo (Palis and Yoder, 2001).

5.1.2. Definitive hematopoiesis

The second phase is the definitive hematopoiesis, which is subdivided into two waves. First of these waves begins on E8.25 with erythroid and myeloid progenitors (EMP) production (Figure 1). EMPs emerge from the vascular endothelial cells of the blood island capillaries of the yolk sac as a cluster of cells. Cells then detach from the capillaries to enter in the systemic circulation and seed in the fetal liver at around E10 (Palis et al., 1999; Palis et al., 2001; McGrath et al., 2003). During this wave B and T lymphoid progenitors also start to develop in the intra and extra-embryonic regions. *De novo* EMPs and lymphoid progenitor generation occurs in the yolk sac, chorio-allantoic/placenta and the intra-embryonic region around the aorta (Yoshimoto et al., 2012). Although this early wave of definitive hematopoiesis does not produce HSCs, it is considered as definitive program because the

EMPs differentiate into HC lineages with adult-type features and functions (Swiers et al., 2013; Yoder, 2014).

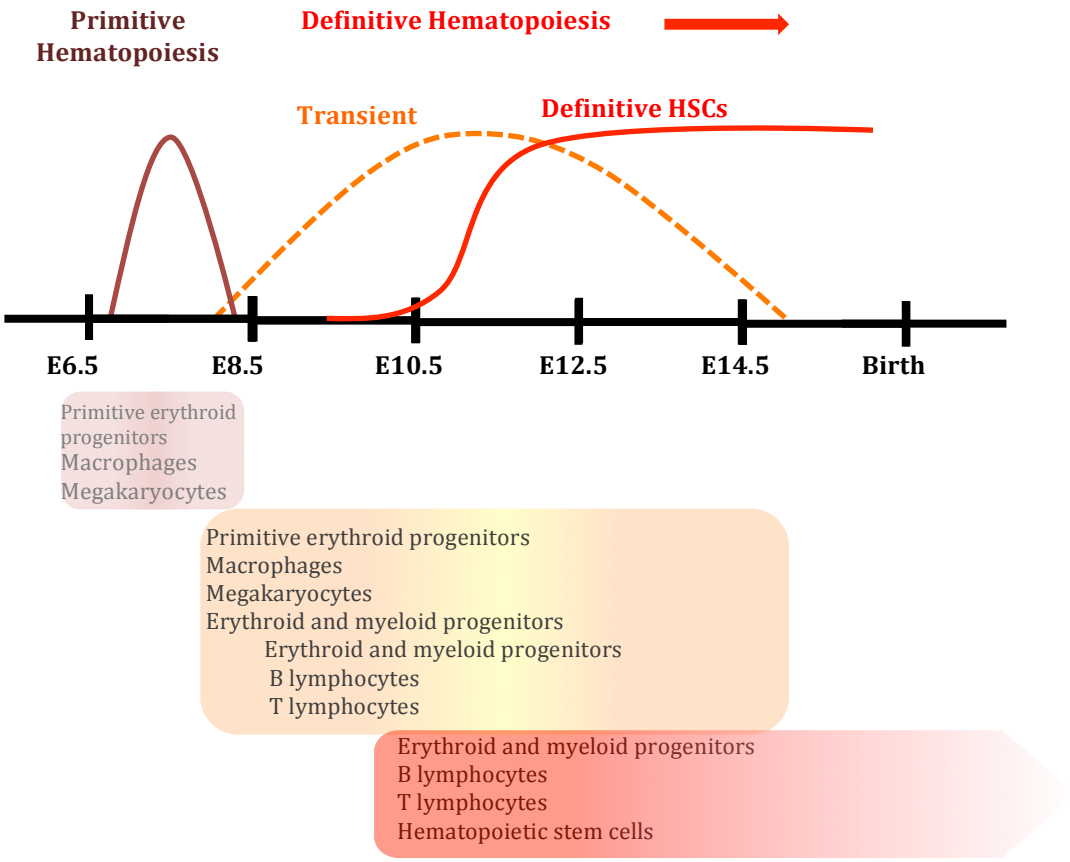


Figure 1: Hematopoiesis during murine embryonic development
Hematopoietic development occurs in two sequential but overlapping programs. Primitive hematopoiesis is transient. It starts at E7.0 and produces primitive erythroid progenitors, embryonic macrophages and megakaryocytes in the yolk sac blood island. The definitive hematopoietic program also starts with a transient wave at E8.5. It produces multi-potent and lineage restricted hematopoietic cells with adult-type morphology. This wave continues through E9.0, when lymphocytes emerge in the yolk sac and AGM region. The next and final wave is responsible for producing definitive HSCs starting at E10.5 of embryonic development. Simultaneous production of EMPs and lymphoid progenitors continues from the yolk sac and AGM region.

Following and final wave of definitive hematopoiesis begins at E10.5 and is marked by the emergence of long-term self-renewing HSCs in the AGM region of the embryo proper (Figure 1, Medvinsky and Dzierzak, 1996). HSCs also appear in other arterial vessels of the embryo, yolk sac and placenta from E11.5 (Kumaravelu et al., 2002; Gekas et al., 2005). Despite the AGM region being the source, differentiation of HSCs do not occur there. These

AGM and yolk sac HSCs and other hematopoietic progenitor cells then migrate and colonize other sites of definitive hematopoiesis, the fetal liver by E11.5 - E12.5 and the bone marrow at E16.5 and contribute to the population of long term repopulating HSCs (Medvinsky et al., 1993; Morrison et al., 1995; Coşkun et al., 2014).

5.2. Development of hematopoietic stem cells

More than ten distinct mature cell types compose the adult hematopoietic system. All these diverse types of HCs are based on one specific cell type i.e., hematopoietic stem cell. Within the system, only HSCs possess the two unique capabilities, multipotency and self-renewal. For HSCs, multi-potency is the ability to differentiate into all functional blood cell types, while self-renewal is the ability to replicate into identical daughter HSCs without undergoing differentiation (Seita and Weissman, 2010).

5.2.1. Site of hematopoietic stem cell emergence

During the murine embryo development, yolk sac produces the first primitive erythrocytes that enter in the circulation and later the definitive EMP and lymphoid progenitors that migrate to the liver (Figure 2). These EMPs can be first identified by expression of CD41 and cKit and later upon maturation they express the pan-hematopoietic marker CD45 (Mikkola et al., 2003). Despite all the plentiful production of hematopoietic progenitors in the yolk sac, there is no convincing evidence of it also being a site for HSC emergence. Explant culture studies showed that early yolk sac explant was devoid of the potential to generate long-term HSCs (Cumano et al., 2001; Medvinsky and Dzierzak, 1996).

The first evidence of embryo proper being the source of HSCs was shown by grafting quail embryos on chicken yolk sac (Dieterlen-Lievre, 1975). It revealed that cells colonizing on the resulting chimeras were embryo derived. Subsequently, intra-embryonic generation of HSCs has been shown conserved among multiple species (Jaffredo et al., 2005). In mice, the region of the embryo proper is first entitled as para-aortic-splanchnopleura (pSP). pSP develop from the lateral plate mesoderm, which is a caudal part of the E8.0 mouse embryo that contains the dorsal aorta, omphalomesenteric artery, gut and splanchnopleural lining. Part of the pSP later becomes the AGM region (Figure 2). Although hematopoietic progenitors were present in the AGM region from E8.5, functional HSCs arise only from E10.5 onward (Jaffredo et al., 2005; Medvinsky and Dzierzak, 1996; Medvinsky et al., 2011). Intra-embryonic origin of HSCs was established when culturing E8.5 pSP region of the pre-circulation embryos (E8.5) as whole tissue explant successfully generated long term HSCs.

Most of the reports now suggest that pSP-AGM region is the initial site of HSC emergence although the subsequent migration and maturation process is essential for the HSCs to acquire self-renewal and reconstitution abilities (Cumano et al., 2001).

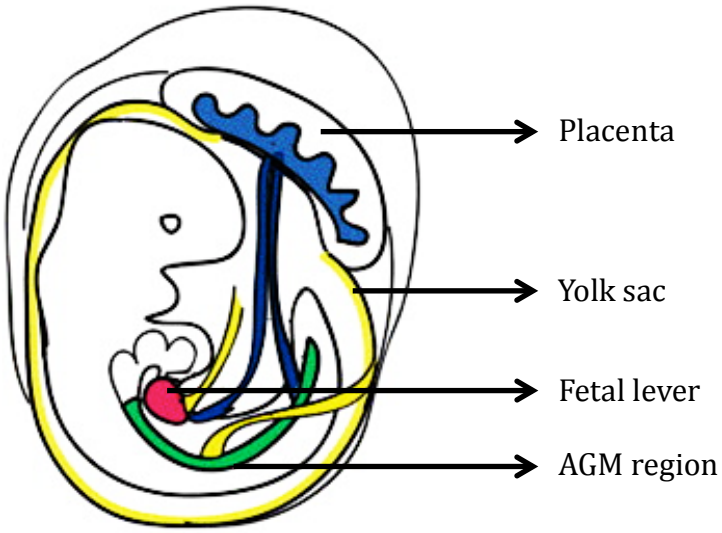


Figure 2: Hematopoietic organs in developing murine embryo
An illustration of hematopoietic organs at E11.0 [Adapted from Mikkola and Orkin, 2006].
Yellow color represents yolk sac; red shows fetal lever; blue is for placental vasculature and green color is representing dorsal aorta.

5.2.2. Markers of emerging hematopoietic stem cells

There is no specific marker that is exclusively expressed by HSCs. The development of HSCs is marked by sets of several combinations of surface markers during the embryonic development. CD41 is known to mark hematopoietic commitment of progenitors and is transiently expressed on the nascent HSCs. Mature HSCs lose CD41 expression (Mikkola et al., 2003; Matsubara et al., 2005). Contrariwise, the pan hematopoietic marker CD45 is not expressed on immature HSCs till E11.5 (Matsubara et al., 2005). Sca1 (Ly6a) also follow similar trend and up-regulated around day 11.5 (De Brujin et al., 2002). The EC associated marker VE-cadherin (cadherin 5) is on the other hand expressed transiently on the emerging HSCs and disappeared from the adult HSC surface (Taoudi et al., 2005). CD31 (Pecam 1), CD34, c-Kit and endomucin are consistently expressed on the HSCs (Matsubara et al., 2005, Mikkola et al., 2006). Table 1 represents a list of markers to identify emerging HSCs along the mouse embryonic development stages.

Table 1: Summary of hematopoietic stem cell markers in developing murine embryo

Embryonic day	Marker
E10.5	$CD45^-$, VE-$cadherin^+$, $CD41^+$, $Runx1^+$, $CD34^+$, $CD31^+$, c-Kit^+, $Sca1^-$, $AA4.1^+$, $Endomucin^+$
E10.5 – E12.5	$CD45^+$, VE-$cadherin^+$, $CD41^{+/-}$, $Runx1^+$, $CD34^+$, $CD31^+$, c-Kit^+, $Sca1^{+/-}$, $AA4.1^+$, $Endomucin^+$
E11.0 – E16.5	$CD45^+$, VE-$cadherin^{+/-}$, $Runx1^+$, $CD34^+$, $CD31^+$, c-Kit^+, $Sca1^+$, $AA4.1^+$, $Endomucin^+$, $CD150^+$, $CD48^-$
Adult HSC	$CD45^+$, $Runx1^+$, $CD34^+$, $CD31^+$, c-Kit^+, $Sca1^{+/-}$, $Endomucin^+$, $AA4.1^+$, $CD150^+$, $CD48^-$

(Ref: Morrison et al., 1995; Okuda et al., 1996; Toudi et al., 2005; De brujin et al., 2002; Mikkola et al., 2003; Matsubara et al., 2005; North et al., 2002; Fehling et al., 2003; Kiel et al., 2005; Chung et al., 2002; Fraser et al., 2002; Bertrand et al., 2005; Ogawa et al., 2001)

5.3. Cellular origin of hematopoietic system

The shared surface markers and the close association of the developing endothelial and hematopoietic lineages have long been observed. Although there have been a lot of effort, the precise cellular origin of HCs is still not completely agreed among researchers. The common precursor of endothelial and hematopoietic lineages was first proposed and termed "hemangioblast" nearly a century ago. Hemangioblast was referred to the mesodermal mass of cells that later emerged into blood island of chick yolk sac (Murray, 1932). Other observations suggested the "hemogenic endothelium", which are specialized subset of ECs i.e., HECs give rise to HCs (Nishikawa et al., 1998b). The co-expression of EC markers on the HC clusters associated with the EC lining of major vessels further suggested a common developmental origin of these two lineages (Jaffredo et al., 2005).

5.3.1. Hemangioblast

Hemangioblasts are defined as the multipotent precursor cells that can differentiate into both hematopoietic and endothelial cells. Due to lack a of specific marker and the fewness of such cells, identification of histological observations and immuno-staining in the embryo was not successful in the earlier studies. The first experimental evidence of the hemangioblast was shown in the mouse ESC culture. ESC derived blast colony forming cells (BL-CFC) were identified as the clonal precursor of HCs and ECs (Choi et al., 1998). During ESC culture, primitive streak mesoderm first arises, which is marked by brachyury (Bry)

expression (Figure 3A). This is followed by production of extra-embryonic mesoderm, which is marked by an upregulation of Flk1 expression and onset of EC specification. Mesoderm derived hemangioblast progenitors express both brachyury and Flk1. Recent reports suggested that $Flk1^+$ hemangioblast first produced the hemogenic endothelium, which then produce CD41 and CD45 expressing hematopoietic cells (Figure 3A; Lancrin et al., 2009; Eilken et al., 2009).

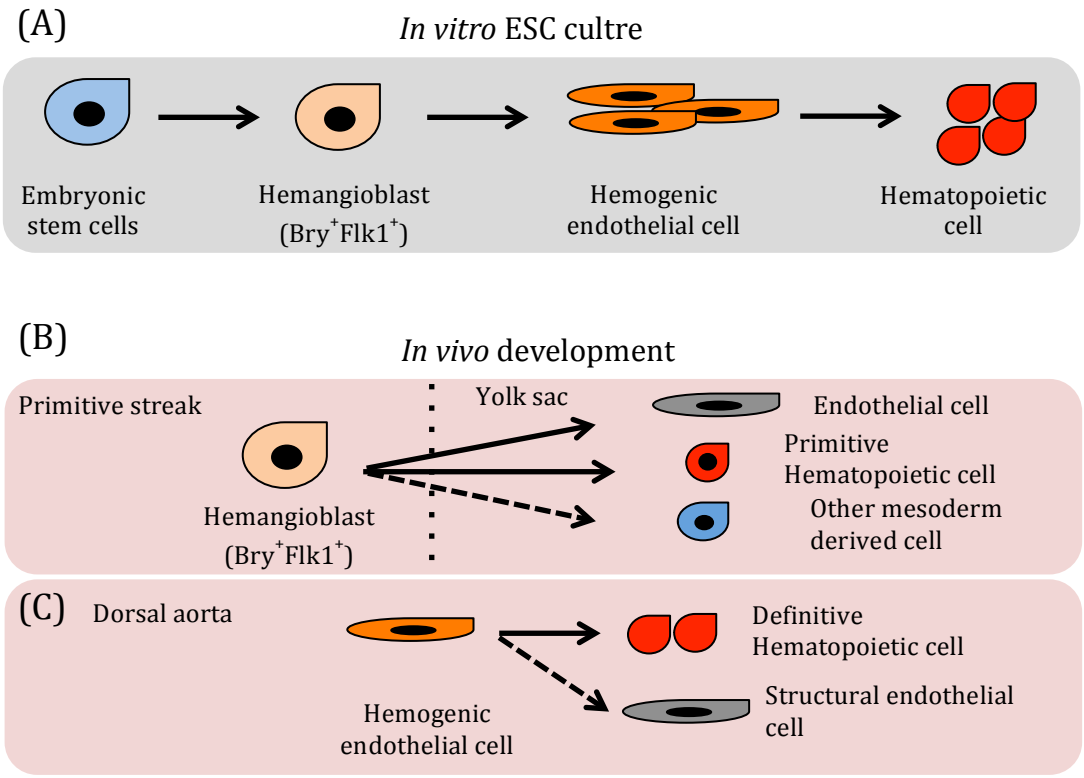


Figure 3: Cellular origins of hematopoietic development
(A) ESC differentiation produces mesoderm derived Bry^+Flk1^+ hemangioblasts. Hemangioblast is capable of generating endothelial and hematopoietic lineages through intermediate HEC stage. (B) Hemangioblasts arise in the primitive strike of murine embryo and then produces HCs and ECs, which later colonize in the yolk sac. (C) Hematopoietic clusters appear from HECs in the wall of major vessels. HCs bud off to enter into the bloodstream inside of the lumen.

Hemagioblast was identified *in vivo* within the posterior primitive streak in the mouse embryo, localized to the gastrulating posterior mesoderm at E7.5. The hemangioblast is equivalent to *in vitro* BL-CFCs and associated with primitive hematopoiesis in the yolk sac. However they could also give rise to smooth muscle cell along with ECs and HCs (Figure 3B; Huber et al., 2004).

5.3.2. Hemogenic endothelial cell

The longstanding idea of hemogenic endothelium suggests that a subset of ECs undergo endothelial-to hematopoietic transition (EHT) to produce hematopoietic cell (HC) lineages. *In vivo* labeling of chick dorsal aorta first experimentally supported this idea, which indicated that hematopoietic cells derive from aortic endothelium (Jaffredo et al., 1998). Other studies in mouse systems also reported the potential of *in vitro* and *in vivo* derived VE-cadherin^{+} ECs to produce hematopoietic lineages (Nishikawa et al., 1998a; Nishikawa et al., 1998b; Ogawa et al., 1999). Strong support in favor of endothelial origin of hematopoietic cells came from the time lapse imaging of live cultures that visualized real time transformation of EC to HCs in the live culture. By tracking morphologically defined immunophenotypic mouse ESC derived EC colonies, it was demonstrated that, in some of the EC colonies, cells begin to express CD41 and then go on to differentiate in CD41^{+}CD45^{+} HCs. However, some EC colonies may not undergo EHT and maintained their EC characteristics suggesting that hemogenic endothelium retain their EC potential and can also contribute to structural endothelium (Eilken et al., 2009).

The hemangioblast should not be confused with HECs. Although hemangioblasts are by definition, bipotential progenitors of HCs and ECs, their potential is not confined for only these two lineages. The Bry^{+}Flk1^{+} hemangioblast cells have also been shown to produce cardiac, skeletal and vascular smooth muscle cells (Motoike et al., 2003; Huber et al., 2004). Hemangioblasts are mesodermal precursor population, which is more associated with yolk sac primitive hematopoiesis. In contrast, HECs are characteristically ECs with the potential to undergo EHT. HECs are associated with AGM hematopoiesis and have shown to produce HSCs.

5.3.3. Hemogenic endothelial origin of hematopoietic stem cells

Many studies now suggest that the origin of HSCs is the HECs of the aortic endothelium. Linage tracing study during early mouse development stages tracked progeny of VE-cadherin^{+} cells by induced VE-cadherin-specific Cre-mediated labeling showed that a portion of adult hematopoietic cells from all hematopoietic lineages was labeled (Zovein et al., 2008). This indicates that their precursor had undergone a hemogenic endothelial stage. Another study opted targeted Cre-mediated deletion of Runx1, which is an essential transcription factor for definitive hematopoietic development in VE-cadherin^{+} cells (Chen et al., 2009). This conditional deletion of Runx1 in VE-cadherin^{+} cells resulted in complete ablation of clonogenic progenitors and HSC formation. In contrast, in Vav-cre mice, in which Runx1 was specifically deleted in the hematopoietic lineages, HSCs were still present. They

suggested that Runx1 deficiency blocks EHT but not the downstream events. Both of these studies solidified the support of the endothelial origin of HSCs.

While it is well accepted that adult HSCs are generated through an EHT event, the locations of the specialized HECs remained unclear. Live imaging technique in the aortic region of zebrafish embryos at different time points of embryonic development showed that hematopoietic cells emerge from the ventral endothelial cells of the dorsal aorta. Studies used transgenic reporter Flk1, cMyb, CD41 and Runx1 embryos and showed that HSCs bud in the sub-aortic mesenchyme and later released in the circulation through the adjacent axial vein (Bertrand et al., 2010; Kissa and Herbomel, 2010; Lam et al., 2010). HECs bend of the aortic endothelium and change their shape to round up and subsequently detach from the neighboring ECs. These studies clearly showed that in the zebrafish embryos, HSCs emerge from the HECs located in the ventral part of the dorsal aorta.

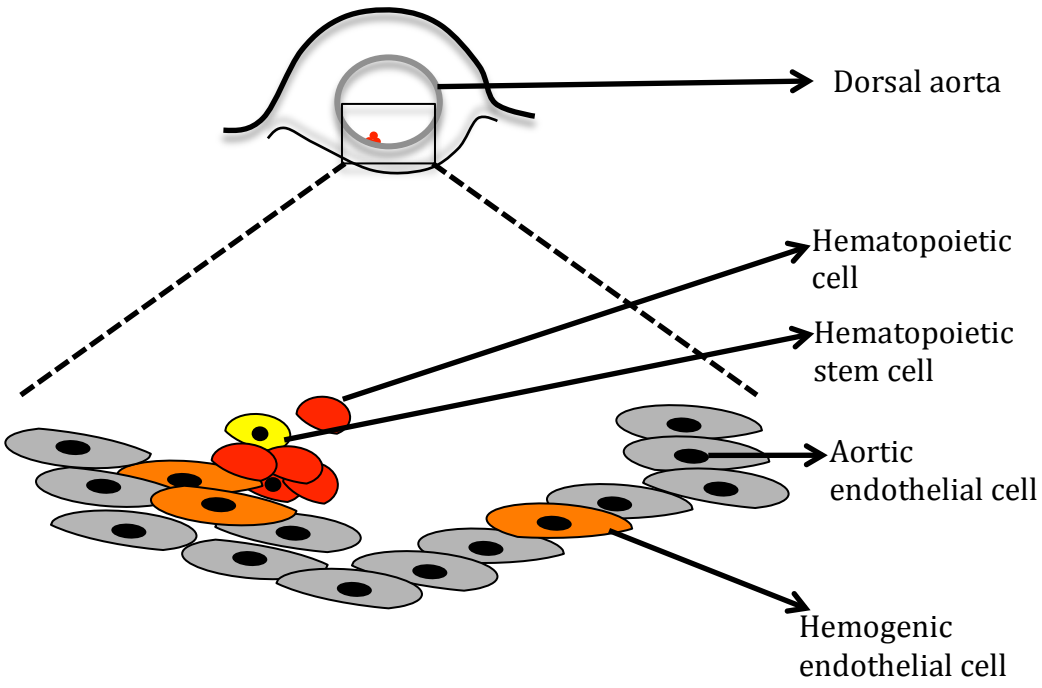


Figure 4. Hemogenic endothelial cells undergo transition to produce HSCs
A schematic representation of a cross section of E10.5 mouse AGM region with the ventral wall of the dorsal aorta boxed and magnified. HC cluster round up and bud out from the HECs (pink) of the dorsal aorta. HCs (red) and HSCs (yellow) are released into the lumen. Non-hemogenic ECs are portrayed gray.

Morphological evidence of hemogenic endothelial origin of HSCs in mouse embryos came from the study of Boisset et al. They used an explant culture of embryo slices of E10.5 mouse AGM region and performed continuous confocal imaging of this samples. Using transgenic embryos expressing GFP under the control of the promoter of HSC marker Ly6A (Sca1) and staining for CD31, they showed real-time observation of the emergence of $CD31^{+}Sca1^{+}$ HSCs from the ventral endothelial cells of the dorsal aorta (Figure 3). HECs round up and bud off the adjacent ECs of the ventral wall and newly formed HSC released into the lumen of the aorta (Boisset et al., 2010).

Recently, an HEC population ($CD45^{-}$VE-cadherin$^{+}CD41^{lo}$ i.e., pre-HSCs) has been shown to be able to generate repopulating HSCs ($CD45^{+}$VE-cadherin$^{+}CD41^{+}$) in an *ex-vivo* co-aggregation culture (Rybstov et al., 2011). Altogether, these studies strongly suggest the HEC origin of HSCs.

5.4. Hemogenic endothelial cell specification and regulation: current understandings

Despite many studies investigated the localization of the HECs, there is no definitive single marker or set of markers are known to distinguish HECs from non-hemogenic ECs. Several studies have taken different approaches to elucidate the phenotypic identity of HECs. Isolated E9.5 mouse VE-cadherin^{+} cell fraction co-expressing EC markers CD34, CD31 and Flk1, have been shown as hemogenic endothelium with functional capability to produce HCs (Nishikawa et al., 1998b). Alpha4-intregin (α4-integrin) has been shown as distinctive marker between hemogenic and non-hemogenic VE-cadherin^{+} cell fraction (Ogawa et al., 1999). Critical role of SCL has been shown to retain hemogenic capability for HECs (Van Handel et al., 2012).

5.4.1. Specification of hemogenic endothelial cells

The molecular mechanisms that govern the specification of HECs from non-hemogenic vascular endothelium are little known. A summary of molecular signals that are currently known to be involved in HEC specification is summarized in Table 2.

Table 2: Summary of signaling pathways and molecules involved in specification of hemogenic endothelial cells from vascular endothelial cells

Molecular signals	Role
Retinoic acid signaling	Retinoic acid signaling is known to regulate endothelial cell specification and development in mice. Retinaldehyde dehydrogenase 2 mutant (*Raldh*$^{-/-}$) embryos exhibit abnormal vasculogenesis (Lai et al., 2003). *Raldh* null embryos were also shown to have a defect in definitive hematopoiesis due to abnormal HEC development although with intact primitive hematopoiesis. Retinoic acid signaling found critical for both yolk sac and AGM hematopoiesis as in both tissues most of the ECs with active retinoic acid signaling possessed HEC characteristics (Chanda et al., 2013).
c-Kit	c-Kit is a receptor tyrosine kinase for Stem cell factor (SCF). c-Kit is an HSC marker and has been shown important for renewal of hematopoietic progenitors (Ogawa et al., 1991). SCF/c-Kit interaction is necessary for definitive hematopoietic development and c-Kit mutations have shown to disrupt HSC development (Mercello et al., 2013; Sattler and Salgia, 2004). c-Kit signaling thus play important but not clearly defined role in HEC specification.
Notch signaling	Notch signaling pathway is well known for its involvement in cell fate control and differentiation of ECs and HCs. There are four Notch family of receptors (Notch 1-4) and five ligands (DLL1, 3, 4 and Jag 1 and 2). EC specific receptor Notch 1 and 4 null embryos showed abnormal vascular development. Chemical inhibitors of Notch caused suppressed HEC specification in the yolk sac. Notch 1 is specifically expressed in the ventral wall of the dorsal aorta and AGM Notch1$^{-/-}$ showed decreased hematopoietic potential. Again, Notch1 induced ESC differentiated to more VE-cadherin^{+} HECs with high HC potential. Notch signaling is suggested to have biphasic effect i.e., controlling both the HEC specification and the EHT event. Other studies also suggested the critical role of Notch signaling in HEC specification and function (Mercello et al., 2013; Jang et al., 2015).
Cell cycle control/p27	p27 is a cell cycle inhibitor which arrests cell cycle in the G1 phase. Rescue experiments showed that p27 could rescue the endothelial cell cycle, HEC specification and the generation of HSCs during hampered retinoic acid and Notch signaling. The mechanism of p27 is suggested to

have downstream of these signaling pathways. There p27 help ECs maintain the bi-potent stage by regulating hemogenic genes and/or altering cell cycle to allow hemogenic transcriptional regulator to function on ECs to become HECs (Mercello et al., 2013).

5.4.2. Regulation of hemogenic endothelial cells to undergo EHT

It is suggested that within the AGM region, emergence of HSC from HECs is a hub of interplay among master transcriptional regulators, their downstream effectors and the extrinsic modifiers of signaling cascades. These activities create a hematopoietic microenvironment or niche, which promote the HSC development from HECs through the EHT. Transcription factors Runx1 and SCL is among the best known transcription factors essential for EHT. Other transcription factors involved in EHT include Hoxa3, Sox17, Etv2 GATA2 and cMyb. The brief roles of the known regulators and signaling pathways are:

Runx1: Transcription factor Runx1 has been focus of large amount of research due to its pivotal role in EHT. Mesenchymal cells in the dorsal aorta in the AGM region, placenta, hematopoietic clusters of the ventral wall of dorsal aorta and vitelline and umbilical arteries express Runx1. Runx1 expression is critical for normal definitive hematopoietic development but not required during primitive hematopoiesis or after EHT (Chen et al., 2009). Runx1 deletion does not prevent the emergence of the CD41 marker on VE-cadherin^{+}CD45^{-} cells but it prevents the transition of these cells to CD41^{+}CD45^{+} cells (Liakhovitskaia et al., 2014). Runx1 has been shown to suppress the endothelial program while activating hematopoietic program as evidenced by sequential emergence of CD41 and CD45 (Lancrin et al., 2009). Runx1 target genes Gfi1/Gfi1b were shown to down-regulate expression of endothelial genes in Runx1-null hemogenic endothelium, thereby promoting a morphological change from flattened EC to round cells (Lancrin et al., 2012). Collectively the evidences now strongly suggest that Runx1 is a critical regulator of HECs to HSC formation.

HoxA3: Hemogenic potential of the ECs is associated with a balance of Runx1 and HoxA3 expression within the endothelium of the dorsal aorta. HoxA3 down-regulation is correlated with the onset of Runx1 in these cells. Conversely re-expression of HoxA3 reactivates and maintains EC program in the HECs (Iacovino et al., 2011).

Sox17: Sox17 is a transcription factor expressed in the AGM HECs and has been implicated in the downstream of HoxA3 (Iacovino et al., 2011). Sox17 is critically required for the

generation of fetal HSCs but not adult HSCs. Sox17 deletion using VE-cadherin-cre or Tie2-cre mice resulted in embryonic lethality. Sox17 overexpression has been shown to down modulate EC markers and expand hemogenic characteristics (Clarke et al., 2015).

SCL: The transcription factor SCL is critically required for HECs to establish. SCL functions upstream of the Runx1. SCL loss of function studies showed an initiation of cardiac program in the yolk sac, indicating SCL role on suppressing cardiac fate. (Lancrin et al., 2009; Van Handel et al., 2012).

Etv2: The transcription factor Etv2 has been reported to mark an enriched population of hemogenic endothelium in both ESC culture and gastrulating embryos. Etv2 is expressed in $Tie2^{+}cKit^{+}$ HECs, rapidly up-regulate CD41 expression and down-regulate EC markers and generate definitive HC progenitors (Wareing et al., 2012).

GATA2: GATA2 is expressed in the pSP/AGM region of the mouse embryo. GATA2 deficient mice die at E10.5 due to defective hematopoiesis. Conditional deletion of a Gata2 cis-regulatory element in the AGM region has been shown to block HSC emergence by reducing the expression of SCL and Runx1, which leads to embryonic lethality around E13.0-14.0 (Gao et al., 2013). Specific deletion of GATA2 by VE-cadherin-cre and Vav-cre has revealed that GATA2 is critical for HSC generation as well as survival (de Pater et al., 2013).

cMyb: Transcription factor cMyb plays important role in HSC emergence and maintenance. Deletion studies showed embryonic lethality at E15.5 due to defective hematopoiesis. AGM explants from $cMyb^{-/-}$ mice also showed 100-fold fewer HSCs (Takakura et al., 2000). Studies in zebrafish also consolidated the cMyb role in HSC generation via EHT in AGM (Zhang et al., 2011).

Notch signaling: In addition to the role of Notch signaling in HEC specification, it is also necessary for HSC development from HECs. Notch signaling mediates EHT by interactions between emerging HSCs expressing Notch receptors and the underlying ECs expressing Notch ligands. Inhibition of Notch signaling by chemical inhibitor DAPT has shown to prevent EHT in cultured E9.5 AGM cells. Consequently Activation of Notch signaling in a modified culture system of E11.0 AGM HECs resulted in production of long term engraft-able HSCs (Hadland et al., 2015).

Wnt/β-catenin signaling: Wnt in a well studied evolutionarily conserved signaling pathway known to regulate several stages of embryonic development. It has been shown that Wnt/β-

catenin necessary for the AGM HECs to produce long term HSCs as well as in the cultured HECs to produce HCs. E11.5 AGM HECs have also been shown to possess high degree of Wnt activity (Ruiz-Herguido et al., 2012).

Apart from the factors and signaling pathways listed in table 3, there are other mediators also known to regulate HSC generation from hemogenic endothelium via EHT. G-protein coupled receptors, Purine signaling, Chomatin remodeling and also hypoxia and inflammation play critical roles in HSC emergence (Gritz and Hirschi, 2016).

5.5. Embryonic stem cells

Embryonic stem cells are pluripotent cells derived from the inner cell mass of blastocyst-stage embryos (Evans and Kaufman, 1981; Martin, 1981). The unique features about ESCs are the capacity of retaining normal karyotypes following extensive passaging in the culture and generating every cell type in the body. The property of ESCs being able to differentiate into multiple lineages in culture allowed studying model embryonic development *in vitro* and the events regulating early lineage induction and specification (Keller, 1995).

5.5.1. Proliferation of embryonic stem cells maintaining pluripotency in culture

ESCs were initially maintained by co-culture with mouse embryonic feeder cell (Evans & Kaufman, 1981; Martin, 1981). Studies showed that the leukemia inhibitory factor (LIF) is one of the key components for maintaining undifferentiated state of ESCs (Williams et al., 1988). Later External signaling pathways such as LIF-gp130-STAT3 (Niwa et al., 1998; Matsuda et al., 1999), BMP-TGF-β-Nodal-Smad (Ying et al., 2003), MAPK-ERK (Burdon et al., 1999), and WNT (Hao et al., 2006; Ogawa et al., 2006) as well as transcription factors *Oct3/4* (Niwa et al., 2000)*, Sox2* (Masui et al., 2007) and *Nanog* (Mitsui et al., 2003) have been reported to play important roles in the self-renewal of mouse ESCs. The interaction of transcription factors including *Oct3/4, Sox2* and *Nanog* form an interaction of network to maintain the pluripotency of ESCs. ESC differentiation is also regulated by the epigenetic modification of chromatin and gene expression (Niwa et al., 2000).

5.5.2. Differentiation of embryonic stem cells in culture

Upon removal of the factors that maintain undifferentiated state of the ESCs, under appropriate conditions ESCs differentiate into lineages of three germ layers: mesoderm, endoderm and ectoderm (Keller, 1995; Smith, 2001). Three general approaches are usually

taken for ESC differentiation in culture. The first method is culturing ESCs to form aggregate into a three dimensional colony called embryoid bodies (EBs). EBs promote cell-cell interactions in the three dimensional colonies which is important to study various developmental program while generation of cytokines and inducing factors inside of the EBs may thwart the desired differentiation.

The second method is to culture ESCs on the layer of stromal cells and the let the ESCs to differentiate in contact with the stromal cells. The most commonly used cell line for this co-culture differentiation studies is mouse OP9 cells (Nakano et al., 1994; Kodama et al., 1994). This co-culture method provides specific growth advantage from the particular cell line used as the stromal cell layer. However undefined factors discharged from the stromal cells may influence the ESC differentiation.

The other method used for ESC differentiation is differentiation in a monolayer on extracellular matrix protein (Nishikawa et al., 1998a). This is more defined condition for the culture and minimizes the stromal cell and surrounding cells' influence. Different proteins influence the differentiation differently hence the use of appropriate matrix protein is critical. ESC differentiation studies allow generating a vast range of cells and thus studying their development *in vitro*.

5.6. *In-vitro* generation of hemogenic endothelial cells

Elucidating the mechanism of hematopoietic differentiation from HECs is crucial to understand the process of HSC development. Despite many studies on the developmental pathway of HSCs have established the endothelial origin of HSCs, the underlying molecular mechanism is yet to be fully understood. The ESC culture system recapitulates development of the HC lineage through mesoderm induction and endothelial differentiation (Ogawa et al., 1999; Endoh et al., 2002; Hashimoto et al., 2007; Lancrin et al., 2009). ESC differentiation system provided the first functional evidence for the existence of common progenitor hemangioblast with endothelial and hematopoietic potential. The common progenitor arises in EBs differentiated from ESCs as a monolayer on collagen IV earlier than hematopoietic progenitors on day 5 (Choi et al., 1998; Nishikawa et al., 1998a).

Recent studies demonstrated the existence of hemogenic endothelium using the mouse ESC differentiation system (Eilken et al., 2009; Lancrin et al., 2009). Plating hemangioblast containing $Flk1^{+}$ mesoderm population in liquid blast condition for two days,

an EC population ($Tie2^{+}$c-$Kit^{+}CD41^{-}$) arises (Lancrin et al., 2009). This population contained EC potential and gave rise to hematopoietic lineages. Equivalent population exists in mouse neural plate stage embryos and E10.5 AGM region. By using time-lapse microscopy, from $Flk1^{+}$VE-$cadherin^{-}$ mesoderm co-cultured with OP9 cells, HECs in the EC colonies were shown to generate HCs (Eilken et al., 2009). These HECs were observed to express VE-cadherin, form tight junction, take up acetylated low-density lipoprotein (Ac-LDL) and reside in an endothelial sheet. These studies suggested HECs are transiently generated for a short time window during the hematopoietic development from the mesoderm.

5.7. Objective of this study

To generate HSCs from ESC culture, it is important to identify a precise HEC population. Although hemogenic potential can be detected in the VE-$cadherin^{+}$ cell population derived from ESCs (Ogawa et al., 1999; Nishikawa et al., 1998a), VE-cadherin is not confined exclusively on ECs. There is no specific marker to distinguish HECs from non-hemogenic ECs and no positional information is available in the ESC culture. Therefore, a functional definition combined with marker expression is necessary to identify HECs in culture. I assume three criteria for identification of ESC-derived HECs. First, HECs in culture should express surface markers and transcription factors that resemble both endothelial and hematopoietic signature. Second, when sorted and cultured in favorable condition, HECs should differentiate to HCs. Third, HECs in culture should retain some EC potential as a proof of their endothelial identity. Such progenitors with a bipotential ability to form HC colonies and EC sheets have been described but their detailed surface markers and regulatory mechanisms remained unknown (Nishikawa et al., 1998a).

The state when a progenitor is bipotential is an important phenomenon as it characterizes the intermediate stage of the HECs that is undergoing EHT by activating hematopoietic program. Manipulating the regulatory mechanism of the bipotential state is a key to HSC induction from ESCs in culture. For this purpose, a population should be selected by specific surface markers which represents HECs. I considered CD41 as one possible candidate, as CD41 expression in VE-$cadherin^{+}$ cells is known to identify the precursor of HSCs in the embryo (Rybstov et al., 2011; Rybstov et al., 2014). Also ESC-derived HECs has been shown to express CD41 at the onset of EHT (Eilken et al., 2009).

I pursued two questions in this study. First, whether CD41 expression on VE-cadherin^{+} cells could identify bipotential HECs and second, how the bipotential state of HECs could be regulated.

In this study, I found that CD45^{-}CD31^{+} VE-cadherin^{+} CD41^{+} cells represent an enriched population of HECs that contain bipotential progenitors of ECs and HCs. These bipotential progenitors could be further enriched in the CXCR4^{+} fraction of this population. I also found that CXCR4 signaling negatively regulates the bipotential state of HECs by suppressing their endothelial potential. This *in vitro* model of regulation of bipotential HECs provide us an insight to control the HEC differentiation toward the induction of transplantable HSCs from ESCs.

6. Materials and Methods

6.1. Cell lines

In this study, KTPU8 ES cell line is used. KTPU8 is a feeder free ES cell line derived from TT2 ES cell line (Yagi et al., 1993). KTPU8 cells were maintained in the undifferentiated state on 0.1% gelatin (Sigma-Aldrich, MO, USA) coated culture dishes in Glasgow Minimum Essential Medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% knockout serum replacement (Life Technologies), 1% fetal calf serum (Japan BioSerum, Fukuyama, Japan), 1mM Sodium Pyruvate (Life Technologies), 1X nonessential amino acids (Life Technologies), 0.1mM 2-Marcaptoethanol (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA) and 1000U/ml recombinant mouse leukemia inhibitory factor (Millipore, Darmstadt, Germany). OP9 stromal cell line (Kodama et al., 1994) was maintained with α-MEM (Life Technologies) supplemented with 10% FCS (Japan BioSerum).

6.2. Mouse

Pregnant ICR mice were purchased from Japan SLC (Shizuoka, Japan). E7.5–8.5 whole embryos or E9.5–10.5 lower trunk of embryos proper and yolk sac were isolated and prepared as a single cell suspension. After sacrificing mice by cervical dislocation, the yolk sac and embryonic body were removed by cutting the uterine membrane and the surrounding tissues were dissected away by using fine forceps. For E9.5–10.5 embryos, the embryonic body is separated from yolk sac. Anterior and caudal parts were cut off from the embryonic body as described Godin et al., 1995 and the embryonic trunk lower than the heart level was pooled. The pooled yolk sac and lower trunk were incubated in dispase II (EIDIA, Tokyo, Japan) at 37°C for 20 minutes. Cell clump was washed and further dissociated by incubation in cell dissociation buffer (Life Technologies) at 37°C for 20 minutes. Finally, single-cell suspension was prepared by gentle pipetting. All animal experiments were performed under the approval of the Institutional Review Board at Kumamoto University.

6.3. Growth factors and inhibitors

Recombinant murine interleukin 3 (IL-3), interleukin 7 (IL-7), stem cell factor (SCF) and FMS-like tyrosine kinase 3 ligand (Flt3L) were purchased from PeproTech (Rocky Hill, NJ, USA). Recombinant mouse CXC chemokine ligand 12 (CXCL12) was purchased from BioLegend (San Diego, CA, USA). Recombinant human erythropoietin (EPO) and granulocyte colony-stimulating factor (G-CSF) were obtained from Kyowa Hakko Kirin

(Tokyo, Japan). The CXC chemokine receptor 4 (CXCR4) antagonist AMD3100 was purchased from Cellagen Technology (San Diego, CA, USA).

6.4. Antibodies

Monoclonal antibodies (mAbs) against CD11b (M1/70), CD19 (6D5), CD31 (390), CD41 (MWReg30), CD45 (30-F11), Gr1 (RB6-6C5), TER119 (TER119) and B220 (RA3-6B2), isotype-matched control mAb (RTK2071) and streptavidin were purchased from BioLegend. Anti-CXCR4 mAb (2B11) was purchased from eBioscience (San Diego, CA, USA). Anti-VE-cadherin mAb (VECD1) (Matsuyoshi et al., 1997) was purified from hybridoma culture supernatant by using CELLine (WHEATON Millville, NJ, USA) prepared and labeled with Biotin or Allophycocyanin (APC) (Dojindo Laboratories, Kumamoto, Japan) as directed by the manufacturer's instruction.

6.5. *In vitro* differentiation of ESCs

A total of 20,000 ESCs were put into a 25 cm2 (T25) culture flask pre-seeded with OP9 stromal cells. Cells were cultured with α-MEM (Life Technologies) supplemented with 10% FCS (Japan BioSerum) and 50 μM 2-mercaptoethanol (induction medium). Medium was changed in every 2 days. Cells were collected by incubation with cell dissociation buffer (Life Technologies) and blocked with normal mouse serum (Millipore) for staining with mAbs.

6.6. *In vitro* differentiation into hematopoietic cells

Cells were co-cultured with OP9 stromal cells in induction medium supplemented with a combination of hematopoietic cytokines. For induction of erythromyloid lineages, IL-3, SCF and G-CSF (20 ng/ml) and EPO (2 IU/ml) were used. For induction of B cells, Flt3L and IL-7 (20 ng/ml) and SCF (50 ng/ml) were used. Medium was changed every 3 days. HC lineages were analyzed by flow cytometry and May-Grunwald Giemsa staining after 7 days for erythromyeloid lineages or 14 days for B cells.

6.7. Cell sorting

Cells were stained with mAbs and washed with Hanks' balanced salt solution containing 1% bovine serum albumin (BSA, Sigma-Aldrich). Unstained, single stained and isotype matched controls were used for gating strategies. Flow cytometry analysis was performed using a BD FACSAria™ III and a BD FACSCanto™ II flow cytometer (Becton Dickinson, NJ, USA). Single cell sorting was performed using the BD FACSAria™ III. Acquired data were analyzed by FlowJo software (FlowJo LLC, Ashland, OR, USA).

6.8. Determination of progenitor frequencies

HC progenitor frequency was determined by limiting dilution or single cell analysis. In limiting dilution analysis, defined numbers of cells (20, 40, 60, 80, 200, 300, 400, 500) were plated on each well of a 96-well plate pre-seeded with OP9 stromal cells (OP9 plates). For each inoculum size, a minimum of 24 wells was seeded. Cells were cultured in the induction medium supplemented with hematopoietic cytokines. Wells containing HCs were scored after 7 days under a microscope. Frequencies were determined by calculating with linear regression analysis on the basis of Poisson distribution and given as the reciprocal of the concentration of test cells that results in 37% negative wells. Cell populations with high progenitor frequencies were subjected to direct measurement by sorting single cells to OP9 plates.

EC progenitor frequency was determined by seeding 1,000 cells in each well of a 6-well plate pre-seeded with OP9 cells and incubating in the induction medium for 4 days. Wells were immunohistochemically stained for scoring EC colonies.

Bipotential progenitor frequency was determined by single cell sorting on OP9 plates. Cells were cultured for 7 days in the induction medium supplemented with hematopoietic cytokines. Wells containing HCs were marked by phase contrast microscopy and ECs were revealed by immunohistochemical staining with anti-VE-cadherin mAb. After staining, each well was analyzed for coexistence of HC and EC colonies by microscopy.

6.9. Immunohistochemistry

Cells were fixed with 2% paraformaldehyde and sequentially stained with anti-VE-cadherin mAb and horseradish peroxidase (HRP)-conjugated donkey anti-rat IgG antibody (Jackson Immuno Research Lab, West Grove, PA, USA). VE-cadherin^{+} EC colonies were revealed using DAB-NiCl substrate solution.

6.10. Immunofluorescence

Cells were fixed with 2% paraformaldehyde, followed by staining with anti-CD45 and TER119 mAbs and FITC-conjugated isolectin B4 (Sigma-Aldrich). HCs were revealed by staining with a goat anti-rat AlexaFluor555 antibody (Life Technologies). Cell nuclei were counterstained with DAPI (4,6-diamidino-2phenylindole). Fluorescence images were taken and processed using a TCS-SP2 laser scanning confocal microscope (Leica, Wetzlar, Germany).

6.11. Determination of CXCL12 level by ELISA

Culture supernatants were centrifuged and stored in –80^{o}C in aliquots. CXCL12 protein level was measured by the SDF1 (CXCL12) Mouse ELISA Kit (Abcam, Cambridge,

MA, USA). Each sample was tested in triplicate. The standard curve was constructed by reconstituting SDF1 standard protein supplied in the kit. The optical density of each well was determined using an iMark^{TM} Microplate Absorbance Reader (BIO-RAD, Hercules, CA, USA) set to 450 nm wavelength.

6.12. Gene expression analysis

Total RNA was extracted from sorted cells using TRIzol Reagent (Invitrogen). RNA was reverse transcribed to cDNA by Superscript II reverse transcriptase (Thermo Fisher Scientific) using random hexamer priming according to the manufacturer's method. Real time PCR reactions were carried out using Power SYBR Green PCR Master Mix (Life Technologies) in an ABI 7300 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). Primers were obtained from Eurofins Genomics (Tokyo, Japan). The list of primers is shown in Table 4.

Table 3: List of PCR primers

	Forward primer	Reverse primer
B2m (146)	CTGACCGGCCTGTATGCTAT	CCGTTCTTCAGCATTTGGAT
Sox17 (119)	TATGGTGTGGGCCAAAGACGAA	CCGCTTCTCTGCCAAGGTCAA
Runx1 (70)	CCTCCTTGAACCACTCCACT	CTGGATCTGCCTGGCATC
Tal1 (150)	ATAGCCTTAGCCAGCCGCTC	GCCGCACTACTTTGGTGTGA
Brachyury (102)	GCTTCAAGGAGCTAACTAACGAG	CCAGCAAGAAAGAGTACATGGC
B2m (98)	TGGTGCTTGTCTCACTGACC	TATGTTCGGCTTCCCATTCT
Etv2 (113)	CAGAGTCCAGCATTCACCAC	AGGAATTGCCACAGCTGAAT
Foxc2 (134)	GCAACCCAACAGCAAACTTTC	GACGGCGTAGCTCGATAGG
Coup TFII (87)	GACTCCGCCGAGTATAGCTG	GCTTTCCACATGGGCTACAT

6.13. Statistical analysis

Statistical analyses were performed using Microsoft Excel and GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA). Values of $p<0.05$ were considered statistically significant. Results are expressed as mean ± SEM.

7. Results

7.1. ESC-derived VE-cadherin$^+$ CD41$^+$ cells have definitive hematopoietic potential

I separated the CD45$^-$ CD31$^+$ VE-cadherin$^+$ cells derived from ESCs co-cultured with OP9 cells based on surface expression of CD41. 14.7±0.7% (n=8) of CD45$^-$ CD31$^+$ VE-cadherin$^+$ cells expressed CD41 on their surface at day 6 culture. This proportion of VE-cadherin$^+$ CD41$^+$ cells is consistent with the finding of a recent study (Hirota and Ogawa, 2015). VE-cadherin$^+$CD41$^+$ subpopulation started to appear from day 5 and reached their peak proportion on day 6 (Figure 5A, B). The expression of CD41 was compared and verified by confirming specific binding of the anti-CD41 antibody using an isotype-matched control antibody (Figure 5C). These results showed that CD45$^-$ CD31$^+$ VE-cadherin$^+$ CD41$^+$ population was differentiated from ESC culture with a peak proportion at day 6.

Next, I compared the hematopoietic potential of CD45$^-$ CD31$^+$ VE-cadherin$^+$ CD41$^+$ cells and CD45$^-$ CD31$^+$ VE-cadherin$^+$ CD41$^-$ cells (represented as V$^+$41$^+$ and V$^+$41$^-$ cells hereafter, respectively). V$^+$41$^+$ and V$^+$41$^-$ cells were sorted on day 6 and re-cultured on OP9 cells in the presence of hematopoietic cytokines. Both V$^+$41$^+$ and V$^+$41$^-$ cells produced HCs in culture, which contained erythrocytes, monocytes/macrophages and granulocytes lineages (Figure 6A, B). There was no significant lineage bias observed between HCs derived from these two populations (Figure 6C). However, in hematopoietic favorable culture condition, V$^+$41$^+$ cells produced a higher number of hematopoietic cells than V$^+$41$^-$ cells (Figure 6E, F, G). V$^+$41$^+$ and V$^+$41$^-$ cells were also able to produce B lymphocytes under culture conditions favorable for lymphopoietic differentiation (Figure 6A, B, D). When investigated for EC potential, V$^+$41$^+$ cells also produced sheet like EC colonies in the culture (Figure 6H). These observations suggested that V$^+$41$^+$ cells had superior hematopoietic potential but not fully committed to hematopoietic lineages, as they retained EC potential.

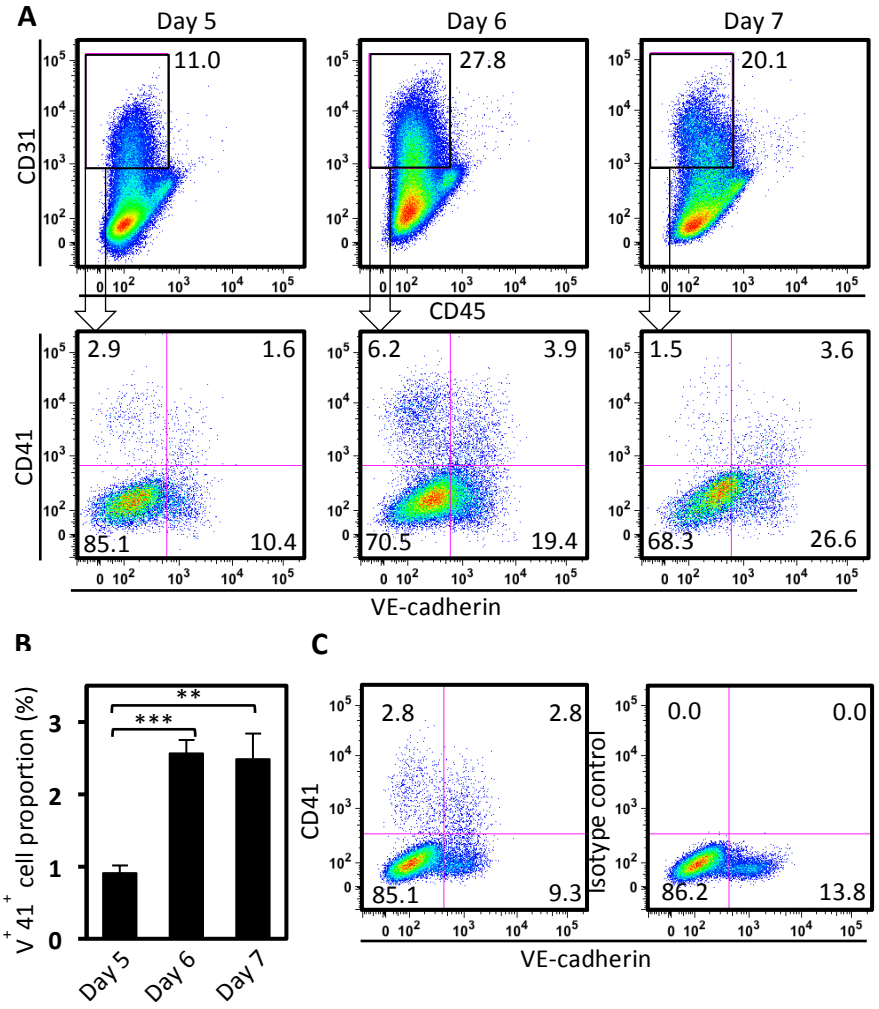


Figure 5: Kinetics of ESC-derived VE-cadherin$^+$ CD41$^+$ cells

(A) Expression of CD41 and VE-cadherin on CD45$^-$ CD31$^+$ cells. ESCs were cultured on OP9 cell layer for 5–7 days and analyzed by FACS. Upper panels show the expression of CD31 and CD45 on live singlet cells. Rectangles indicate the CD45$^-$ CD31$^+$ cell gate. Numbers indicate the percentage of cells in the rectangles. Lower panels show the expression of CD41 and VE-cadherin in the CD45$^-$ CD31$^+$ cells. Numbers indicate the percentage of cells in the quadrants. A representative result from five independent experiments is shown. (B) Proportion of V$^+$41$^+$ cells in total cells. n≥4. *p<0.05, **p<0.01 in Tukey's multiple comparison test. (C) Representative FACS pattern of differentiating ESCs. ESCs were cultured on OP9 cell layer for 6 days and analyzed by FACS. Left panel shows staining pattern of anti-CD41 and anti-VE-cadherin antibodies on CD45$^-$ CD31$^+$ cells. Right panel shows staining pattern of anti-VE-cadherin and isotype-matched control antibody instead of anti-CD41. Numbers indicate the percentage of cells in the quadrants. A representative result from three independent experiments is shown.

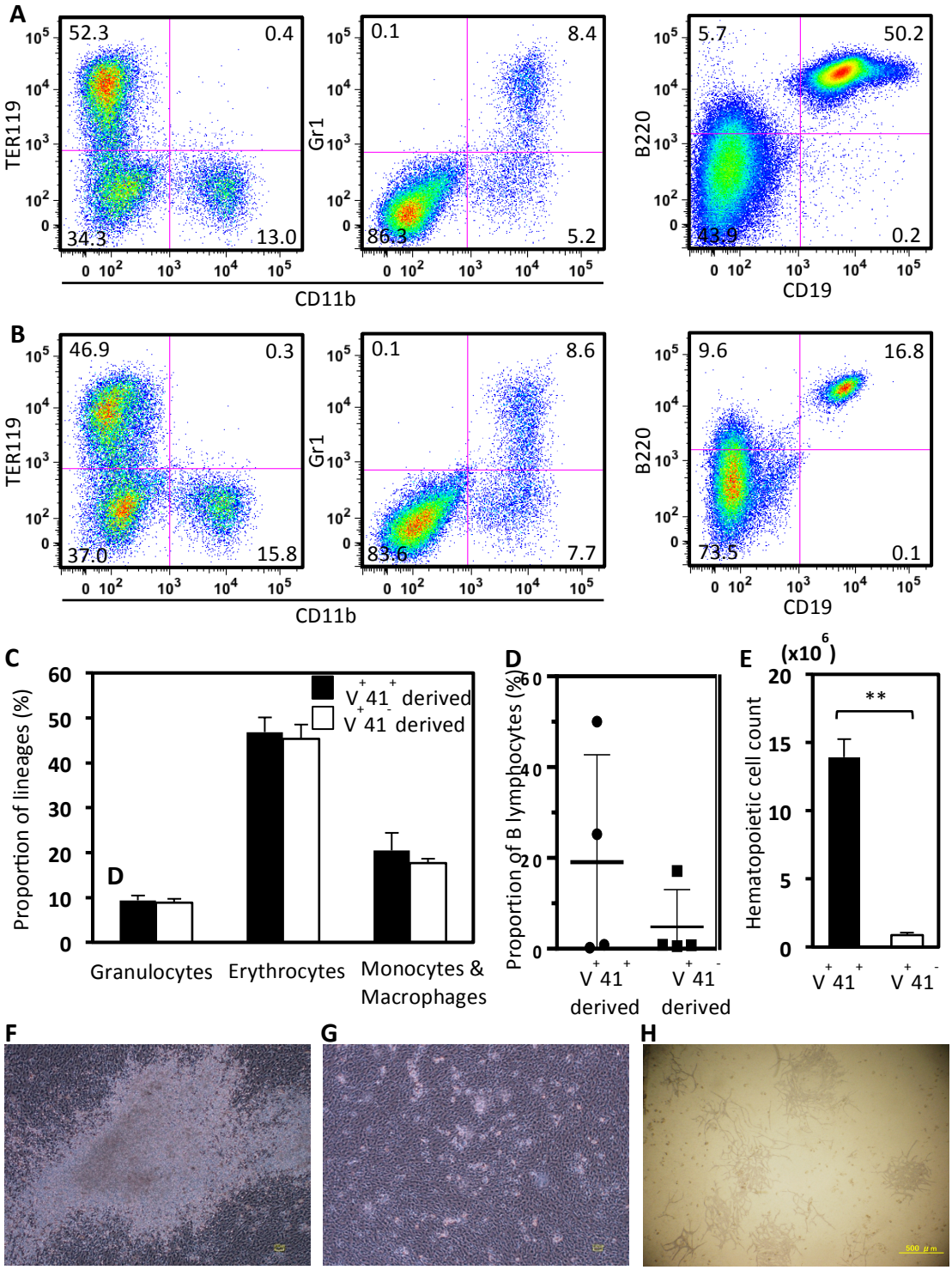


Figure 6. Hematopoietic and endothelial potential of ESC derived VE-cadherin$^+$ CD41$^+$ and VE-cadherin$^+$ CD41$^-$ cells

(A) Hematopoietic lineages derived from V$^+$41$^+$ cells. ESCs were cultured on OP9 cell layer for 6 days. V$^+$41$^+$ cells were sorted and cultured on OP9 cell layer in either the erythromyeloid or B lymphoid condition. TER119$^+$ erythrocytes (left panel), CD11b$^+$Gr-1$^-$ macrophages (middle panel), Gr1$^+$CD11b$^+$ granulocytes (middle panel) and CD19$^+$B220$^+$ B lymphocytes (right panel) were detected by FACS. A representative result from more than three independent experiments is shown. (B) Hematopoietic lineages derived from V$^+$41$^-$ cells. V$^+$41$^-$ cells were sorted from day 6 ESC culture and hemogenic

potential was analyzed. TER119$^+$ erythrocytes (left panel), CD11b$^+$Gr1$^-$ macrophages (middle panel), Gr1$^+$CD11b$^+$ granulocytes (middle panel) and CD19$^+$B220$^+$ B lymphocytes (right panel) were detected by FACS. A representative result from three independent experiments is shown. (C) Proportions of HC lineages derived from V$^+$41$^+$ cells and V$^+$41$^-$ cells. No significant difference was observed in the proportion between V$^+$41$^+$ cells and V$^+$41$^-$ cells by Student's T test. n=3. (D) Proportions of B lymphocytes derived from V$^+$41$^+$ cells and V$^+$41$^-$ cells. Thick horizontal bars indicate the mean of four independent experiments. No significant difference was observed in the proportion between V$^+$41$^+$ cells and V$^+$41$^-$ cells by Student's T test. n=4. (E) Hemogenic potential of day 6 V$^+$41$^+$ and V$^+$41$^-$ cells. A total of 12,000 cells from each fraction were cultured with OP9 cells and hematopoietic cytokines. HCs were counted after 7 days. n=4. $^{**}p<0.01$ in Student's T test. (F) HC colonies produced from V$^+$41$^+$ cells. A total of 1,000 V$^+$41$^+$ cells were sorted on OP9 cell layer and cultured with hematopoietic cytokines for 7 days. Bar indicates 100 mm. (G) HC colonies produced from V$^+$41$^-$ cells. A total of 1,000 V$^+$41$^-$ cells were sorted on OP9 cell layer and cultured with hematopoietic cytokines for 7 days. Bar indicates 100 mm. (H) EC colonies produced from V$^+$41$^+$ cells. A total of 1,000 V$^+$41$^+$ cells were sorted on OP9 cell layer and cultured for 4 days. Cells were immunohistochemically stained with anti-VE-cadherin antibody. Bar indicates 500 mm.

As V$^+$41$^+$ cells have potential to differentiate in to both HCs and ECs in culture, I investigated the expression of transcription factors in V$^+$41$^+$ and V$^+$41$^-$ cells. Both population of V$^+$41$^+$ and V$^+$41$^-$ cells expressed transcription factors of endothelial signature. Transcription factors express in endothelial cells including HECs, i.e. Sox17 (Corada et al., 2013; Bos et al., 2015) and Etv2 (Lee et al., 2008; Wareing et al., 2012), were expressed in both V$^+$41$^+$ and V$^+$41$^-$ cells without any significant difference (Figure 7A). Transcription factor Coup-TFII expression was lower than Sox17 and Etv2 levels in both populations (Figure 7A). Expression level of Foxc2, which is essential for arterial specification (Seo et al., 2006), was lower in V$^+$41$^+$ cells compared with V$^+$41$^-$ cells but still higher than undifferentiated ESCs (Figure 7B). In contrast, the transcription factors involved in hematopoietic specification of ECs were expressed in different levels between these two populations. Runx1, a transcription factor involved in hematopoietic specification (Okuda et al., 1996; Okada et al., 1998; Wang et al., 1996; Yokomizo et al., 2001), was expressed 10-fold higher in V$^+$41$^+$ cells than V$^+$41$^-$ cells (Figure 7C). Transcription factor Tal1 level was also found increased two-fold in V$^+$41$^+$ cells compared with V$^+$41$^-$ cells (Figure 7C). The expression pattern of transcription factors showed that, V$^+$41$^+$ cells express transcription factors of both hematopoietic and endothelial signature.

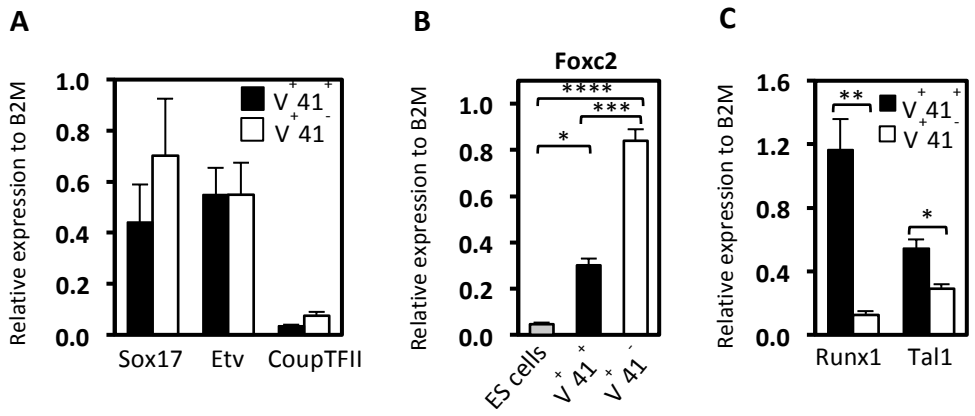


Figure 7. Expression of transcription factors in ESC derived VE-cadherin$^+$CD41$^+$ and VE-cadherin$^+$CD41$^-$ cells

(A) Expression of Sox17, Etv2 and Coup-TFII in V^+41^+ and V^+41^- cells. Expression level was normalized to that of beta-2-microglobulin. n≥3. No significant difference in Student's T test. (B) Foxc2 expression in undifferentiated ESCs, V^+41^+ cells and V^+41^- cells. n=3. *p<0.05, ***p<0.001, ****p<0.0001 in Tukey's multiple comparison test. (C) Expression of Runx1 and Tal1 in V^+41^+ and V^+41^- cells. n≥3. *p<0.05, **p<0.01 in Student's T test.

Altogether, these results demonstrated that ESC-derived V^+41^+ cells have a high hematopoietic potential to produce definitive HC lineages including B lymphocytes, yet retain a capacity of endothelial differentiation.

7.2. VE-cadherin$^+$CD41$^+$ cells contain bipotential progenitors of hematopoietic and endothelial cells

To compare the differentiation potential, I measured the progenitor frequencies among the VE-cadherin and CD41 subpopulations. The hematopoietic progenitor frequency was measured in V^+41^+ cells sorted from days 5 to 7 in culture. Progenitor frequency peaked at 1 in 9 V^+41^+ cells on day 6 and decreased thereafter (Table 4). At day 6, V^+41^- cells showed a 165-fold lower frequency than V^+41^+ cells (Table 4). Hematopoietic progenitor frequency of V^-41^+ cells was also measured during the same culture period. V^-41^+ cells were found to possess a four-fold lower frequency than that of V^+41^+ cells (Table 4).

The cell populations were then measured for their endothelial progenitor frequencies. In addition to their high hemogenic potential, V^+41^+ cells also possessed a high frequency of endothelial progenitors, as 1 in 5 cells produced EC colonies (Table 4). Endothelial progenitor frequency was stable across day 5 to 6 and slightly declined on day 7. In contrast to their

inferior hematopoietic progenitor frequency, Day 6 V^+41^- cells showed a comparable endothelial progenitor frequency as that of the V^+41^+ cells (Table 4). In comparison with the frequencies of V^+41^+ and V^+41^- cells, V^-41^+ cells showed only 177-fold lower endothelial progenitors that that of the V^+41^+ cells (Table 4). These results suggested that both hematopoietic and endothelial progenitors are highly enriched in V^+41^+ cells with the highest proportion of the progenitors at day 6. V^+41^- cells contained mostly endothelial progenitors and few hematopoietic progenitors. On the other hand, V^-41^+ cells contained mostly committed hematopoietic progenitors and very few endothelial progenitors, which is in line with the previous reports (Hashimoto et al., 2007).

Table 4. Progenitor frequencies in the cell populations derived from ES cells

Cell populations[a]	Progenitor frequencies (Number of independent experiments; Total number of wells tested)			Proportion of bipotential progenitors in HC progenitors
	Hematopoietic	Endothelial	Bipotential	
Day 5 V^+41^+	1/12 (4; 384[b])	1/5 (3; 18[d])	1/58 (3; 288[b])	19.2%
Day 6 V^+41^+	1/9 (6; 672[b])	1/5 (4; 24[d])	1/38 (5; 576[b])	20.8%
Day 7 V^+41^+	1/32 (3; 288[b])	1/8 (3; 18[d])	1/144 (3;288[b])	22.2%
Day 6 V^+41^-	1/1485 (3; 288[c])	1/6 (4; 24[d])		
Day 6 V^-41^+	1/39 (4; 384[c])	1/887 (3; 15[d])		

(a) ES cells were cultured on OP9 cell layer for 5–7 days. $CD45^-CD31^+$ cells were separated according to the expression of CD41 and VE-cadherin by FACS. VE-cadherin$^+$CD41$^+$ population is denoted by V^+41^+, VE-cadherin$^+$CD41$^-$ by V^+41^- and VE-cadherin$^-$CD41$^+$ by V^-41^+. Progenitor frequencies in the sorted cell populations were determined.

(b) Frequency was measured by single cell analysis. Single cells were deposited into each well of a 96-well OP9 plate. Cells were cultured in the presence of hematopoietic cytokines. After 7 days, cultures were fixed and stained with VE-cadherin antibody. Wells containing both an endothelial cell colony and hematopoietic cells were scored as bipotential progenitors.

(c) Frequency was measured by limiting dilution analysis. For VE-cadherin$^+$ CD41$^-$ cells, 200, 300, 400 and 500 cells per well were seeded on 96-well OP9 plates. For VE-cadherin$^-$ CD41$^+$ cells, 20, 40, 60 and 80 cells per well were seeded on 96-well OP9 plates. For each cell density, 24 wells were tested. After 7 days of culture with hematopoietic cytokines, wells containing hematopoietic cells were scored for determination of hematopoietic progenitor frequency.

(d) Frequency was measured by low density bulk culture. A total of 1,000 sorted cells were inoculated in each well of 6-well OP9 plates. After 4 days, cultures were fixed and stained with VE-cadherin antibody. Number of endothelial cell colonies was scored.

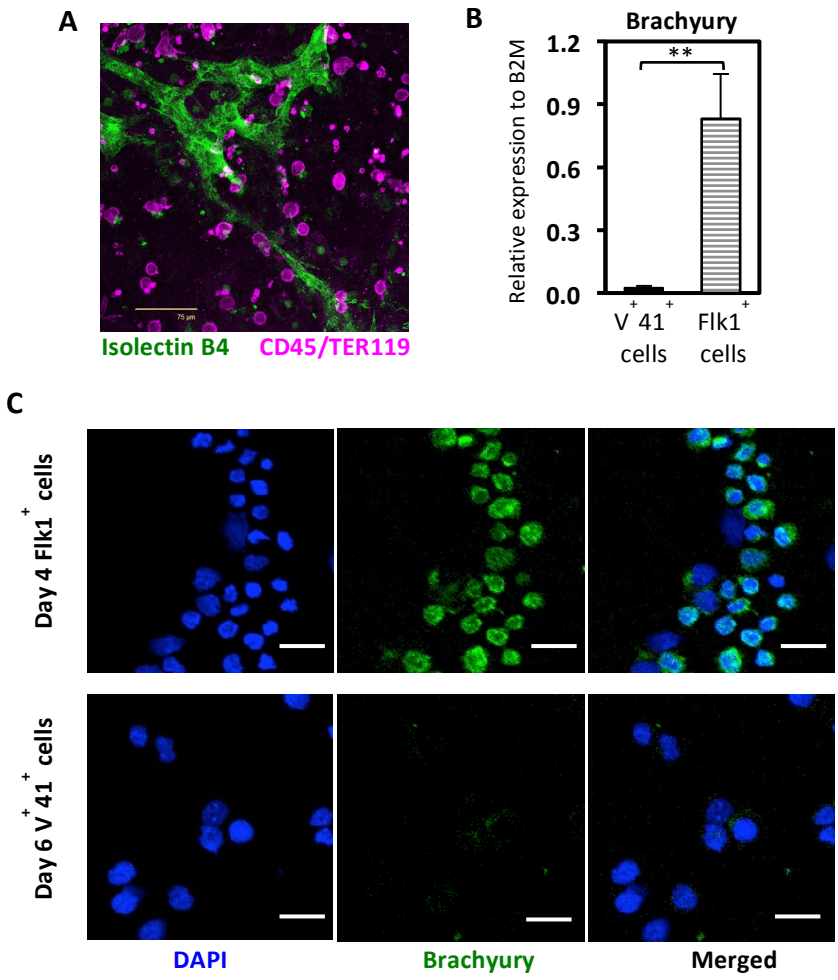


Figure 8. ESC-derived VE-cadherin$^+$CD41$^+$ cells contain bipotential hemogenic endothelial progenitors

(A) HCs and an EC colony produced from a single V$^+$41$^+$ cell. Day 6 V$^+$41$^+$ cells were individually deposited into wells of OP9 plate and cultured for 7 days with hematopoietic cytokines. Cultures were stained with isolectin B4 (green), anti-CD45 and anti-TER119 mAbs (magenta). Photomicrographs were taken using a TCS-SP2 confocal microscope. The scale bar indicates 75 mm. (B) Brachyury expression in day 6 V$^+$41$^+$ cells and day 4 Flk-1$^+$ cells. n=4. **p<0.01 in Student's T test. (C) Brachyury immunofluorescence staining. ESC-derived day 4 Flk-1$^+$ and day 6 V$^+$41$^+$ cells were cyto-spotted on glass slides. Specimens were fixed and stained with anti-Brachyury antibody (Abcam, ab20680, green). Nuclei were counterstained with DAPI (blue). Microphotographs were taken using a TCS-SP2 confocal microscope. Scale bars indicate 25 mm.

As V$^+$41$^+$ cells contained high frequency of both hematopoietic and endothelial progenitors, it urged me to examine whether this population contains bipotential progenitors. Bipotential progenitor is the cell that generates both HC and EC colonies from a single cell. To investigate this, single cells from the V$^+$41$^+$ population were sorted on each well of 96-well plates and cultured with OP9 cells in the presence of hematopoietic cytokines. After culturing for 7 days, I detected some wells containing both HC and EC colonies derived from

single cells (Figure 8A). Such wells were scored as bipotential progenitors. Bipotential progenitors were found across day 5 to 7 and constantly and the frequency was 1 in 38 cells from day 6 V^+41^+ cells. Around 20% of hematopoietic progenitors were detected as bipotential and this proportion was consistent from day 5 to 7 of the culture (Table 4).

Table 5. Frequency of brachyury expression

Population	Cells counted	Nuclear stained bright cells	Frequency of nuclear stained cells
Day 4 $Flk1^+$ cells	2760	984	1/2.80
Day 6 V^+41^+ cells	2219	12	1/184.92

Cells from each population were cyto-spotted on glass slides. Specimens were fixed and stained with anti-Brachyury antibody. Nuclei were counterstained with DAPI. Cells were then counted by taking microphotographs of different fields on each slide using a TCS-SP2 confocal microscope. Frequency was calculated as the ratio of total number of cells counted and number of cells stained.

As the mesoderm derived hemangioblast cells could also be bipotential for EC and HC lineages (Nishikawa et al., 1998a), I checked the *brachyury* expression in V^+41^+ cells. V^+41^+ cells did not express *brachyury*, which is a marker of mesodermal cells as evidenced by the expression in Flk-1^+ cells in day 4 culture (Figure 8B). To verify the fact that the bipotential progenitors were not a contamination of hemangioblast cells, I checked the *brachyury* expression in individual V^+41^+ cells and day 4 Flk-1^+ cells by immunofluorescence staining (Figure 8C). The frequency of Brachyury-expressing cells in the Flk-1^+ cells were expectedly high (~1/3) whereas, the frequency in V^+41^+ cell population was lower than that of the frequency of bipotential progenitors (1/185 vs. 1/38, Table 5). These results suggested that V^+41^+ cells contained bipotential progenitors that generate HC and EC lineages from single cells and they are a cell population distinct from immature mesodermal cells.

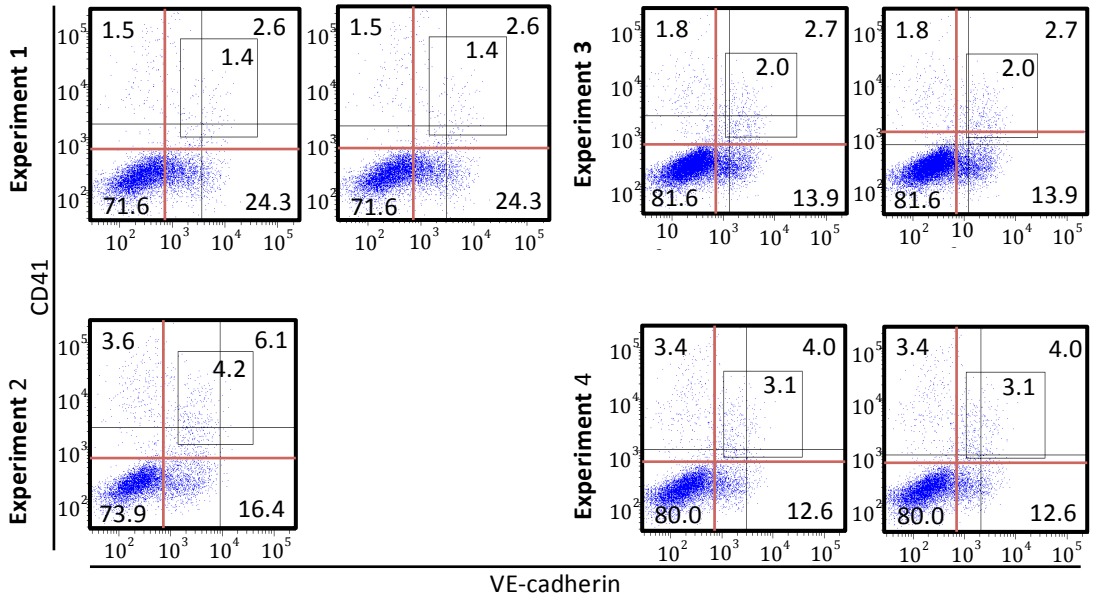


Figure 9. VE-cadherin and CD41 expression on the bipotential progenitors

Expression of VE-cadherin and CD41 in CD45$^-$ CD31$^+$ cells derived from ESCs. ESCs were cultured on OP9 cell layer for 6 days. Single V$^+$41$^+$ cells were sorted on OP9 plates using the index-sorting mode of FACSAria^{TM} III. Bipotential progenitors were marked as the wells containing both HCs and ECs after culturing for 7 days with hematopoietic cytokines. Expression level of VE-cadherin and CD41 was analyzed for individual bipotential progenitors by index-sorting data. Seven individual bipotential progenitors were analyzed from four independent index-sorting experiments and depicted in separate panels. Thick red lines indicate quadrant for negative and positive area determined by negative controls. Numbers indicate the percentage of cells in the quadrants. The rectangles in the VE-cadherin$^+$ CD41$^+$ quadrants indicate the sorting gate. Numbers in the sorting gates indicate the percentage of cells. The section point between the two thin black lines inside the sorting gate represents the position of the respective bipotential progenitor.

The bipotential progenitors were an important feature of V$^+$41$^+$ cells. To investigate whether the bipotential progenitors are evenly distributed in the V$^+$41$^+$ cells and show a significant level of CD41 and VE-cadherin expression, I used the index-sorting method. By using index-sorting, the wells identified as bipotential can be traced back to check the intensity of surface marker expression on the specific cell. Index-sorting experiments showed that the VE-cadherin expression on the bipotential progenitors was evenly distributed i.e., they expressed from high to low level of VE-cadherin (Figure 9). However, the level CD41 expression was constantly found low (Figure 9).

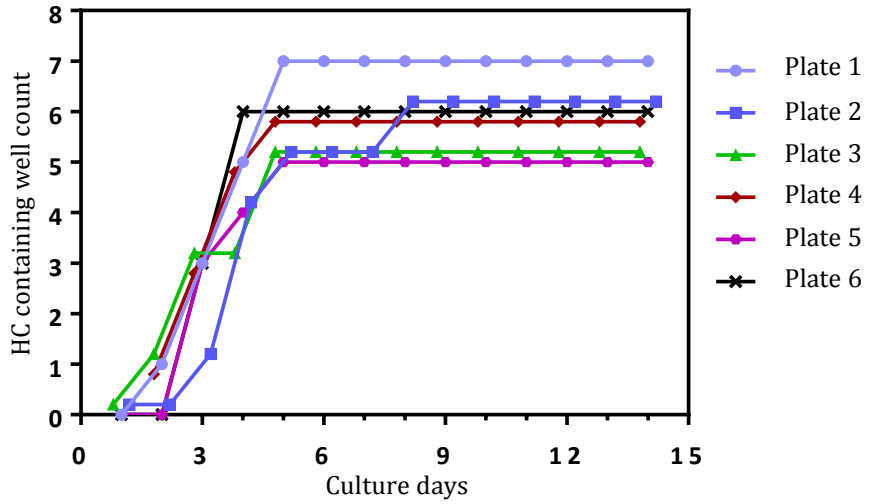


Figure 10. Kinetics of emergence of HC colonies from VE-cadherin$^+$ CD41$^+$ cells
Kinetic analysis of HC differentiation from V$^+$41$^+$ cells. ESCs were cultured on OP9 cell layer for 6 days. Single V$^+$41$^+$ cells were sorted on OP9 plates and cultured for 14 days with hematopoietic cytokines. Each plate was observed in every 24 hours to monitor the emergence of HC colonies. Results of six plates from two independent experiments are presented as a cumulative count of HC-positive wells in each plate.

In addition to their high hematopoietic potential, V$^+$41$^+$ cells retained the endothelial potential as well. To check the V$^+$41$^+$ cell derived ECs, whether they are bona fide ECs or the ECs that undergoing delayed hematopoietic differentiation, I checked the kinetics of HC differentiation of V$^+$41$^+$ cells. Kinetic analysis revealed that most of HC colonies emerged by day 5 of single cell culture and almost no new HC colony containing well was detected after the 7-day culture (Figure 10). This result suggested that EC colonies derived from V$^+$41$^+$ cells do not retain hemogenic potentials (Figure 10).

Altogether these observations indicate that V$^+$41$^+$ cells fulfilled the standards of HECs. V$^+$41$^+$ cells express surface markers and transcription factors of both EC and HC signatures. Most importantly V$^+$41$^+$ cells include the bipotential EC and HC progenitors. I thus propose that V$^+$41$^+$ cells are hemogenic endothelial cells derived *in vitro* from ESCs.

7.3. CXCR4 signal modulates the bipotential state of VE-cadherin$^+$CD41$^+$ cells

Enrichment of the bipotential progenitor frequency in the V$^+$41$^+$ cells is necessary to understand the regulatory mechanisms of the bipotential state of HECs. I sought to further enrich the bipotential progenitors in the V$^+$41$^+$ population by examining the surface expression of different markers. Results showed that CXCR4, the α-chemokine receptor was expressed on 20.6±1.7% (n=11) of V$^+$41$^+$ cells (Figure 11A). Frequencies determined by single cell analyses showed that HC and EC progenitors were evenly distributed in the CXCR4$^+$ and CXCR4$^-$ fractions in the V$^+$41$^+$ population (Figure 11B). This finding is consistent with the fact that CXCR4 expressing cells were uniformly distributed in the V$^+$41$^+$ cells.

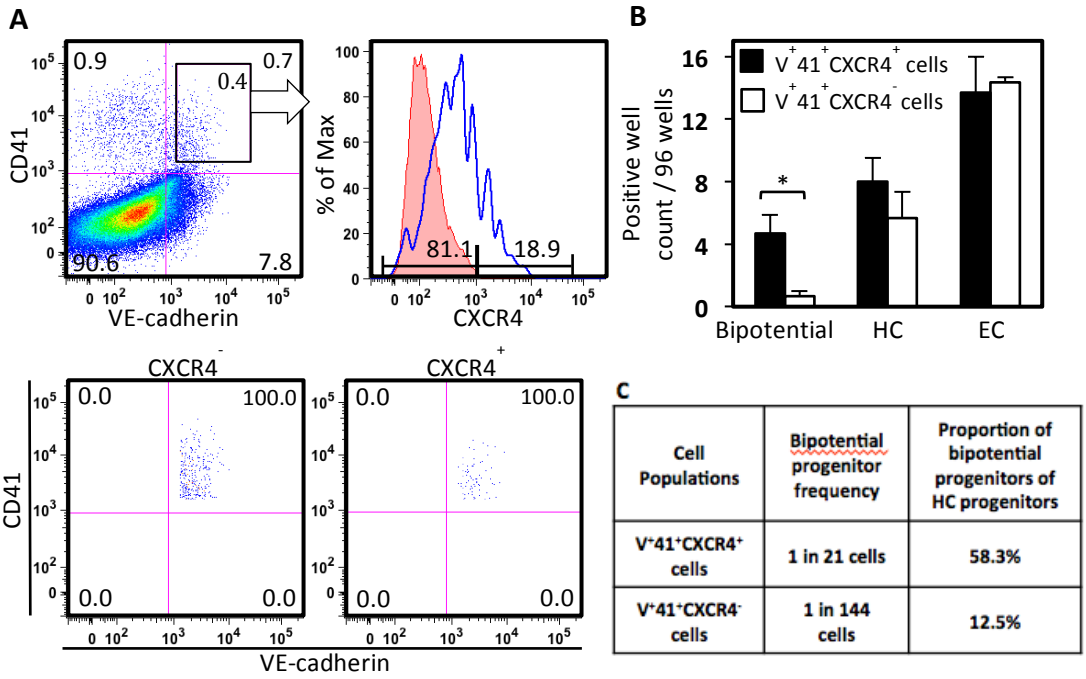


Figure 11. Bipotential hemogenic endothelial cells express CXCR4

(A) CXCR4 expression on V$^+$41$^+$ cells. ESCs were cultured on OP9 cell layer for 6 days and analyzed by FACS. Top left panel shows expression of CD41 and VE-cadherin on CD45$^-$ cells. Numbers indicate the percentage of cells in the quadrants. Top right panel shows expression of CXCR4 on the V$^+$41$^+$ cells. Thick blue line represents fluorescence intensity of CXCR4 staining and red area represents the unstained control. Numbers indicate the percentage of CXCR4$^+$ and CXCR4$^-$ cells. Bottom left and right panels show the expression pattern of VE-cadherin and CD41 on the CXCR4$^-$ and CXCR4$^+$ cells, respectively. A representative result from three independent experiments is shown. (B) Progenitor frequencies in the CXCR4$^+$ and CXCR4$^-$ fractions. Single cells of CXCR4$^+$ and CXCR4$^-$ fractions were sorted on each well of 96-well OP9 plate and cultured with hematopoietic cytokines for 7 days. Cultures were stained with anti-VE-cadherin antibody for the detection of ECs. n=3. *p<0.05 in Student's T test. (C) Bipotential progenitor frequency in CXCR4$^+$ and CXCR4$^-$ fractions. A total of 288 wells from three independent experiments were examined for each population.

The expression levels of VE-cadherin and CD41 are not significantly different between the $CXCR4^+$ and $CXCR4^-$ fractions (Figure 11A). In contrast, the frequency of bipotential progenitors was significantly different, which was four-fold higher in the $CXCR4^+$ fraction than the $CXCR4^-$ fraction of V^+41^+ cells (Figure 11B, C). In the $CXCR4^+$ fraction, more than half of the HC progenitors showed bipotential state (Figure 11C). These results suggest that CXCR4 expression marks most of the bipotential progenitors in the V^+41^+ cells. $V^+41^+CXCR4^+$ cells thus represent a further enriched population containing bipotential HECs.

As the bipotential progenitors were more concentrated in the CXCR4 expressing fraction of the V^+41^+ cells, I checked the functional role of the CXCR4 receptor on the progenitors. For this purpose, CXCL12, the ligand of CXCR4, was exogenously added in the culture during all 6 days of ESC differentiation (Figure 12A). The addition of exogenous CXCL12 did not affect the cell growth as the average cell count from treated and untreated flasks were not significantly different (Figure 13A). The proportion of V^+41^+ cells were also unaffected by the CXCL12 treatment (Figure 13B). Also no significant change in the proportion of $CXCR4^+$ fraction in V^+41^+ cells was observed (Figure 13C).

Surprisingly, however, the CXCL12 addition did affect the bipotential progenitors. The frequency of bipotential progenitors in the $CXCR4^+$ fraction was reduced to one-fourth than that of the untreated cultures (Figure 12A). Although the frequency of HC progenitors was unaffected, the CXCL12 treatment also reduced the EC progenitor frequency in the $CXCR4^+$ fraction of the V^+41^+ cells (Figure 12A). These results showed that CXCL12 addition in the ESC differentiation period decreases the frequency of bipotential and EC progenitors.

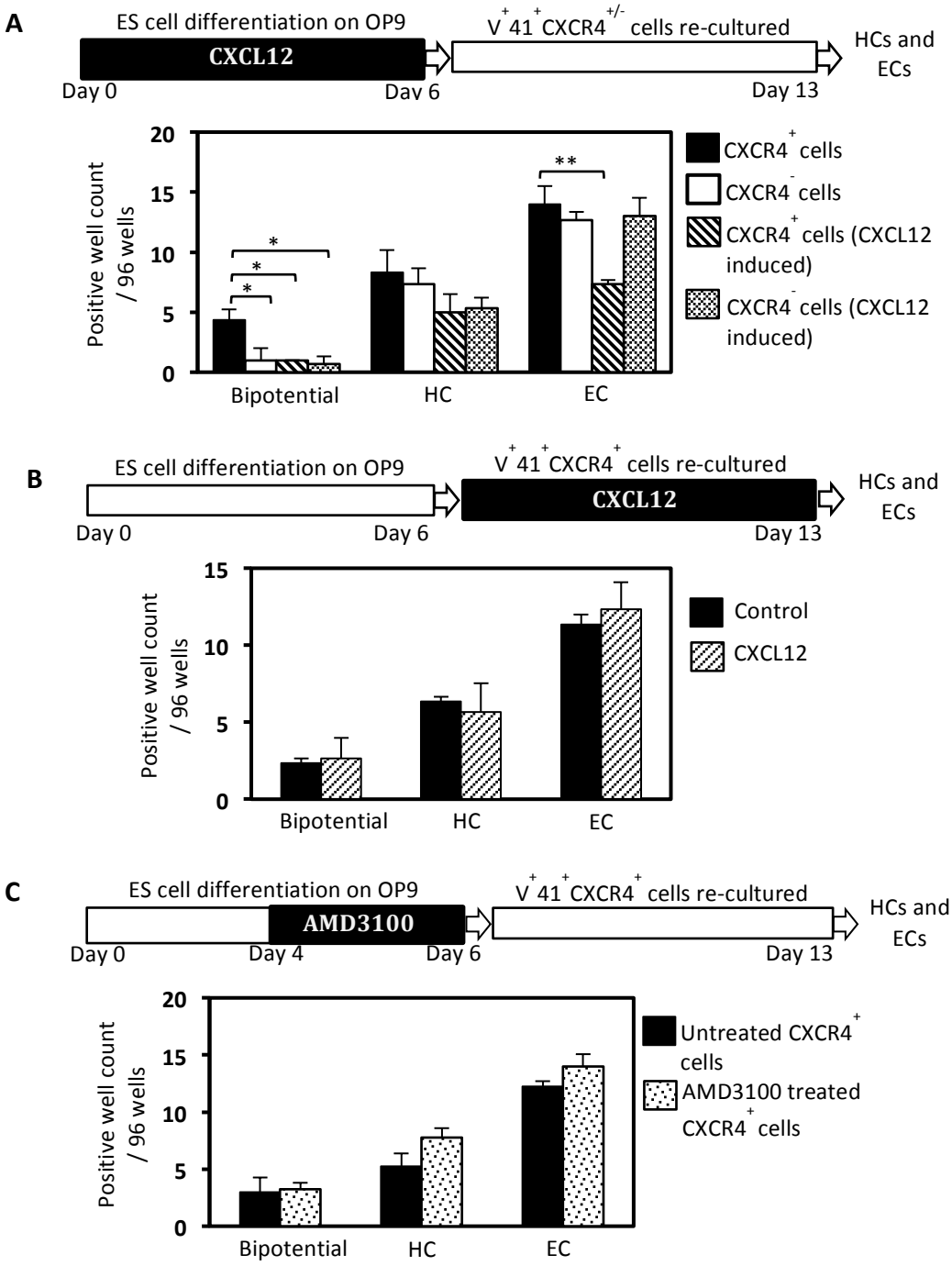


Figure 12. CXCR4 signaling reduces the frequency of bipotential progenitors

(A) Effect of CXCL12 treatment on the development of progenitors. ESCs were cultured on OP9 cell layer with or without 20 ng/ml CXCL12 for 6 days. Single cells of $CXCR4^+$ and $CXCR4^-$ fractions of V^+41^+ cells were sorted on each well of 96-well OP9 plates and cultured for 7 days. Progenitor frequency of each sample was compared with that of untreated $CXCR4^+$ cells using Dunnett's multiple comparison test. n=3. *p<0.05, **p<0.01. (B) Effect of CXCL12 treatment on the differentiation of HCs and ECs. Single cells of the $CXCR4^+$ fraction of V^+41^+ cells were sorted on each well of 96-well OP9 plates and cultured with or without 20 ng/ml CXCL12 for 7 days. No significant difference in progenitor frequency was observed between CXCL12-treated and untreated cultures by Student's T test. n=3. (C) Effect of CXCR4 inhibition on the development of progenitors. CXCR4 antagonist AMD3100 was added at the concentration of 50 nM during day 4–6 of ESC differentiation. Single cells of the $CXCR4^+$ fraction of V^+41^+ cells were sorted on each well of 96-well OP9 plates and cultured for 7 days. No significant difference in progenitor frequency was observed between untreated and AMD3100-treated $CXCR4^+$ cells by Student's T test. n=4.

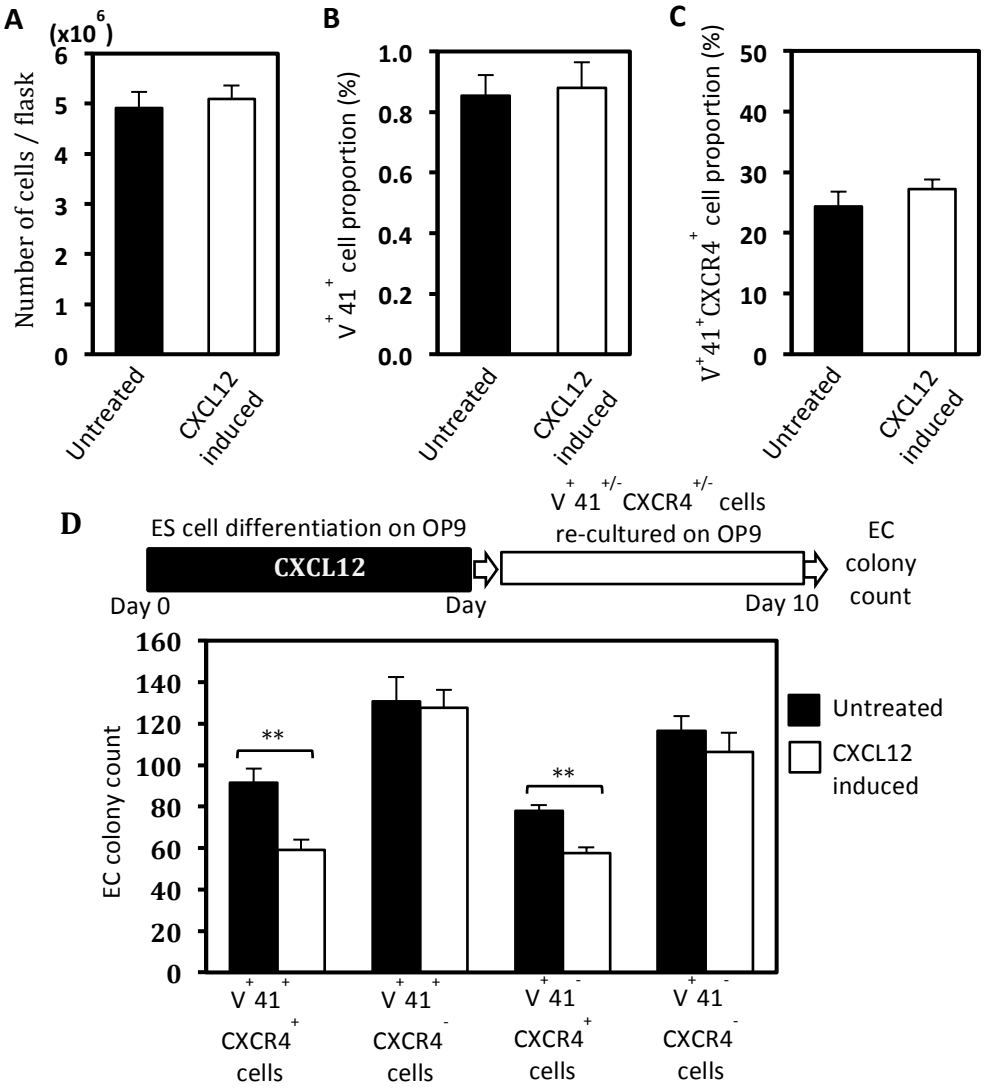


Figure 13. Effects of modulating CXCR4 signaling on ESC differentiation and EC potential
(A) Effect of exogenous CXCL12 addition on cell growth. CXCL12 (20 ng/ml) was added during ESC differentiation on OP9 cell layer. No significant difference in total cell count at day 6 was observed between untreated and CXCL12-treated cultures by Student's T test. n=5. (B) Effect of CXCL12 addition on the proportion of V^+41^+ cells. No significant difference was observed. n=5. (C) Effect of CXCL12 addition on the proportion of $CXCR4^+$ cells. No significant difference was observed. n=5. (D) Effect of CXCL12 addition during the ESC differentiation on the potential to form EC colonies. ESCs were cultured on OP9 cell layer for 6 days with and without exogenous CXCL12. $CXCR4^+$ and $CXCR4^-$ cells were sorted from both V^+41^+ and V^+41^- populations. 1000 sorted cells were then cultured on OP9 cell layer for 4 days. EC colonies were revealed by immunohistochemical staining with anti-VE-cadherin antibody, and the numbers of colonies were counted. EC count from each population was compared in the untreated and CXCL12 induced groups. n=4. $^{**}p<0.01$ in Student's T test.

Whether this suppressive effect of CXCL12 is specific for the $V^+41^+CXCR4^+$ cells or it affects all CXCR4 expressing EC progenitors was investigated next. Exogenous CXCL12 was added during ESC differentiation for all 6 days and $CXCR4^+$ and $CXCR4^-$ fractions were checked from both V^+41^+ and V^+41^- cells for EC potential. The frequency of EC progenitors was reduced in the $CXCR4^+$ fractions from both V^+41^+ and V^+41^- cells. $CXCR4^-$ fractions were however, remained unaffected (Figure 13D).

To investigate whether the exogenous CXCL12 affect the frequency of the isolated progenitors, CXCL12 was added during the EC/HC differentiation culture of sorted $CXCR4^+$ cells (Figure 12B). This would also prevent any carryover of the CXCL12 in the cultures of sorted $CXCR4^+$ cells and thereby influencing the differentiation of ECs and reducing the detection of bipotential cells. However, the frequencies of bipotential progenitors as well as EC and HC progenitors were not changed significantly by the post-sorting addition of exogenous CXCL12 (Figure 12B). This result indicated that CXCL12-CXCR4 interaction affects during the generation of the progenitors but does not influence the differentiation and maturation.

Altogether these results suggest that CXCR4/CXCL12 signaling attenuates the endothelial potential of CXCR4-expressing progenitors including HECs. , CXCR4/CXCL12 signaling thus negatively modulate the bipotential state of HECs.

To check whether inhibiting the endogenous CXCL12 in the culture had any effect on progenitor frequencies, I examined the effect AMD3100, a chemical antagonist of CXCR4 receptor (Hatse et al., 2002). AMD3100 was added in the last 2 days of ESC differentiation (Figure 12C), as during this period, ECs have been shown to be differentiatied from mesoderm (Endoh et al., 2002; Hashimoto et al., 2007). Although the duration of treatment was not the total culture period, the average number of cells retrieved per flask decreased in a dose-dependent manner at day 6 (Figure 14A). This suggests that the AMD3100 have cytotoxicity over cell growth. The proportion of V^+41^- and V^+41^+ cells remained stable irrespective of the concentration of AMD3100 (Figure 14B). However, the proportion of the $CXCR4^+$ fraction in V^+41^+ cells increased two-fold at the concentration of 50 nM but decreased in higher concentrations (Figure 14C). Nevertheless, the frequency of bipotential and EC and HC progenitors in the $CXCR4^+$ fraction remained unaffected by the treatment of 50 nM AMD3100 (Figure 12C). This result showed that blocking the endogenous CXCR4 signaling did not change the progenitor frequencies.

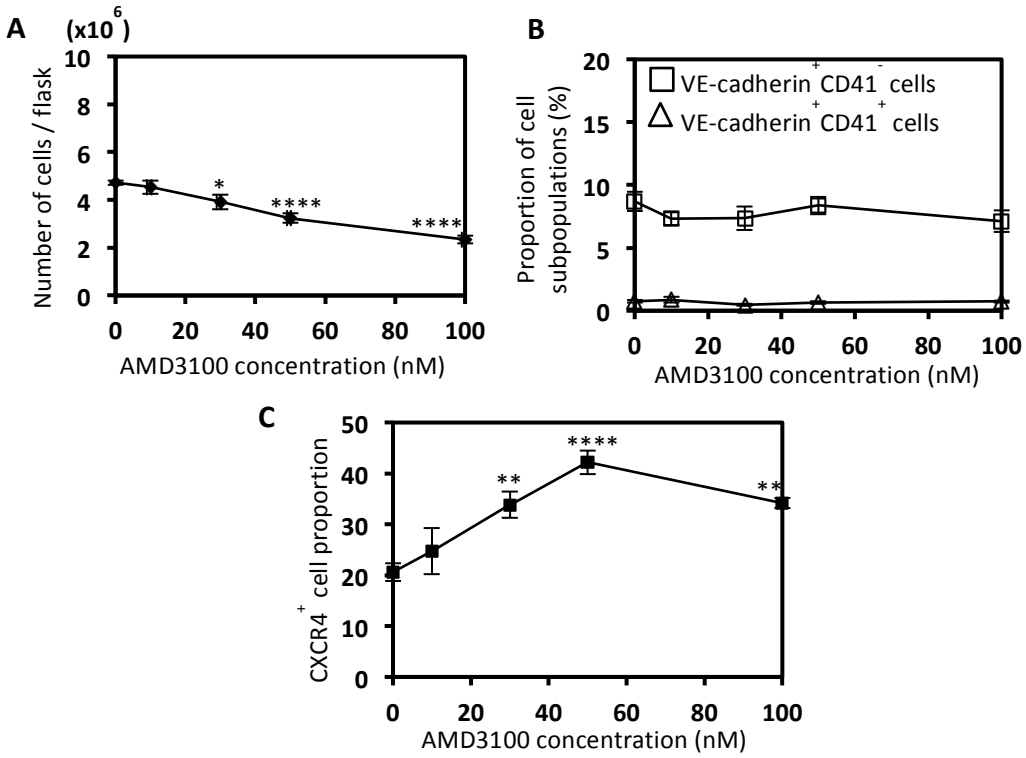


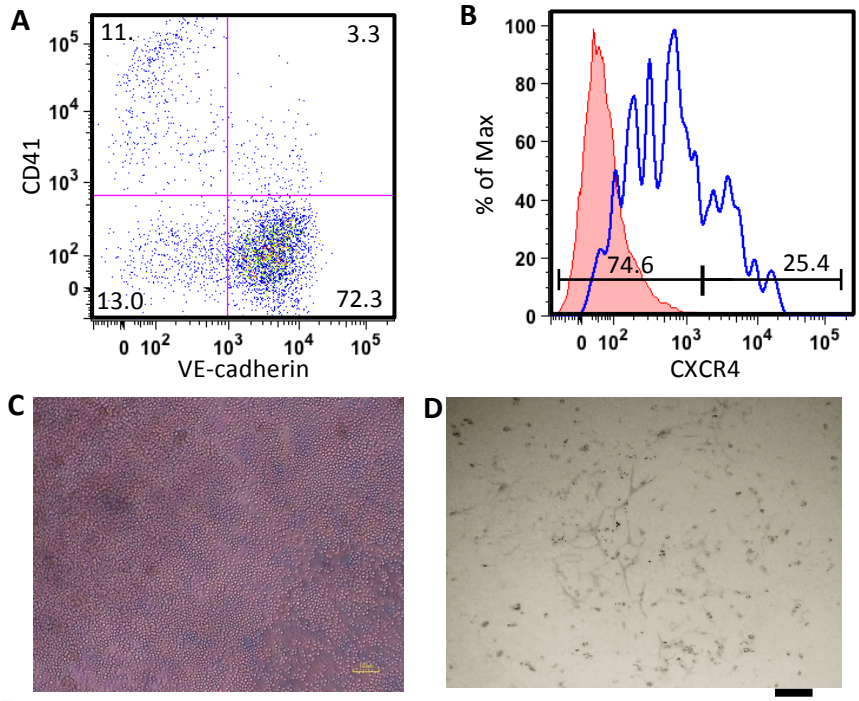
Figure 14. Effects of antagonizing CXCR4 signaling on ESC differentiation

(A) Dose-dependent reduction of cell growth by AMD3100 treatment. Different concentrations of AMD3100 were added during day 4–6 of ESC differentiation on OP9 cell layer. The number of cells harvested from each flask after 6 days was compared with untreated control flask using Dunnett's multiple comparison test. n≥3. *p<0.05, ****p<0.0001. (B) Proportion of V^+41^+ and V^+41^- cells induced with different concentrations of AMD3100. Proportion was compared with that of untreated control using Dunnett's multiple comparison test. n≥3. No significant difference was observed. (C) Proportion of the $CXCR4^+$ fraction in V^+41^+ cells. Proportion was compared with that of untreated control using Dunnett's multiple comparison test. n≥3. **p<0.01, ****p<0.0001.

I measured the concentration of endogenous CXCL12 using ELISA assays. The concentration of CXCL12 in the day 6 culture supernatant was found 487±94 pg/ml (n=3). These results may suggest that the endogenous level of CXCL12 was low than the level requires saturating the CXCR4 receptors. At this endogenous concentration of CXCL12, the effect of AMD3100 antagonist on the generation of progenitors was not noticeable.

7.4. Bipotential progenitors are less frequent in mouse embryos

Whether the bipotential progenitors can be detected *in vivo*, was the next question. To investigate this, I collected phenotypically equivalent V^+41^+ cells from the lower trunk of E10.5 mouse embryos (Figure 15A). E10.5 V^+41^+ cells were analyzed in single cell culture. Embryo-derived V^+41^+ cells contained a higher frequency of HC progenitors compared with ESC-derived V^+41^+ cells (Table 6, Figure 15C). In contrast, E10.5 V^+41^+ cells produced only faint and scattered EC colonies (Figure 15D), and the frequency was lower than ESC-derived V^+41^+ cells (Table 6). Moreover, wells with coexisting HC and EC colonies were extremely rare, only 2 out of 768 cells such example was found. This result suggested the rareness of bipotential progenitors in the embryos.



E

Cell Population	Hematopoietic progenitor frequency	Endothelial progenitor frequency	Bipotential progenitor frequency
E10.5 $V^+41^+CXCR4^+$ cells	1 in 8 cells	1 in 30 cells	<1 in 576 cells

Figure 15. Bipotential progenitors are not detected in the E10.5 embryos

(A) Expression of CD41 and VE-cadherin on $CD45^-$ $CD31^+$ cells isolated from mouse embryos. Single cell suspension was prepared from lower trunk of E10.5 embryos and analyzed by FACS. Numbers indicate the percentage of cells in the quadrants. A representative result from six independent experiments is shown. (B) CXCR4 expression on the E10.5 V^+41^+ cells. Thick blue line represents fluorescence intensity of CXCR4 staining and the red area represents the unstained control. Numbers

indicate the percentage of $CXCR4^+$ and $CXCR4^-$ cells. A representative result from three independent experiments is shown. (C) HCs derived from E10.5 V^+41^+ cells. Single E10.5 V^+41^+ cells were deposited into each well of 96-well OP9 plate and cultured with hematopoietic cytokines for 7 days. Bar indicates 100 mm. (D) EC colonies differentiated from E10.5 V^+41^+ cells. A total of 1,000 E10.5 V^+41^+ cells were inoculated in each well of 6-well OP9 plates. ECs were analyzed after 4 days by immunohistochemical staining with anti-VE-cadherin antibody. Bar indicates 100 mm. (E) Progenitor frequencies in the $CXCR4^+$ fraction of E10.5 V^+41^+ cells determined by single cell analysis. A total of 576 wells from three independent experiments were examined.

Table 6. Progenitor frequencies in the cell populations isolated from embryos

Cell populations[a]	Progenitor frequencies (Number of independent experiments; Total number of wells tested)			Proportion of bipotential progenitors in HC progenitors
	Hematopoietic	Endothelial	Bipotential	
E10.5 (EP) V^+41^+	1/4 (6; 768[b])	1/16 (3; 6[d])	1/384 (6; 768[b])	1%
E10.5 (EP) V^+41^-	<1/4800 (3; 288[c])	1/17 (3; 12[d])		
E10.5 (EP) V^-41^+	1/25 (3; 288[c])	1/10000 (3; 10[d])		
E9.5 (EP) V^+41^+	1/3 (3; 480[b])	1/96 (3; 480[b])		
E9.5 (YS) V^+41^+	1/3 (3; 384[b])	1/192 (3; 384[b])		
E8.5 (YS + EP) V^+41^+	1/6 (3; 576[b])	1/576 (3; 576[b])		

(a) E8.5 whole embryos or E9.5–10.5 lower trunk of embryos proper (EP) and yolk sac (YS) were collected. $CD45^-CD31^+$ cells were separated according to the expression of CD41 and VE-cadherin by FACS. VE-$cadherin^+CD41^+$ population is denoted by V^+41^+, VE-$cadherin^+CD41^-$ by V^+41^- and VE-$cadherin^-CD41^+$ by V^-41^+. Progenitor frequencies in the sorted cell populations were determined.

(b) Frequency was measured by single cell analysis. Single cells were deposited into each well of a 96-well OP9 plate. Cells were cultured in the presence of hematopoietic cytokines. After 7 days, cultures were fixed and stained with VE-cadherin antibody. Wells containing both an endothelial cell colony and hematopoietic cells were scored as bipotential progenitors.

(c) Frequency was measured by limiting dilution analysis. For VE-$cadherin^+$ $CD41^-$ cells, 20, 40, 60 and 80 cells per well were seeded on 96-well OP9 plates. For VE-$cadherin^-$ $CD41^+$ cells, 10, 20, 30 and 35 cells per well were seeded on 96-well OP9 plates. For each cell density, 24 wells were tested. After 7 days of culture with hematopoietic cytokines, wells containing hematopoietic cells were scored for determination of hematopoietic progenitor frequency.

(d) Frequency was measured by low density bulk culture. A total of 1,000 sorted cells were inoculated in each well of 6-well OP9 plates. After 5 days, cultures were fixed and stained with VE-cadherin antibody. Number of endothelial cell colonies was scored.

Expression pattern of CXCR4 on E10.5 V^+41^+ cells was similar to that of the ESC-derived V^+41^+ cells as the E10.5 V^+41^+ cells could also be separated by their surface expression of CXCR4 (Figure 15B). However, the selective enrichment in the CXCR4 expressing cells was not evident. A total of 576 cells from the $CXCR4^+$ population were analyzed, but no bipotential progenitors were found (Figure 15E).

Other phenotypically equivalent populations i.e. V^+41^- cells and V^-41^+ cells were detectable in the embryo-derived cells. E10.5 V^+41^- cells mostly contained EC progenitors and E10.5 V^-41^+ cells contained HC progenitors as expected (Table 6).

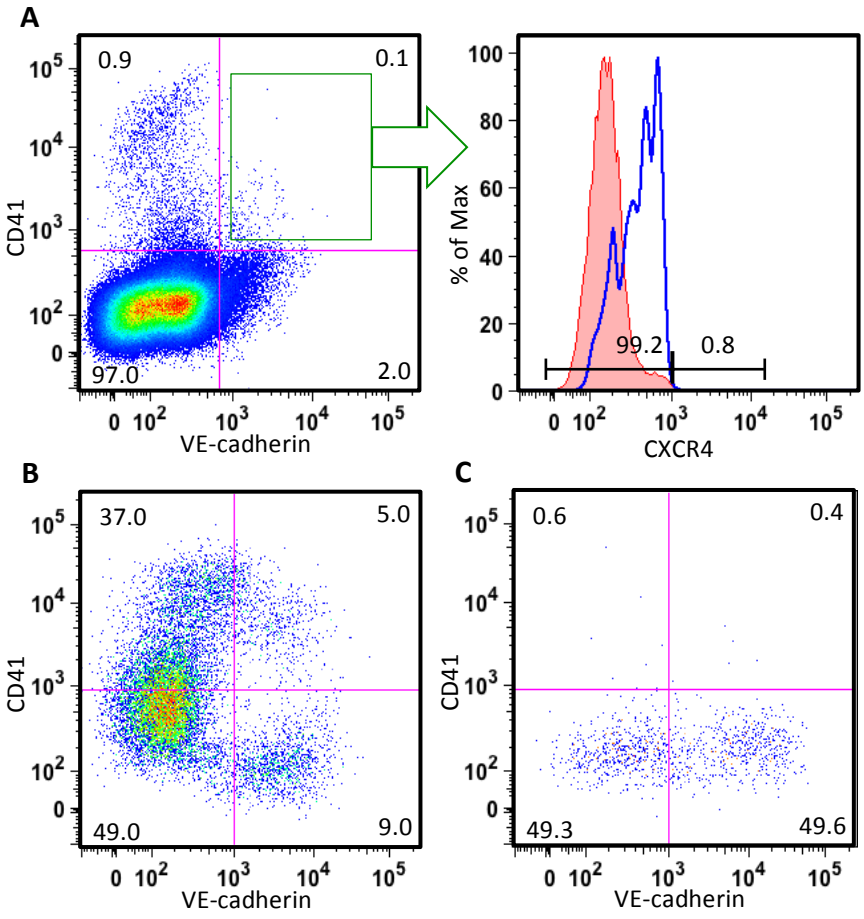


Figure 16. Bipotential progenitors are not detected in earlier embryonic populations
(A) Representative FACS pattern of E9.5 embryonic cells. Expression of CD41 and VE-cadherin on $CD45^-$ cells is shown in the left panel. Numbers indicate the percentage of cells in the quadrants. Right panel shows the staining pattern of anti-CXCR4 antibody on V^+41^+ cells. The thick blue line represents fluorescence intensity of CXCR4 staining and the red area represents the negative control. Numbers indicate the percentage of $CXCR4^+$ and $CXCR4^-$ cells. A representative result from three independent experiments is shown. (B) Expression of CD41 and VE-cadherin on $CD45^-$ $CD31^+$ cells in the E8.5 whole mouse embryos. Numbers indicate the percentage of cells in the quadrants. A representative

result from three independent experiments is shown. (C) Expression of CD41 and VE-cadherin on $CD45^-$ $CD31^+$ cells in the E7.5 whole mouse embryos. Numbers indicate the percentage of cells in the quadrants. A representative result from three independent experiments is shown.

Phenotypically equivalent V^+41^+ cells were also detected from E9.5 embryo proper and yolk sack cells. Both E9.5 embryo proper and yolk sac derived V^+41^+ cells were found to contain high potential for hematopoietic differentiation and very low endothelial potential (Table 6). Bipotential progenitor was not found in any E9.5 cells (Table 6). CXCR4 expressing cells were not detected in the E9.5 embryonic V^+41^+ cells (Figure 16A).

To investigate whether earlier embryo than E9.5 contain bipotential progenitors, I checked the V^+41^+ cells from E8.5 whole embryo (Figure 16B). However, E8.5 V^+41^+ cells also contained few EC progenitor and no bipotential progenitors were detected (Table 6). V^+41^+ cells were not detected in E7.5 whole embryos (Figure 16C).

Altogether these observations showed that bipotential progenitors are rarely detected in the embryos.

8. Discussion

The responsibility of constant maintenance and immune protection of the whole body cells is attributed on blood cells. HSCs are the cells that make constant renewal of blood possible by producing billions of new blood cells everyday. HSCs can self-renew, differentiate to a variety of specialized cells and mobilize out of the bone marrow into circulating blood while they make up only 1 in 100,000 cells in the blood stream. HSC transplantation is now routinely used to treat cancers and other immune disorders. Because of the tremendous importance of HSCs in therapy, *in-vitro* generation of HSCs from ESC culture have been one of the most exciting research area. Although murine ESCs in culture, given the right growth factors can produce almost every different blood cell type, generation of long term transplantable HSCs is a goal have not been achieved yet.

The fact that HSCs are derived from specialized endothelial cells i.e., HECs is now well accepted. However, the mechanism of EHT and the state of the transiting HECs is very important but still not clearly understood. For this purpose, it is necessary to narrow down the ESC derived EC population to an enriched level where it is possible to modulate their fate and thus understanding their regulatory mechanism of EHT.

The first question approached in this study was whether CD41 expression on VE-cadherin^{+} cells can identify HECs in the ESC culture system. The functional definition of HEC is, a cell that has endothelial characteristics with hemogenic potential. In the current study, I studied $V^{+}41^{+}$ cells differentiated from ESC cultured with OP9 stromal cells. $V^{+}41^{+}$ cells were found to possess 165 times more hematopoietic progenitors than that of the $V^{+}41^{-}$ cells. $V^{+}41^{+}$ cells are thus showed that CD41 expression enriches the hemogenic potential. The superior hemogenic potential of $V^{+}41^{+}$ cells was further supported by the observation that $V^{+}41^{+}$ cells expressed Runx1 at a 10-fold greater level compared with $V^{+}41^{-}$ cells. Bos *et al.* suggested that, increased expression of Runx1 characterizes the determination of hematopoietic fate in ECs of the embryonic aorta (Bos et al., 2015), which is in line with the results in this study. However, further studies with genetically tagged Runx1-expressing cells will be required to directly correlate Runx1 expression with hemogenic potential (Ng et al., 2010).

Unlike the hemogenic potential, which is concentrated in the $V^{+}41^{+}$ cells but not in the $V^{+}41^{-}$ cells, the ability to form EC colonies was evenly distributed in both populations. The expression pattern of transcription factors also supported this finding. The transcription

factors Sox17 and Etv2 were expressed at a similar level in both populations. Interestingly, however, the expression level of Foxc2 was lower in V^+41^+ cells than the level in V^+41^- cells. This might be associated with the gaining of hemogenic potential by the V^+41^+ cells. The results however, indicate that V^+41^+ cells are highly hemogenic and yet retained their endothelial identity. However, it could also be possible V^+41^+ cells represent a mixture of individual progenitors of both hematopoietic and endothelial lineages. Therefore, to fulfill the criteria of HEC definition, V^+41^+ cells should possess bipotential progenitors.

In this study, I demonstrated the existence of bipotential progenitors in V^+41^+ cells that generate both EC and HC colonies from a single cell in culture. Bipotential progenitors make up one out of five HC progenitors in the V^+41^+ cell population, which is a relatively high frequency. The real frequency of bipotential progenitors may even be higher than that estimated in this study because some progenitors could potentially take up hematopoietic fate before the first cell division. Such a progenitor is thus could not be counted as bipotential.

Nishikawa *et al*. reported that one out of 18 cells of ESC differentiated Flk-1^+ VE-cadherin$^+$ cells produces HCs in the single-cell culture (Nishikawa et al., 1998a). Additionally, one out of 3.6 of those HC progenitors was bipotential, and thus produced coexisting EC and HC colonies from one single progenitor. Although Nishikawa's report described these frequencies by examining the whole VE-cadherin$^+$ cell population, the observation appears to be consistent with results in this study. In this study, I found that the hemogenic potential was not evenly distributed in the VE-cadherin$^+$ cells. The CD41$^+$ subfraction of VE-cadherin$^+$ cells almost exclusively contained the hematopoietic and as well as the bipotential progenitors. Therefore, the results suggest that a major portion of VE-cadherin$^+$ cells, which acquired hematopoietic fate, is endowed with endothelial potential.

Conversely, the EC potential was evenly in the total VE-cadherin$^+$ cell population. Both V^+41^+ and V^+41^- cells have similar progenitor frequencies for ECs. The ECs differentiated from V^+41^+ cells may well be bipotential progenitor as well, which would later differentiate to HCs. However, the kinetic study of hematopoietic differentiation in this study showed that almost no delayed increase in the HC frequency. Therefore, the results suggest that ECs derived form V^+41^+ cells were bonafide endothelial cells.

An EC with the capacity to differentiate to HC lineages is considered as HEC. In embryo, the identity of an HEC can be proved by its morphology as well as anatomical location e.g., ventral wall of the dorsal aorta in AGM region. However, such a histological identification is not available for HECs in culture. Therefore, in order to show the endothelial

identity of the bipotential progenitors in the V^+41^+ population, it is necessary to distinguish them from the immature mesodermal cells, which could be contaminated during cell sorting and identified as a bipotential HEC. In this study, V^+41^+ cells were investigated for the expression of *brachyury*, which is a benchmark of mesodermal cells (Fehling et al., 2003). V^+41^+ cells were found to express very low level of *brachyury* in the real time PCR analysis compared to the $Flk1^+$ mesodermal cells. This data is supported by the immunofluorescence staining analyses, which showed that *brachyury* expression is abundant in the $Flk1^+$ cells whereas in V^+41^+ cells it was 5 times lower than the frequency of bipotential progenitors. As it has been suggested that in the ESC culture, mesoderm induction and endothelial differentiation occurs sequentially (Hashimoto et al., 2007), a bipotential progenitors of mesodermal origin would not express a meaningful level of VE-cadherin and CD41. The index-sorting analysis in this study traced back and confirmed that the bipotential progenitors express significant level of VE-cadherin and CD41 on their surface. Taken together, I would like to conclude that CD41 expression on VE-$cadherin^+$ cells identifies the HEC population in the ESC culture system.

The second question asked in this study was how the bipotential state of HECs is regulated by external signals. The results showed that bipotential progenitors could be further enriched, as they were more concentrated in the CXCR4 expressing fraction of the V^+41^+ cells. Although only one fifth of the V^+41^+ cells are CXCR4 expressing, more than half of HC progenitors in $V^+41^+CXCR4^+$ cells possessed the ability to produce EC colonies. Interestingly, the addition of CXCR4 ligand, CXCL12, during ESC differentiation to $V^+41^+CXCR4^+$ cells reduced the frequency of bipotential progenitors and unipotent EC progenitors in the $CXCR4^+$ cell. CXCL12 addition did not influence the frequency of HC progenitors. There are two possible ways to explain this finding. First, The CXCL12-CXCR4 interaction independently regulates the bipotential progenitors and unipotent EC progenitors via separate mechanisms. Second, endothelial differentiation of progenitors are suppresses by the CXCL12, thereby reducing both bipotential progenitors and EC progenitors. Although there is not evidence to distinguish these two possibilities in this study, I prefer the second explanation, as it appears to be more straightforward.

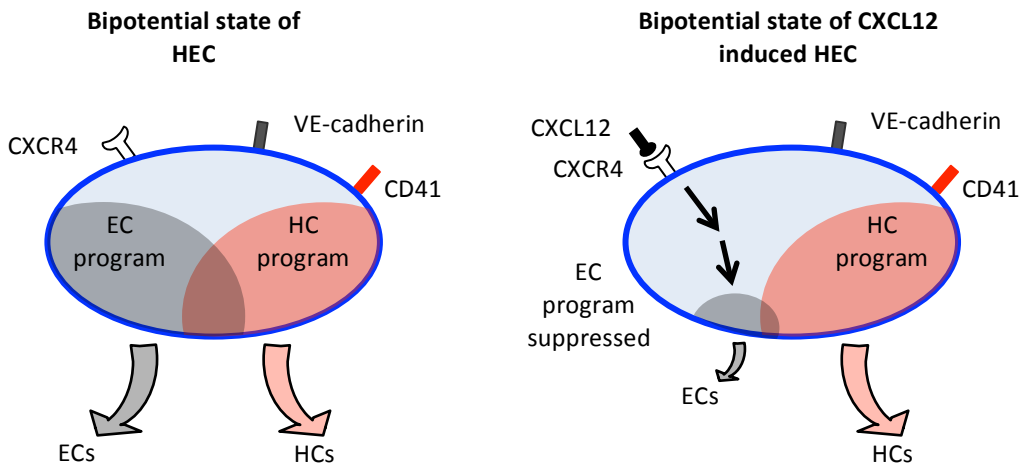


Figure 17: A model of cell fate control in the bipotential hemogenic endothelial cells
$CD45^{-}VE\text{-}cadherin^{+}CD41^{+}CXCR4^{+}$ cells derived from ESCs represent a hemogenic endothelial cell (HEC) population. HECs encompass the differentiation programs for both the endothelial cell (EC) and hematopoietic cell (HC) lineages within individual cells. A balance between these two programs provides an HEC with a bipotential state that allows the progeny of the cell to differentiate into both ECs and HCs. Fate of the bipotential HECs can be regulated by extrinsic factors. CXCL12/CXCR4 signaling suppresses the EC program independently of the hematopoietic fate. The CXCL12-induced suppression of EC fate makes HECs undetectable as bipotential progenitors.

The effect of CXCL12 on the frequencies of bipotential progenitors and EC progenitors may indicate that CXCL12-CXCR4 interaction inhibits the progenitors from forming EC colonies, which reduces the frequency of these progenitors. However, this possibility is unlikely, as the results showed that EC colony formation was not affected by the addition of CXCL12 after sorting of $V^{+}41^{+}CXCR4^{+}$. Alternatively, the negative effect of CXCL12 may indicate that CXCL12-CXCR4 interaction abrogates the commitment of bipotential progenitors and unipotent EC progenitors toward endothelial colony-forming cells.

Exogenous addition of CXCL12 was necessary to exert the suppressive effect on the frequencies of bipotential and EC progenitors. Chemical inhibition of the endogenous CXCR4 signaling that is present in the OP9 co-culture by AMD3100 did not affect any of the HC, EC and bipotential progenitor frequencies. This could suggest that endogenous CXCR4 signaling could be bypassed by some other mechanism. Although, CXCR4 is the primary receptor, it has been shown that CXCL12 can also bind receptor CXCR7 (Burns et al., 2006). As AMD3100 is a competitive antagonist of CXCL12 and the inhibitory effect is strictly confined to the CXCR4 (Hatse et al., 2002), this possibility cannot be entirely ruled out. However, there is no known report, which suggest that CXCR7 can rescue the CXCR4

signaling effect on differentiation. Moreover, in this study, the endogenous level of CXCL12 present in the OP9 co-culture of ESC differentiation was in the sub-Nano gram level. Therefore this results may suggest that the endogenous CXCL12 level is low to saturate the CXCR4 receptors thus the effect of inhibition was insignificant.

CXCL12-CXCR4 signaling is known to regulate formation of vascular network during organogenesis such as in the developing gastrointestinal tract and kidney, especially by promoting the remodeling of blood vessels to higher order structures (Tachibana et al., 1998; Takabatake et al., 2009). CXCL12-CXCR4 interaction was shown to regulate the morphology of endothelial tip cells in retinal vascular development at the cellular level (Strasser et al., 2010). Other studies have demonstrated that CXCR4 signaling regulate the migration of ECs during the formation of arteries in regenerating zebrafish fins as well as in the developing mouse limb skin (Xu et al., 2014; Li et al., 2013). To my knowledge, no direct evidence showing the regulation of EC specification and/or differentiation by CXCR4 signaling has been reported. However, several reports have proposed roles of CXCR4 signaling in the regulation of differentiation of other cell types. CXCR4 signaling upregulate of *MyoD* and *myogenin* expression in the C2C12 myoblast cell line, thereby induce the myogenic differentiation (Melchiona et al., 2010). Oligodendrocyte differentiation and re-myelination in a cuprizone-mediated demyelination model has been shown to be highly dependent on CXCR4 signaling (Patel et al., 2010).

Considering these known regulatory functions in cell differentiation, CXCR4 signaling is also suggested to control the acquisition of endothelial fate of ESC-derived progenitors. The CXCR4 regulation however, acts as suppressive rather than facilitating the EC differentiation. The CXCL12 mediated suppression of EC potential was not specific on the bipotential HECs but also on unipotent EC progenitors. The effect exerted independent of CD41 expression. Therefore, CXCR4 signaling appears to work in a common mechanism in both bipotential HECs and general EC progenitors. The endothelial and hemogenic program is supposed to coexist in equilibrium in a bipotential HEC (Figure 17). From the observations in this study, it can be suggested that endothelial and hemogenic programs can be independently regulated by external signals. CXCL12/CXCR4 signaling down-regulate the endothelial potential (Figure 17). At this point the signaling molecules that are involved in the regulate of maintain the hemogenic potential is not identified and left for future studies.

V^+41^+ cells equivalent to ESC derived cells were detectable in the yolk sac and embryo proper of mouse embryos from E8.5. V^+41^+ cells from either yolk sac or embryo proper from E8.5, E9.5 and E10.5 contained higher numbers of HC progenitors than ESC-

derived V^+41^+ cells. On the other hand, embryonic V^+41^+ cells produced smaller EC colonies at a lower frequency with close to no bipotential progenitors compared with ESC-derived counterpart. CXCR4 expression in V^+41^+ cells was not detectable before E10.5 and the enrichment of bipotential progenitors was not evident in embryonic $V^+41^+CXCR4^+$ population. Therefore, despite the common surface markers between the embryonic population and ESC-derived V^+41^+ cells, the differentiation potential of the embryonic V^+41^+ population is different and suggested to be more biased toward hematopoietic commitment. These characteristics of embryonic V^+41^+ cells are consistent with the previous observations (Rybstov et al., 2011).

The external signals regulating the differentiation is important for bipotential progenitors. Eilken *et al.* demonstrated that Flk-1^+ mesodermal cells isolated from E7.5 mouse embryos and re-cultured on the OP9 cell layer, Flk-1^+ cells differentiated into HCs by transiting through an intermediate hemogenic endothelial stage (Eilken et al., 2009). In the real-time imaging analysis of the culture, a sheet-like colony of ECs has shown to be generated from primary mesodermal cells. The ECs incorporated acetylated low-density lipoprotein and observed to express CD41. Both $CD41^+$ free-floating HCs and $CD41^-$ adherent sheet ECs observed to be differentiated from these EC colonies. This observation implies that given the microenvironment provided by the OP9 cell layer, primary mesodermal cells could produce $CD41^+$ bipotential HECs in the culture.

It can be assumed that the microenvironment provided by the OP9 stromal cells is responsible for allowing HECs to keep the bipotential state for an extended and detectable period of time. This can possibly explain the relatively high frequency of bipotential HECs observed in ESC-derived V^+41^+ cells. In embryonic microenvironment, however, due to difference in the signaling the bipotential state of embryonic HECs seems to be too transient to detect. The differentiation potential of *in vivo* V^+41^+ cells resembles the CXCL12-induced reduction of bipotential progenitors and EC progenitors in the ESC-derived V^+41^+ cells. This suggests that the EC program must be dominant enough to maintain equilibrium with the HC program so that they can be experimentally identified (Figure 17). It is plausible that the bipotential state in the embryonic environment is very transient and regulated by interplay of several signaling cascades. Whether CXCR4 signaling suppresses the EC potential of the bipotential progenitors, rendering their transient existence is remaining for future studies.

9. Concluding remarks

The first observation of hematopoietic activity in the wall of developing vertebrate aorta was made a century ago (Jordan, 1916; Sabin 1917). Since then a tremendous amount of work has been performed to understand the hematopoietic development from the vasculature. It is now established that hematopoietic and vascular lineages do not segregate until late in embryogenesis. Cells of the definitive hematopoiesis and HSCs are derived from the cells that already acquired endothelial phenotype i.e., HECs. The transition state of HECs when a progenitor has the potential to differentiate in either endothelial or hematopoietic lineage is the bipotential state. The bipotential state of HECs holds the key to the development of HSCs *in vitro*. In this study, the ESC derived $CD45^{-}CD31^{+}$ VE-$cadherin^{+}$ $CD41^{+}$ cells are shown as the HECs that contain a substantial total of bipotential progenitors. Bipotential progenitors were further enriched in the CXCR4 expressing fraction of these cells.

The development of HSCs from the HECs is a complex event controlled by a number of different developmental signals. Here I showed that, the hematopoietic and endothelial potential of the ESC derived bipotential HEC progenitors are independently regulated. CXCR4 signaling regulates the endothelial fate by suppressing endothelial potential. Identifying these regulatory signals is important for modulating the HEC differentiation. Further study will be required to reveal the mechanism by which the hematopoietic fate of the bipotential progenitors can be independently regulated. Combination of these mechanisms will allow us to modulate the bipotential HECs and control their differentiation to induce the first transplantable HSCs *in vitro*.

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