Investigating the Stage-Specific Roles of NOTCH Signaling during Hemato-endothelial Development from Human Pluripotent Stem Cells

By

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ABSTRACT

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Under the supervision of Professor Igor I. Slukvin

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The technology surrounding human pluripotent stem cells (hPSCs), including human embryonic and induced pluripotent stem cells (hESCs and hiPSCs) have opened the opportunity to generate blood cells in vitro for research and therapeutic applications. However, the effectiveness of hPSC-derived hematopoietic stem cells (HSCs) and their progeny depends on their long-term engraftment potential, which has thus far been unobtainable. The ability to generate long-term engrafting definitive HSCs (LT-dHSCs) in vitro depends on our ability to recapitulate in vivo development. The current understanding of mammalian hematopoietic development is that there are two waves: The transient extra-embryonic primitive wave that starts in blood islands of the yolk sac, and the life-long intra-embryonic definitive wave that emerge from hemogenic endothelium (HE) in the dorsal aorta by the process of endothelial-to-hematopoietic transition (EHT). Molecular profiling analyses comparing hPSC-derived HPs to de novo LT-dHSCs have identified aberrant NOTCH signaling activation, a critical signaling pathway found to be required for definitive hematopoiesis in animal models, though exactly what role NOTCH signaling plays during HE development, EHT, and LT-dHSC specification have been contended. While specific stages analogous to in vivo murine hematopoietic development were previously identified during hPSC differentiation by co-culturing hPSCs on the OP9 mouse stromal cell line, the undefined and variable conditions of this method impedes the ability to identify, isolate, and investigate individual signaling pathways during hemato-endothelial differentiation. Thus, we have created a directed chemically defined two-dimensional differentiation platform that reproduces all of the developmental stages of hPSC hematopoietic-endothelial differentiation. In addition, we have identified that Tenascin C is critical for
definitive hematopoiesis from hPSCs. Next, we adapted our defined directed differentiation platform in order to manipulate NOTCH signaling by using an immobilized DLL1-Fc ligand for increased activation of NOTCH, and the small molecule γ-secretase inhibitor, DAPT, to inhibit NOTCH. We found that hPSC-derived D4 CD144⁺CD43⁻CD73⁻DLL4⁻ HEP in secondary culture conditions with DLL1-Fc increases EHT and HP expansion, while adding DAPT decreases hematopoietic activity. Kinetic analysis of EHT following culture of HE with NOTCH signaling modulators revealed that NOTCH activation increases a unique population of CD144⁺CD43⁻CD73⁻ arterial-like HE that are DLL4⁺Notch⁺hi and only undergoes EHT with increased NOTCH activation. Continued NOTCH activation post-EHT increased CD34⁺CD43⁺ HP expansion while maintaining CFC-GEMM potential, increased T-cell potential, and α- and β-globin expressing erythrocyte potential. Overall, the methods developed in this work have created a defined platform on which to study the isolated effects of the NOTCH signaling pathway. We have identified that NOTCH activation increases arterial HE specification, is necessary for subsequent EHT, and facilitates definitive-type HP specification and expansion. In conclusion, we are one step closer to generating human HSCs from hPSCs with long-term engraftment and multilineage potential.
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<th>Description</th>
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<tbody>
<tr>
<td>7-AAD</td>
<td>7-Aminoactinomycin D</td>
</tr>
<tr>
<td>α-MEM</td>
<td>α Minimum Essential Medium</td>
</tr>
<tr>
<td>ACT</td>
<td>Adoptive Cell Transfer</td>
</tr>
<tr>
<td>ActA</td>
<td>Activin A</td>
</tr>
<tr>
<td>AGM</td>
<td>Aorta-Gonado-Mesonephros</td>
</tr>
<tr>
<td>AHP</td>
<td>Angiogenic Hematopoietic Progenitor</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>APLNR</td>
<td>Apelin Receptor</td>
</tr>
<tr>
<td>BM</td>
<td>Bone Marrow</td>
</tr>
<tr>
<td>BMP4</td>
<td>Bone Morphogenic Protein 4</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAR</td>
<td>Chimeric Antigen Receptor</td>
</tr>
<tr>
<td>CB</td>
<td>Cord Blood</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CDLC</td>
<td>Chemically Defined Lipid Concentrate</td>
</tr>
<tr>
<td>CFC</td>
<td>Colony Forming Cell</td>
</tr>
<tr>
<td>CFC-E</td>
<td>Colony Forming Cell-Erythrocyte</td>
</tr>
<tr>
<td>CFC-G</td>
<td>Colony Forming Cell-Granulocyte</td>
</tr>
<tr>
<td>CFC-GEMM</td>
<td>Colony Forming Cell-Granulocyte/Granulocyte/Macrophage/Megakaryocyte</td>
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<td>CFC-GM</td>
<td>Colony Forming Cell-Granulocyte/Macrophage</td>
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<td>CFC-M</td>
<td>Colony Forming Cell-Macrophage</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>Collagen IV</td>
</tr>
<tr>
<td>CXCR4</td>
<td>CXC Chemokine Receptor 4 (Fusin)</td>
</tr>
<tr>
<td>Cy</td>
<td>Cyanine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4'-6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DLL1</td>
<td>Delta-like Ligand 1</td>
</tr>
<tr>
<td>DLL1-Fc</td>
<td>Delta-like Ligand 1-IgG conjugate</td>
</tr>
<tr>
<td>DLL4</td>
<td>Delta-like Ligand 4</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxycycline</td>
</tr>
<tr>
<td>E8</td>
<td>Essential 8</td>
</tr>
<tr>
<td>EB</td>
<td>Embryoid Body</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial Cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epithelial Growth Factor</td>
</tr>
<tr>
<td>EHT</td>
<td>Endothelial-to-Hematopoietic Transition</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>F12</td>
<td>Ham's F-12 Media</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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FGF2 Fibroblast Growth Factor 2
FITC Fluorescein Isothiocyanate
FLT3L Fms-like tyrosine kinase 3 ligand
GFP Green Fluorescent Protein
GVHD Graft-Versus-Host-Disease
HB Hemangioblast
HB-CFC Hemangioblast Colony-Forming Cell
HE Hemogenic Endothelium
HEP Hemogenic Endothelial Progenitor
hESC human Embryonic Stem Cell
hiPSC human induced Pluripotent Stem Cell
HP Hematopoietic Progenitor
hPSC human Pluripotent Stem Cell
HSC Hematopoietic Stem Cell
HSCT Hematopoietic Stem Cell Transplant
HVMP Hematovascular Mesoderm Precursor
ICM Inner Cell Mass
IF9S IMDM/F12+9 Supplements
IGF-I Insulin-like Growth Factor I
IGF-II Insulin-like Growth Factor II
IL-3 Interleukin 3
IL-6 Interleukin 6
IL-7 Interleukin 7
IMDM Iscove's modified Dulbecco's Medium
KDR Kinase Domain Receptor (VEGFR2, FLK-1, CD308)
LT-dHSC Long-Term Engrafting Definitive Hematopoietic Stem Cell
LiCl Lithium Chloride
Lin Lineage
mAb monoclonal Antibody
MACS Magnetically Activated Cell Sorting
MB Mesenchymoangioblast
mESC mouse Embryonic Stem Cell
MPP Multipotent Progenitor
mPSC mouse Pluripotent Stem Cell
MTG Monothioglycerol
non-HEP non-Hemogenic Endothelial Progenitor
NSGW Nod/SCID/IL2-γ chain^-/-/W41^-/-
p-Sp para-Splanchnopleura
PAGE Polyacrylamide Gel Electrophoresis
PB Peripheral Blood
PBMC Peripheral Blood Mononuclear Cell
PBS Phosphate Buffer Saline
PDGFRα Platelet Derived Growth Factor α (CD140α)
PE Phycoerythrin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>PerCP</td>
<td>Peridinin Chlorophyll</td>
</tr>
<tr>
<td>PM</td>
<td>Primitive Mesoderm</td>
</tr>
<tr>
<td>PVA</td>
<td>Polyvinyl Alcohol</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative Real Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated Protein Kinase</td>
</tr>
<tr>
<td>RPL13a</td>
<td>Ribosomal protein L13A</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem Cell Factor</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>TenC</td>
<td>Tenascin C</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor-β</td>
</tr>
<tr>
<td>TPO</td>
<td>Thrombopoietin</td>
</tr>
<tr>
<td>VE-Cadherin</td>
<td>Vascular Endothelial Cadherin</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VTN</td>
<td>Vitronectin</td>
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CHAPTER 1: GENERAL INTRODUCTION
1.1 History and Therapeutic Applications of Hematopoietic Cell Transplant and Transfusion

Blood transfusions and hematopoietic stem cell transplants are common methods of treatment for a wide range of hematological diseases and disorders today, and have been the objects of much research since their initial development. The history of blood transfusion and hematopoietic cell transplant dates back to 1818 when a British physiologist injected a patient suffering from internal bleeding with blood from several donors (Baskett, 2002; Holland, 1998). In 1959, the first hematopoietic stem cell transplant (HSCT) was conducted to treat end-stage leukemia (Thomas et al., 1959). These early procedures have been supplemented by a number of additional discoveries: the discovery of blood groups in the 1900’s (Heal et al., 2005), the identification and genotyping of the 5 major Human Leukocyte Antigen complexes HLA-A, -B, -C, -DR, and -DQ (Van Rood et al., 1958; Van Rood and Van Leeuwen, 1963), the establishment of the first blood banks beginning in 1914 (Hess and Schmidt, 2000), and the registration of nearly 27 million HSC donors have contributed to the accessibility of blood transfusions and HSCT therapies (Gragert et al., 2014; Juric et al., 2016). Bone marrow extraction is being replaced by apheresis of mobilized HSCs in the peripheral blood (PB) using cytokines (e.g. granulocyte-colony stimulating factor) or CXCR4 antagonists (e.g. plerixafor), making donations easier and safer. (DiPersio et al., 2009; Dreger et al., 1996; Horwitz et al., 2012; Schmitz et al., 1996). In the United States alone, over 6 million units of blood are used for transfusion every year to treat blood loss and assist in surgery (Whitaker et al., 2016), whereas almost 50,000 HSCTs are performed annually to treat a wide range of diseases, including hematological malignancies, non-hematological malignancies, and hematological genetic disorders (Copelan,
Cancers ranging from multiple myeloma, Hodgkin’s disease, neuroblastoma and ovarian cancer, can be now treated with myeloablation chemotherapy followed by autologous HSCT from purified HSCs that have little to no risk of contaminating leukemia stem cells (LSCs) (Brenner et al., 1993; Copelan, 2006; Krishnan et al., 2000; Krivanova et al., 2004; Lilleby et al., 2006; Stewart et al., 2001). Similarly, hematological malignancies such as acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML), chronic lymphocytic leukemia (CLL), can be treated with chemotherapy followed by allogeneic HSCT, which takes advantage of the donor’s low level immunogenicity to target leukemia initiating stem cells (Martino et al., 2002; Pulsipher et al., 2004; Schetelig et al., 2003; van Rood et al., 2012). In addition, allogeneic HSCT can also be used to treat inherited metabolic disorders such as osteoporosis or leukodystrophy, inherited hemaglobinopathies such as aplastic anemia, Fanconi’s anemia, Diamond-Blackfan anemia, and sickle cell anemia, and bone marrow failure states that cause severe combined immunodeficiency (SCID) (Copelan, 2006; Delfini et al., 1986; Di Bartolomeo et al., 1991; Rabusin et al., 2000; Sykes and Nikolic, 2005; Tani, 2016). Recently, HSCs with a dominant negative mutation of CCR5 were transplanted into HIV-positive patients and successfully cleared the patient of the virus (Allers et al., 2011; Hutter et al., 2009).

Early allogeneic HSCT revealed a previously undetermined benefit to transplanting contaminating lymphocytes. Mouse models demonstrated that transferring cytotoxic CD8⁺ T cells (CTLs) with tumor-recognizing T-cell receptors (TCR) can eliminate certain tumors with unique antigens (Dudley and Rosenberg, 2003; Perica et al., 2015). In combination, recent advancements in cellular immunology and molecular biology have introduced a new type of hematopoietic transplant: Adoptive cell transfer (ACT). Pre-clinical trials of ACT began when
interleukin-2 (IL-2) was found to be a potent stimulator of proliferation in CTLs (Muul et al., 1987; Topalian et al., 1987). Today, ACT has been successfully used to treat patients with melanoma (Chapuis et al., 2012; Rosenberg et al., 1994) and Epstein-Barr-virus-induced lymphoproliferative diseases (Brentjens et al., 2011; Rooney et al., 1998). Recent advancements in the human genome modification, particularly with CRISPR/Cas9, have expanded the possibilities of genetically engineering T-cells expressing chimeric antigen receptors (CARs) to recognize and destroy tumor cells instead of relying on purified tumor-specific CTLs from patients (Bilal et al., 2015; Jakobisiak and Golab, 2010; Liu et al., 2016; Morgan and Kakarla, 2014). Cytotoxic T lymphocytes with anti-CD19 recognizing TCRs have been engineered and were successfully used to clear patients with ALL (Davila et al., 2014; Pegram et al., 2015).

Despite great progress in the field of therapeutic hematopoietic transplants and transfusions, as of 2016 the sole source of all hematopoietic cells is still donations. Relying on donors has been proven to carry certain risks, and is limited to donor availability (Lai et al., 2016; Merz et al., 2016; Ramsey et al., 2015). When blood transfusions became more common in the mid-1900s, blood-borne diseases such as hepatitis (first described in 1943, reviewed (Holland, 1998)) and HIV (discovered in 1985, reviewed (Ward et al., 1988)) became a concern. Today, other pathogens such as Zika viruses (Arellanos-Soto et al., 2015; Kashima et al., 2016) and prion diseases (Hunter et al., 2002) are being screened for in donated blood samples. While finding HLA-matched HSC donors has become easier with increased donor registration, this is generally limited to specific ethnicities that have access to established and common registries (Mitchell et al., 1997; Schwake et al., 2005). Thus, despite improved HLA-screening, allogeneic HSCTs that are not 100% matches can still lead to graft-versus-host disease (GvHD). In addition, while HSC purification techniques with Fluorescence Activated Cell Sorting (FACS) have
improved, we still have not determined the exact phenotype of long-term engrafting HSCs that can be used to distinguish them from short-term engrafting HSCs and LSCs (Bonnet and Dick, 1997; Plaks et al., 2015; Wang and Dick, 2005). Thus, patients being treated with autologous HSCT for multiple myeloma and Hodgkin’s disease still risk cancer relapse from contaminating LSCs (Stewart et al., 2001).

Another limitation to current hematopoietic therapies dependent on HSCs and their progeny is the fact that HSCs are quiescent and biologically low in number (Catlin et al., 2011; Shepherd et al., 2004). The genetic modification of quiescent cells is extremely inefficient and often leads to cell death (Mitchell et al., 2004). The advent of CRISPR/Cas9 technology has made genetic modification easier, but the limiting factor to generate gene-corrected HSCs for patients with various inherited hematological diseases and some metabolic disorders is the fact that HSCs are quiescent and inducing their division leads to exhaustion and loss of long-term engraftment potential. Culturing HSCs without losing their ability to engraft has only recently been demonstrated (Ohishi et al., 2002), but this method is still in clinical trials. Other described methods of expansion are limited, often leading to exhaustion and insufficient engraftment (Gothot et al., 1998; Riviere et al., 2012). While human HSCs have been genetically manipulated to knockdown CCR5 with CRISPR/Cas9 and was shown to confer HIV resistance, this was only shown experimentally and was not used for therapy (Li et al., 2015).

There is a wide range of therapeutic applications and benefits if it were possible to generate and engineer HSCs and their progeny in vitro. Recently, this concept became a real possibility with the generation of human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs).
1.2 History and Background of Mammalian Pluripotent Stem Cells

The derivation of mouse embryonic stem cells (mESCs) in 1981 by Gail Martin (Martin, 1981) and Evans and Kaufmann (Evans and Kaufman, 1981) had vast impact on developmental biology, genetics, pathology, and medicine. Mouse ESCs are derived from the inner cell mass of pre-implantation blastocysts and can be indefinitely propagated \textit{in vitro} in an undifferentiated state while maintaining their ability to differentiate into all three primary germ layers – the endoderm, mesoderm, and ectoderm. The ability to culture mESCs created the opportunity to generate transgenic mice by manipulating the cells \textit{in vitro}, injecting the cells with targeted genetic mutations into the blastocyst of another mouse embryo, and implanting it into a pseudo-pregnant female (Doetschman et al., 1987; Thomas and Capecchi, 1987; Thomas et al., 1986). Various transgenic mouse lines have since been generated to elucidate specific gene functions, model human genetic diseases, and serve as a platform for toxicity and drug testing (Manis, 2007).

The culture conditions to grow and maintain pluripotency of mESCs stemmed from a 1965 study in which Cole, Edwards, and Paul established a rabbit ESC line, the first mammalian ESC line, cultured on irradiated “feeder” fibroblast in media containing fetal calf serum (FCS) (Cole et al., 1965, 1966). Initially, mESC culture also depended on feeder cells and FCS (Evans and Kaufman, 1981; Martin, 1981). Utilizing this knowledge of conditions required for mammalian ESC culture, Jamie Thomson was able to establish the first non-human primate ESC (rhESC) line from rhesus macaque blastocysts in 1995 (Thomson et al., 1995). In 1998, Thomson and colleagues succeeded in deriving the first human ESC lines from discarded IVF embryos (Thomson et al., 1998). These human ESC lines had normal karyotype unlike
previously used human embryonic carcinoma lines, the ability to propagate indefinitely, and maintain their ability to differentiated into all three germ layers in a teratoma when grafted into immunocompromised mice. While initial culture systems for hESCs required the use of irradiated mouse embryonic fibroblasts and serum-containing media, the current protocol utilizes a completely chemically defined, animal protein-free culture system, established by the Thomson lab in 2011 (Chen et al., 2011a). The chemically defined Essential 8 (E8) media and the recombinant extracellular membrane (ECM) protein vitronectin (VTN) can easily be made under good manufacturing process (GMP) guidelines for clinical applications. The derivation of hESCs and the ability to culture them in vitro in a chemically defined culture system contributed to progressing hESC technology towards clinical applications.

The next landmark discovery in regenerative medicine was made in 2007 when Shinya Yamanaka reported the generation of mouse induced pluripotent stem cells (iPSCs) from mouse embryonic fibroblasts and tail tip fibroblasts (Takahashi et al., 2007). By ectopically expressing four key pluripotency factors, Oct4, Sox2, Klf4, and c-Myc (OSKM, Yamanaka factors), somatic cells were “reprogrammed” to a state of pluripotency with characteristics similar to mESCs. These mouse iPSCs are capable of dividing indefinitely and contributing to all three germ layers of fertile mice when injected into a blastocyst and implanted into a pseudo-pregnant female (Takahashi et al., 2007).

In the same year, Thomson and colleagues independently demonstrated that human foreskin fibroblasts can also be reprogrammed into a pluripotent state using OCT4, SOX2, NANOG, and LIN28 (OSNL, Thomson factors) (Yu et al., 2007). Again, these human induced pluripotent stem cells (hiPSCs) were shown to multiply indefinitely and contribute to all three germ layers in teratomas when grafted into immunocompromised mice. Soon after, other human
somatic cell types such as amniotic fluid cells (Liu et al., 2012), keratinocytes (Aasen et al., 2008), bone marrow mononuclear cells (Hu et al., 2011), and cord blood cells (Giorgetti et al., 2009) were shown to be reprogrammable into hiPSCs. While initial studies utilized viral transduction of the pluripotency factors for iPSC reprogramming, recent studies have generated hiPSCs using non-viral and non-integrating episomal vectors (Hu et al., 2011; Yu et al., 2009) in E8/VTN (Chen et al., 2011a), providing a promising new GMP-approved source of hPSCs for clinical applications (Baghbaderani et al., 2015).
1.3 Potential Research and Therapeutic Applications of Human Pluripotent Stem Cells

There are a number of key potential research and therapeutic applications of hPSCs. Both mouse ESCs and iPSCs have been shown to maintain their ability to develop into a full adult mouse when injected into a blastocyst and implanted into a pseudo-pregnant female (Doetschman et al., 1987; Thomas and Capecchi, 1987; Thomas et al., 1986). In combination with the ability of hPSCs to develop teratomas in immunocompromised mice (Chen et al., 2011a; Thomson et al., 1998; Yu et al., 2007), there is strong evidence that hPSCs can be differentiated into any adult cell type, including HSCs and their progeny, for HSCT and transfusion. Indeed, there are a number of directed differentiation studies that have generated multiple hematopoietic lineages, including erythromegakaryocyte (Dias et al., 2011; Takayama et al., 2008), myeloid (Choi et al., 2011; Choi et al., 2009b), NK cells (Hermanson et al., 2016; Knorr and Kaufman, 2010; Ni et al., 2011; Woll et al., 2009), and T-lymphocyte (Kennedy et al., 2012b; Themeli et al., 2013; Vizcardo et al., 2013) lineages with some therapeutic potential (Kaufman, 2009).

Because hPSCs propagate indefinitely, they are a potential source for an unlimited supply of therapeutic hematopoietic cells. An unlimited supply of HSCs also increases access for research applications for the study of hematology and immunology. One key animal model currently being used to study human hematology and immunology in vivo is the humanized mouse model, in which an immunocompromised mouse is injected with human HSCs which reconstitutes a human immune system. The current method of generating humanized mice requires donations of precious cord blood HSCs which would otherwise be used for clinical applications, and drastically limits the ability to generate and use humanized mice. The ability to generate an unlimited supply of human HSCs from hPSCs increases access to the humanized mouse model,
and could very well expand their research applications (Ito et al., 2012; McIntosh and Brown, 2015; Shultz et al., 2012; Shultz et al., 2007).

The ability to generate hematopoietic cells from hPSCs in vitro will also free us from reliance on donors for HSCT and blood transfusions, and can make these procedure safer in two ways: First, generating hematopoietic cells in vitro virtually eliminates possible infection of blood-borne pathogens (Lai et al., 2016; Merz et al., 2016; Ramsey et al., 2015). Today, there are already GMP-certified methods to generate hiPSC-derived mesenchymal stem cells (MSCs) for transplant that are guaranteed to be pathogen-free and non-toxic for potential patients (Sabapathy and Kumar, 2016). Second, directed differentiation of hPSCs into specific lineages increases the purity of the cells being transplanted or transfused, which decreases the concern for unwanted-CTL carryover that causes GvHD in patients requiring allogeneic HSCT and also eliminates the possibility of re-transplanting contaminating LSCs for patients receiving autologous HSCT (Stewart et al., 2001; Themeli et al., 2013).

The advent of hiPSCs has created the possibility of generating HSCs and their progeny for autologous transplant or transfusion. While the number of registered HSC donors has increased the likelihood of HLA-matched HSCT for Caucasian haplotypes, patients who have rare haplotypes, who are of mixed ethnicities, or who are underrepresented in the community are at a disadvantage (Chalela et al., 2014; Schwake et al., 2005). In addition, being able to generate hiPSCs from a wide variety of somatic cells means that hiPSCs can be generated from normal, non-neoplastic cell sources for patients with leukemia-initiating mutations in their hematopoietic cells (Hu et al., 2011). These iPSCs can then be differentiated into HSCs and be used as a healthy, non-malignant source of autologous HSCT (Slukvin, 2013b). While the immunogenicity of iPSC-derived differentiated cells is still under investigation, recent evidence shows that non-
viral methods of reprogramming and utilizing immune-privileged sources of somatic cells to generate hiPSCs can limit immunogenic responses. These findings may contribute to our ability to produce hiPSC-derived therapeutic cells that can be safely injected into recipients (Araki et al., 2013; Wang et al., 2014; Zhao et al., 2011; Zhao et al., 2015).

Because iPSCs are genotypically identical with the patients’ originating somatic cells, iPSCs generated from patients with inherited diseases will also carry the same mutation (Hu et al., 2011). These iPSCs can be differentiated into hematopoietic cells to model the disease and screen for new drugs in vitro or in vivo using the humanized mouse model (Suknuntha et al., 2015). A recent study by Doulatov and colleagues used hiPSCs generated from patients with Diamond-Blackfan anemia to screen for drugs that can alleviate the condition (Doulatov et al., 2017). This is particularly useful in cases where the same genetic mutation in mice does not result in the same disease phenotype.

In addition to modeling inherited diseases, there is also a new potential to correct the mutation that causes the disease phenotype. Gene therapy on HSCs has had very low success rates in the past because HSCs are quiescent and do not divide. Homologous recombination requires cells to be dividing to replace the mutated endogenous genome with the corrected DNA fragment. The fact that iPSCs are readily dividing makes them more amenable to genetic modification via homologous recombination. The genetically corrected iPSCs can then be differentiated into HSCs and their progeny used for autologous HSCT or transfusion (Slukvin, 2013b).

In some cases, causing a mutation can cure the disease. We could potentially genetically engineer hiPSCs with specific chimeric antigen T-cell receptors that can then be differentiated into CAR-T cells and be used for adoptive cell transfer therapy (Themeli et al., 2015). Not only
could this technology treat cancer, we could also potentially generate CAR-T cells to recognize viral, bacterial, or fungal pathogenic antigens that have previously evaded immune responses (Bilal et al., 2015; Liu et al., 2016). In addition, recent studies have shown that transplanting HSCs with a CCR5 mutation can be a viable cure for HIV/AIDS (Allers et al., 2011; Hutter et al., 2009; Kang et al., 2015; Li et al., 2015). The HIV-1 enters and replicates inside CD4\textsuperscript{+} T-helper cells by binding to the CD4/CCR5 complex and initiating endocytosis. Hematopoietic stem cells with the CCR5 mutation give rise to progeny without CCR5 expression on the cell surface and are not as easily infected by HIV-1, effectively giving the host immune system a chance to clear the virus (Allers et al., 2011; Hutter et al., 2009). The efficiency was low when researchers tried to mutate HSCs directly (Li et al., 2015), but targeting specific genes in hiPSCs is much more efficient (Kang et al., 2015; Themeli et al., 2013).

Today, hESC-derived cells are being tested in clinical trials to treat diabetes (Trounson and DeWitt, 2016), spinal cord injury (Chapman and Scala, 2012), and myocardial infarction (Menasche et al., 2015), while hiPSC-derived retinal pigment epithelium are currently in clinical phase I and II trials to treat age-related macular degeneration in Japan (Miyagishima et al., 2016; Trounson and DeWitt, 2016). However, all of the aforementioned research and therapeutic applications of hPSC-derived hematopoietic cells can only be achieved if we can generate long-term engrafting definitive HSCs (LT-dHSCs). However, we have yet to successfully generate hPSC-derived HSCs \textit{in vitro} with long-term engraftment potential (Chadwick et al., 2003; Ditadi et al., 2015; Doulatov et al., 2013; Kennedy et al., 2012b; Uenishi et al., 2014; Vodyanik et al., 2006). Hematopoietic stem cell engraftment in immunocompromised mice and generating a humanized mouse are critical to determining the efficacy of human HSCs (Amabile et al., 2013; Ivanovs et al., 2011; McIntosh et al., 2015; Notta et al., 2011; Slukvin, 2016a). However, the
lack of success in engraftment into immunocompromised mice suggests that our current hPSC-
derived hematopoietic cells are not definitive HSCs with long-term engraftment potential, and
cannot currently be used for therapeutic applications (Ditadi et al., 2017; Rowe et al., 2016;
Slukvin, 2013b). To understand why hPSC-derived HSCs are not capable of engrafting
immunocompromised mice to reconstitute a human immune system, a better understanding of the
complexities of mammalian and human hematopoietic development is required.
1.4 History and Background of Avian and Murine Hemato-endothelial Development

The inability to generate long-term engrafting HSCs has been associated with the complexity of in vivo mammalian Hemato-endothelial development. Studies utilizing avian and murine embryos show there are two independent waves of hematopoiesis during vertebrate embryogenesis (Clements and Traver, 2013; Medvinsky et al., 2011): The primitive wave, which initiates in the extra-embryonic yolk sac and transiently produces limited lineages of blood cell types, and the definitive wave, which emerges from within the embryo conceptus and produces HSCs with multilineage and long-term engraftment potentials (Dzierzak and Speck, 2008; Medvinsky et al., 2011). While the origins of each wave of hematopoiesis can be distinguished anatomically, the progeny of both hematopoietic progenitor (HP) cells overlap, and, unlike solid organ tissues, get distributed throughout the embryo through circulation. In addition, while specific cell surface markers that are exclusively expressed on emerging LT-dHSCs have increasingly been identified in mice (Kiel et al., 2005; Kim et al., 2006; Oguro et al., 2013; Yilmaz et al., 2006), these markers cannot be used to identify emerging LT-dHSCs in humans (Larochelle et al., 2011). The ability to replicate human definitive HSC development with hPSCs will depend on increasing our understanding of mammalian embryonic hemato-endothelial development.

The extra-embryonic yolk sac was identified as the first site of avian hematopoiesis in 1920 when Sabin showed primitive erythrocytes emerging concurrently with the developing vasculature of chick yolk sacs (reviewed (Dzierzak and Medvinsky, 2008)). It was suggested that a mesodermal precursor from the primitive streak, called “hemangioblasts,” migrates to the yolk sac to form blood islands, which produce both endothelial and hematopoietic cells. Indeed,
murine embryonic studies identified cells in the posterior primitive streak at E8.25 that express both the mesodermal marker Brachyury (T) and the endothelial and emerging hematopoietic marker KDR (Flk-1, VEGFR2, CD309) (Huber et al., 2004). Primitive erythroid colony-forming cells (EryP-CFCs) were detectable as early as E7.25 from the mid-primitive streak stage, before the development of blood islands, proving the hematopoietic commitment of mesodermal precursors that give rise to hemangioblasts (Palis et al., 1999). Early yolk sac-derived hematopoiesis prior to E8.0 only produces large nucleated erythrocytes expressing the embryonic globins ε-, γ-, and βH1-globins (Palis, 2014; Yi et al., 2006), smaller megakaryocytes with limited thrombocyte production (Tober et al., 2007; Xu et al., 2001), and macrophages that mature without progressing through a monocyte stage (Naito et al., 1989; Takahashi et al., 1989). Critically, hematopoietic cells isolated from the yolk sac prior to E11.5 were incapable of producing lymphoid lineages or repopulating immunocompromised mice (Medvinsky and Dzierzak, 1996; Muller et al., 1994).

Originally, it was thought that the yolk sac was the only source of hematopoiesis and the progeny existed through adulthood by emigrating to the fetal liver and eventually the bone marrow (Moore and Metcalf, 1970). However, in 1975, Deiterlen-Lievre showed experimentally for the first time that an intraembryonic wave of hematopoiesis emerges independently by utilizing chimeric avian embryos (Dieterlen-Lievre, 1975). By grafting quail embryos onto chicken yolk sacs prior to vascularization, Deiterlen-Lievre showed that chick yolk sac-derived hematopoietic cells were incapable of colonizing the spleen and thymus of the quail embryo. Later, Dieterlen-Lievre determined that before circulation commences with the beating heart, the walls of the aorta in developing chick embryos contain hematopoietic cells with macrophage-colony forming cells (CFC-M), granulocyte-colony forming cells (CFC-G), granulocyte- and
macrophage-colony forming cells (CFC-GM), and erythroblast-colony forming cells (BFU-Es) (Cormier and Dieterlen-Lievre, 1988). This was the first evidence of the origins of LT-dHSCs.

Murine embryo studies utilizing cell fate mapping (Kanatsu and Nishikawa, 1996; Kinder et al., 1999), single cell marking (Lawson et al., 1991), and genetic marking (Zovein et al., 2008; Zovein et al., 2010) also showed evidence for the separation of mesodermal lineages that give rise to extra-embryonic yolk sac hematopoiesis and intra-embryonic hematopoiesis. Furthermore, hematopoietic cells that emerge from within the embryo conceptus were found to have potent repopulating capabilities when transplanted into immunocompromised mice (Medvinsky and Dzierzak, 1996; Muller et al., 1994). Finally, transplantation assays conducted on various regions of the murine embryo from E8 to E12 identified that the E10.5 Aorta-Gonado-Mesonephros (AGM) region contained the first LT-dHSCs with high repopulating capabilities in immunocompromised mice, along with adult erythroid, myeloid, and lymphoid multilineage potential (de Bruijn et al., 2000a; Medvinsky and Dzierzak, 1996; Muller et al., 1994).

Recently, LT-dHSCs were found to emerge from specialized endothelial cells lining the ventral wall of the dorsal aorta (DA) (Bertrand et al., 2010; Dzierzak and Speck, 2008; Medvinsky et al., 2011; Yokomizo and Dzierzak, 2010). These CD144⁺ (VE-Cadherin, CDH5) hemogenic endothelial cells (HE) undergo a process of endothelial-to-hematopoietic transition (EHT) and release non-adherent CD45⁺ hematopoietic cells into the lumen of the aorta (Bertrand et al., 2010; Boisset and Robin, 2010; Boisset et al., 2010; Eilken et al., 2009; Kissa and Herbomel, 2010; Lam et al., 2010). Hemogenic endothelium has also been found in the arteries of the yolk sac (de Bruijn et al., 2000a), placenta (Gekas et al., 2005; Ottersbach and Dzierzak, 2005), and the embryonic head (Li et al., 2012), but never in venous vasculature, suggesting that HE and arterial endothelium are closely related developmentally (Chen et al., 2016; Zhou et al.,
2016). Indeed, multiple transgenic mouse studies have shown that genes associated with arterial specification such as Notch1 (Kumano et al., 2003; Robert-Moreno et al., 2005; Robert-Moreno et al., 2008) and EphrinB2 (Chen et al., 2016) are necessary for LT-dHSC emergence from the DA. However, other studies have suggested that HE and arterial endothelium are distinct lineages and have individual programs in response to different degrees of NOTCH activation (Gama-Norton et al., 2015). There is strong evidence that the Runt-related transcription factor 1 (Runx1) is a critical transcription factor expressed in the HE of the DA required for EHT, as conditional deletion of Runx1 in CD144+ endothelial cells during embryonic development completely inhibits the development of LT-dHSCs (Chen et al., 2009). Critically, Runx1-knockout mice still have extra-embryonic yolk sac hematopoiesis (Yokomizo et al., 2008), which indicates a critical role specifically during definitive hematopoiesis (Lam et al., 2010; North et al., 2002). More specifically, a Runx1 enhancer 23.5 kb downstream of the start codon in exon 1 was found to be activate specifically among the HE that give rise to definitive HSCs in the AGM (Bee et al., 2009; Nottingham et al., 2007).

While many hematopoietic cells can be detected in the lumen of the aorta at E10.5, not all of them are LT-dHSCs (Guiu et al., 2013). Some studies have shown that only 2-3 LT-dHSCs exist per embryo equivalent at this stage (Kumaravelu et al., 2002; Taoudi et al., 2008; Zhou et al., 2016). More recently, specific CD144+ progenitors from the E9 para-splanchnopleura (p-Sp, the primordial AGM) have been shown to have engraftment capabilities, but only when injected into neonates (Yoder et al., 1997a; Yoder et al., 1997b). These results suggest that definitive HSC specification may initiate before EHT, possibly during hemogenic endothelial specification, or that these “pre-HSCs” require a fetal environment to mature (Ditadi et al., 2017). Furthermore, in vitro culture of E9-E10 AGM explants has revealed that LT-dHSCs undergo stepwise
maturation from pro-HSCs that are Runx1^+CD144^+CD117^-CD41^-CD43^-CD45^-, to Type-I pre-
HSCs that gain CD43 expression, to Type-II pre-HSCs that gain CD45 expression, and then
finally to definitive HSCs that lose CD41 expression (Rybtsov et al., 2014; Rybtsov et al., 2011).
Recent studies have revealed that the process of maturation from Type-I pre-HSCs to Type-II
pre-HSCs is dependent on NOTCH activation, but maturation from Type-II pre-HSCs to
definitive HSCs is NOTCH independent (Souilhol et al., 2016b). Other signaling pathways
implicated in definitive HSC specification in the dorsal aorta include hedgehog (Baron, 2001),
TGFβ (Monteiro et al., 2016b), retinoic acid (Chanda et al., 2013; Dou et al., 2016; Ghiaur et al.,
2013), inflammation (He and Liu, 2016; He et al., 2015; Li et al., 2014), hormone (Heo et al.,
2015; Kim et al., 2016), and blood flow-induced shear stress (Kim et al., 2015; North et al.,
2009) signaling pathways.

Following emergence in the lumen of the aorta, the murine LT-dHSCs migrate to the
fetal liver (FL) where they proliferate without exhaustion (Ema et al., 1998; Paige et al., 1984).
Fetal liver explant studies have found no evidence of HE in the hepatic vasculature, but have
found over 50 definitive LT-dHSCs per embryo equivalent as early as E12 and give rise to adult
erythromegakaryocytic, myeloid, and lymphoid lineages when transplanted into
immunocompromised mice (Ema et al., 1998; Ema and Nakauchi, 2000; Paige et al., 1984;
Rebel et al., 1996b). These fetal liver HSCs have also been shown to have greater proliferative
capabilities and serial engraftment potential compared to their adult bone marrow counterparts
(Harrison et al., 1997; Micklem et al., 1972; Rebel et al., 1996a). In fact, by E16, the number of
definitive HSCs expand over 38-fold in the fetal liver to about the same number as found in adult
bone marrow (Ema and Nakauchi, 2000; Rebel et al., 1996b). Recent research has identified
specific stromal cells in the stem cell niche of the FL that provide critical signaling cues for the
rapid proliferation of LT-dHSCs (Chou and Lodish, 2010; Khan et al., 2016; Zhang et al., 2006; Zhang and Lodish, 2004). Specifically, Nestin$^+$NG2$^+$ pericytes and Neuropilin1$^+$EphrinB2$^+$ arterial endothelium in the HSC niche of the FL proliferate with the associated LT-dHSCs (Khan et al., 2016). These Nestin$^+$NG2$^+$ pericytes were necessary for LT-dHSC expansion in the fetal liver, and may contribute unique signaling cues to induce rapid proliferation (unlike the bone marrow HSC niche). The Neuropilin1$^+$EphrinB2$^+$ arterial endothelium eventually transitions into EphrinB4$^+$ venous endothelium as the umbilical inlet closes at birth, and may contribute to the emigration of the definitive HSCs out of the FL and to the bone marrow (Khan et al., 2016).

From embryonic stage E15, circulating LT-dHSCs begin to colonize the bone marrow, specifically in the axial skeleton of the flat bones (Dzierzak and Speck, 2008; Medvinsky et al., 2011; Sasine et al., 2016). Three-dimensional imaging of the marrow has revealed that the circulating LT-dHSCs migrate to regions that are high in the chemokine CXCL12 (SDF-1, CXCR4/CD184 ligand) (Sipkins et al., 2005; Xie et al., 2009). Originally, bone marrow osteoblasts and mesenchymal stromal cells were thought to be the critical niche cells for HSC maintenance (Dexter et al., 1977; Lord et al., 1975; Taichman and Emerson, 1994). However, because extra-medullary hematopoiesis has been found to be associated with endothelial stem cell niches (Taniguchi et al., 1996), and vascular regeneration is necessary prior to HSCT for successful HSC engraftment and reconstitution (Fliedner et al., 1961; Knospe et al., 1972), it was newly hypothesized that perhaps endothelial cells were also implicated in LT-dHSCs maintenance. It was not until the combination of improved imaging techniques (Sipkins et al., 2005; Xie et al., 2009) and the discovery of SLAM family of cell surface receptors specific to murine LT-dHSCs (Kiel et al., 2005; Oguro et al., 2013) that researchers found evidence for the role of endothelial cells and pericytes in the HSC niche. In addition, while CXCL12-deletion in
bone marrow osteoblasts did not affect definitive HSC maintenance, CXCL12-deletion in
endothelial cells markedly impaired definitive HSC maintenance and decreased the LT-dHSC
pool in the bone marrow (Ding and Morrison, 2013; Greenbaum et al., 2013). In addition to
CXCL12 signaling, endothelial cells in the HSC niche of the bone marrow were found to
produce SCF (Ding et al., 2012), pleiotrophin (Himburg et al., 2012; Himburg et al., 2010a),
epidermal growth factor (EGF) (Doan et al., 2013a; Doan et al., 2013b), Angiopoeitin-1(Arai et
al., 2004), Wnt agonists (Luis et al., 2011; Malhotra and Kincade, 2009), and were also found to
express the NOTCH ligand, Jagged-1 (Butler et al., 2010; Poulos et al., 2013). While NOTCH
signaling has also been shown to be dispensable for normal hematopoiesis in mice (Maillard et
al., 2008), recent studies have also shown that Jagged-1-induced NOTCH signaling is essential
for hematopoietic regeneration (Poulos et al., 2013). Recent studies have also found that
quiescent and active LT-dHSCs reside in different niches within the bone marrow (Kunisaki et
al., 2013). Quiescent LT-dHSCs were found to reside in arterioles with rare NG2+ pericytes,
while the cycling LT-dHSCs were found to migrate to LeptinR+ perisinusoidal niches (Kunisaki
et al., 2013). These studies show that, like the FL, the HSC niche in the bone marrow also consist
of specialized endothelial cells that provide important signaling cues for LT-dHSC maintenance.
It is in these endothelial niches in the bone marrow where LT-dHSCs reside quiescently for the
life of the animal (Delassus and Cumano, 1996).

Mammalian hemato-endothelial development is vastly complex, and there is still a lot to
be learned. For obvious ethical reasons, little direct research exists on human embryonic hemato-
endothelial development. Avian and murine studies have provided invaluable insights in the
primitive and definitive waves of hematopoietic development. The knowledge gained from non-
human studies has been extrapolated to create a model of human hematopoietic development and
utilized to study human specimens that have been available for research. Recently, Ivanovs et al. discovered the presence of hematopoietic cells capable of long-term multilineage engraftment exclusively in the human AGM of Carnegie stages 14 and 15, at least five days before the yolk sac equivalent to murine developmental stage E9 and E10. Similar to the mouse AGM, only one to two LT-dHSCs were detected per embryo equivalent (Ivanovs et al., 2011). During the lifetime of both humans and mice, LT-dHSCs are known to be mostly quiescent, but while human LT-dHSCs are thought to divide more slowly compared to mouse LT-dHSCs, studies estimate that LT-dHSCs in both species divide the same number of times during a lifetime (Abkowitz et al., 1996; Catlin et al., 2011; Shepherd et al., 2004; Wilson et al., 2008).

There are some limitations to relying solely on animal models to elucidate human hematopoietic development, as a few critical differences between mouse and human hematopoiesis have been reported (Doulatov et al., 2012; Haley, 2003; Mestas and Hughes, 2004). Mouse erythrocytes begin to suppress $\gamma$-globin in the erythrocytes during fetal liver hematopoiesis, while humans suppress $\gamma$-globin in the erythrocytes when the site of hematopoiesis moves to the bone marrow after birth (Sankaran et al., 2009). As mentioned earlier, the SLAM family markers can be used to distinguish definitive HSCs in the mouse, but do not identify human definitive HSCs (Kiel et al., 2005; Larochelle et al., 2011). However, Notta et al. were able to identify that the CD34$^+$CD38$^-$CD45RA$^-$CD90$^+$CD49f$^+$Rho$^{lo}$ phenotype can purify human LT-dHSCs from cord blood samples and can reconstitute immunocompromised mice from a single cell (Notta et al., 2011). In addition, while NOTCH signaling has been found to be largely dispensable for mouse hematopoietic maintenance in the bone marrow niche (Maillard et al., 2008; Souilhol et al., 2016b), human LT-dHSCs have been shown to maintain long-term engraftment potential when cultured in the presence of NOTCH
ligands (Karanu et al., 2001; Ohishi et al., 2002; Shojaei et al., 2005; Suzuki et al., 2006).

Finally, there are several differences in human and mouse adult immunology, such as the balance of lymphocytes versus neutrophils (Doeing et al., 2003) and the molecular mechanisms involved in lymphoid differentiation (Noguchi et al., 1993; Puel et al., 1998).

The humanized mouse model has been an invaluable tool for investigating human hematology and immunology (Shultz et al., 2012; Shultz et al., 2007). Various strains of immunocompromised mice, including ones that do not require irradiation before human LT-dHSC injection, have been generated to further increase the investigative potential and utility of humanized mice (Ito et al., 2012; McIntosh and Brown, 2015). Unfortunately, the generation of humanized mice is still limited to human LT-dHSCs availability, mostly from cord blood donations or other controversial fetal sources. In addition, while the generation of humanized mice can be used to determine the functionality of human LT-dHSCs, they cannot be used to investigate human hemato-endothelial development before LT-dHSC specification. Thus, the ability to generate LT-dHSCs from hPSCs in vitro will not only be important for therapeutic applications, but can also be used to study human hemato-endothelial development, hematology, and immunology as well (Sackett et al., 2016).
1.5 Methods of Human Pluripotent Stem Cell Differentiation into Hematopoietic Progenitors

Mouse ESC differentiation studies have shown promising evidence that in vitro differentiation recapitulates the stages of in vivo long-term engrafting definitive hematopoietic stem cell development, from mesoderm induction, hemogenic endothelial specification, and endothelial-to-hematopoietic transition into hematopoietic progenitors. While scientists have been unable to generate LT-dHSCs from directed differentiation of even mouse PSCs, other methods of hematopoietic induction, including ectopic expression of specific transcription factors (Kyba et al., 2002) and direct reprogramming (Doulatov et al., 2013), have generated PSC-derived hematopoietic progenitors with some engraftment capabilities. These studies, and the fact that mouse PSCs can be re-injected into blastocysts to generate mice, suggest that generating LT-dHSCs is possible from hPSCs. In addition, studies isolating and re-injecting hematopoietic progenitors from hPSC-derived teratomas have shown some engraftment capabilities (Amabile et al., 2013; Suzuki et al., 2013).

Currently, three main strategies exist for differentiating hPSCs into hematopoietic progenitors, and each have been critical in developing a model for human hemato-endothelial development from hPSCs.

The first method involves co-culturing undifferentiated hPSCs with murine bone marrow stromal cells in the presence of serum-containing media. While multiple different murine bone marrow stromal cells have been tested, Vodyanik et al showed that the OP9 cell line is the most efficient at hemato-endothelial induction (Vodyanik et al., 2006). The OP9 bone marrow stromal cell line lacks macrophage colony-stimulating factor (M-CSF) (Nakano et al., 1994; Vodyanik et
al., 2005), but can, with the use of very specific fetal bovine serum (FBS), generate hematopoietic progenitors from hPSCs without any additional growth factors or cytokines (Choi et al., 2009a). Additionally, this co-culture system has been used to identify specific stages of differentiation of hPSCs from primitive mesoderm, hematovascular mesoderm precursors (HVMP), HE progenitors distinguishable from non-HE progenitors, and finally HPs (Choi et al., 2012a). The OP9 co-culture system has been shown to generate HPs with lymphoid lineage capabilities, including T-cell, B-cell, and NK-cell lineages when cultured on human DLL1- or DLL4-expressing OP9 (Vodyanik et al., 2005; Vodyanik et al., 2006). Genetic profiling analysis has also revealed unique molecular signatures in OP9 stromal cells compared to other murine bone marrow stromal cell lines that do not support hematopoietic differentiation of hPSCs as efficiently (Uenishi et al., 2014).

While much knowledge has been gained through the use of the stromal cell co-culture, there are a few significant limitations with this system. The efficiency of hPSC differentiation on OP9 stromal cells is heavily dependent on stromal cell density, specific FBS lots, and starting colony sizes of the hPSCs. As such, isolating, controlling, and investigating the effect of specific signaling pathways during hPSC differentiation into hemato-endothelial lineages is difficult. In addition, the therapeutic potential of this system is limited due to the reliance on xenogeneic material. Human ESCs co-cultured on mouse feeder cells in the presence of serum and non-human serum substitutes have been reported to absorb high levels of the nonhuman immunogenic sialic acid Neu5Gc (Martin et al., 2005), an antigen that healthy humans produce antibodies for and launch an immune response against. While xenogen-free hPSC maintenance methods have been established, defined methods of differentiation is necessary to produce hPSC-derived hematopoietic cells with therapeutic potential.
The second method involves the generation of three-dimensional hPSC aggregates called embryoid bodies (EBs) and either culturing them in serum-containing media or treating them with stage-specific cytokines, morphogens, and small molecules to promote hemato-endothelial differentiation. Several methods have been used to generate EBs, including culturing single-cell suspensions of hPSCs in low-attachment micro-well plates, hanging droplets of media with specific densities of hPSCs on the lids of culture dishes, and, more recently, making consistently sized aggregates by centrifuging a defined number of hPSCs in round-bottom 96-well plates (Ng et al., 2005; Ye et al., 2009). Traditional media used in these protocols relied on serum (Thomson et al., 1998), though recent progress has established serum-free media (Ferrell et al., 2015; Kattman et al., 2011; Lengerke et al., 2009; Park et al., 2008; Pick et al., 2007). Some researchers argue that the generation of EBs from hPSCs in suspension culture recapitulates the environment of a blastocyst’s inner cell mass and more closely resembles in vivo development (Itskovitz-Eldor et al., 2000). Indeed, hPSC-derived EBs spontaneously express Brachyury and eventually produce CD34⁺CD45⁺ HP cells (Lengerke et al., 2009). The efficiency of spontaneous hematopoietic differentiation of hPSC-derived EBs has been improved by adding bone-morphogenetic protein 4 (BMP4) and Wnt-agonists to the culture to increase the induction of mesoderm, VEGF to increase angiogenesis, and finally, hematopoietic cytokines to improve hematopoiesis (Ditadi et al., 2015; Kattman et al., 2011; Kennedy et al., 2012b; Kennedy et al., 2007; Sturgeon et al., 2014).

The EB method of differentiation greatly improved our understanding of specific cell signaling pathways involved in hemato-endothelial differentiation from hPSCs. However, it is still not without limitations. Despite improved consistency in EB-size formation, there is still variation between each EB, and the efficiency of hematopoietic differentiation is not the same
across all EBs (Itskovitz-Eldor et al., 2000; Ng et al., 2005; Sturgeon et al., 2014). In addition, the naturally complex nature of cystic EBs and the relatively slow differentiation limits our ability to isolate, control, and investigate specific cell signaling pathways during differentiation, much like the use of stromal feeder cells. While BMP4, Wnt signaling pathways, and FGF signaling pathways have been studied under EBs during mesoderm induction, the EBs themselves produce growth factors and morphogens which make it harder to determine whether the addition of one factor increases the expression of others (Kattman et al., 2011; Kennedy et al., 2007; Sturgeon et al., 2014). This limits the ability to isolate and determine the effect of individual signaling pathways during EB-based hPSC differentiation. As such, different results looking at the effect of the NOTCH signaling pathway, which requires cell-to-cell interaction, have been reported during HE specification, EHT, and hematopoietic differentiation from hPSC-derived EBs (Ditadi et al., 2015; Lee et al., 2013).

Utilizing the knowledge gained from the hemato-endothelial differentiation methods of murine bone marrow stromal cells and the stage specific treatment of growth factors led to the development of a two-dimensional, directed differentiation protocol on ECM-coated plates. Some studies utilize Matrigel, the mouse sarcoma cell line-derived ECM, to plate cells on, but recent studies have found human collagen IV, fibronectin, and laminin are sufficient ECM proteins that can support mesoderm induction and promote hemato-endothelial differentiation (Lian et al., 2014; Niwa et al., 2011; Park et al., 2010; Salvagiotto et al., 2011; Wang et al., 2012b). The media has also become more defined, with serum-free and animal-component-free media commercially available (https://www.stemcell.com/products/stemdiff-hematopoietic-kit.html). While previous two-dimensional differentiation protocols started with hPSC colonies, which introduces variation, the discovery of a Rho kinase inhibitor promoting survival of
singularized hPSCs has created an opportunity to study the differentiation of singularized hPSCs into hemato-endothelial lineages (Chen et al., 2011a; Emre et al., 2010; Takehara et al., 2008). In addition, the two-dimensional method also decreases differentiation time while increasing the hemato-endothelial differentiation efficiency (Park et al., 2010; Salvagiotto et al., 2011; Uenishi et al., 2014; Wang et al., 2012b). The use of stage-specific addition of growth factors, morphogens, small molecules, and cytokines has made the differentiation of hPSCs into hemato-endothelial lineages efficient, chemically defined, easier to manipulate, and thus, has created a platform on which to study the effects of specific cell signaling pathways during hPSC differentiation into hemato-endothelial lineages.

The two-dimensional method of hPSC differentiation is not without limitations. The intrinsic nature of a two-dimensional model disregards the fact that in vivo development occurs in a three-dimensional environment. While the potential ability to control and regulate every signaling pathway on the differentiating cells is an advantage, a greater understanding of all signaling pathways involved in hemato-endothelial development is necessary to recapitulate in vivo environment in such a minimalist platform. Nonetheless, developing a completely chemically defined, feeder- and EB-free method of differentiation has the greatest therapeutic potential (Baghbaderani et al., 2015).

All of these methods of differentiation have been critical in developing a model of human hemato-endothelial development from hPSCs. In order to generate human LT-dHSCs in vitro, we must be able to induce each developmental stage that leads specifically to LT-dHSCs. While primitive versus definitive hematopoiesis can be easily distinguished by anatomical location in vivo, that advantage does not exist for in vitro cultures, and so specifically inducing the correct developmental ontogeny is critical (Sturgeon et al., 2014). Therefore, a model of human hemato-
endothelial development is necessary to reproduce *in vivo* development in a chemically defined, feeder- and EB-free system where the stage specific addition of growth factors, morphogens, cytokines, and small molecules can be used to generate LT-dHSCs from hPSCs.
1.6 Modeling Human Hemato-endothelial Development with Human Pluripotent Stem Cells

The most comprehensive model of hemato-endothelial development from hPSCs described to date has been from OP9 stromal cell co-culture and EB-differentiation (Choi et al., 2012a; Sturgeon et al., 2014). In both of these models, the first stage involves initiating differentiation from a pluripotent state towards primitive mesoderm. Recent studies have identified Apelin Receptor (APLNR) as a pan-mesodermal cell surface marker expressed on all Brachyury+ primitive mesoderm cells, which closely resemble the primitive streak in developing mouse embryos (Vodyanik et al., 2010). The mesoderm that eventually gives rise to hemato-endothelial lineages has been specifically described as APLNR+KDR+ (CD309, Flk-1, VEGFR2) and PDGFRα+ (CD140a) (Choi et al., 2012a; Sturgeon et al., 2014; Wang et al., 2012b). Studies using mouse ESCs and human EB differentiation have found that high levels of BMP4 and low levels of fibroblast growth factor-2 (FGF2) can induce the expression of Wnt3a and differentiation into mesodermal lineage (Kattman et al., 2011; Lian et al., 2013). Other studies have shown that GSK3b (a Wnt-signaling inhibitor) inhibition alone can initiate mesoderm patterning in human and non-human primate PSCs (D'Souza et al., 2016; Lian et al., 2013). The mouse and human PSCs exposed to high levels of BMP4 have been shown to undergo an epithelial-to-mesenchymal transition, which closely resembles the formation of the primitive streak and the transition from epiblast to mesoderm in the developing mouse embryo (Kattman et al., 2011; Yu et al., 2011).

The APLNR+KDR+PDGFRα+ primitive mesoderm continues to differentiate into lateral-plate like mesoderm cells which bifurcate into $^{EHM}Lin^A$APLNR+KDR$^{hi}$PDGFRα$^{lo}$
hematovascular mesoderm precursors (HVMP) (Choi et al., 2012a) and $^{\text{EHM} \text{Lin}^{-}}$ APLNR$^{+}$KDR$^{lo}$PDGFR$\alpha^{hi}$ cardiac mesoderm precursors (Kattman et al., 2011). The bifurcation can be influenced with the modulation of the Wnt signaling pathway. Inhibition of Wnt signaling at this stage has been found to induce cardiac mesoderm, while continued activation of Wnt signaling promotes HVMP (Lian et al., 2014; Lian et al., 2013).

The hematovascular mesoderm precursor begins to differentiate into hemogenic endothelial progenitors (HEP) that have the phenotype CD144$^{+}$CD31$^{+}$CD73$^{-}$CD43$^{-}$DLL4$^{-}$. Vascular endothelial growth factor (VEGF) has been found to be a critical growth factor necessary to induce the differentiation from HVMP to HEP, as well as the proliferation and survival dependent on FGF2 (Park et al., 2010; Salvagiotto et al., 2011; Sturgeon et al., 2014). Other studies have found that TGF$\beta$ inhibition can increase the efficiency of HEP induction (Bai et al., 2013; Wang et al., 2012b).

From the CD144$^{+}$CD31$^{+}$CD73$^{-}$CD43$^{-}$DLL4$^{-}$ HEPs, three distinct endothelial populations with varying degrees of hemogenic and angiogenic potential have been found to emerge (Choi et al., 2012a). While the CD144$^{+}$CD31$^{+}$CD73$^{-}$CD43$^{-}$ HEPs continue to proliferate, maintaining both hemogenic and angiogenic potential, non-HE population can also be detected with the upregulation of CD73. Others have found that this population of non-HE cells can be divided into CD73$^{med}$CD184$^{+}$DLL4$^{+}$ arterial and CD73$^{hi}$CD184$^{-}$ venous endothelium. This bifurcation between arterial and venous non-HE can be influenced by levels of NOTCH, mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase (PI3K) signaling pathways, with NOTCH and MAPK activation promoting arterial specification and PI3K activation promoting venous specification (Ditadi et al., 2015; Hong et al., 2006). In addition to HE and non-HE populations, an angiogenic hematopoietic progenitor population (AHP), identified by the
CD144⁺CD31⁺CD73⁻CD43⁺ phenotype, emerges. These hematopoietic progenitors are unique in that they form hematopoietic colonies only in a specialized FGF2-containing methylcellulose culture, but retain angiogenic potential and form sheets of endothelium in endothelial-specific culture (Choi et al., 2012a). While the exact identity of these AHPs are still being investigated, they have close resemblance to embryonic EMP hematopoiesis found in the between stages of primitive and definitive hematopoiesis.

Next, the CD144⁺CD31⁺CD73⁻CD43⁻ HE undergo endothelial-to-hematopoietic transition. In hPSC differentiation cultures, this stage has largely been found to be NOTCH dependent, as the treatment with γ-secretase inhibitors such as DAPT inhibits EHT (Ditadi et al., 2015). However, mouse AGM studies have found confounding evidence in the role of NOTCH signaling at this stage. Other signaling pathways implicated in hPSC cultures, include hedgehog (HH) signaling, hormone signaling, shear stress-induced CREB signaling, retinoic acid signaling, and inflammatory signaling pathways, have been investigated (Giorgetti et al., 2017). While RUNX1 and GATA2 have definitely been shown to be critical in EHT, there is still a lot yet to be uncovered about unique transitional event (Lim et al., 2013; Wahlster and Daley, 2016).

Finally, lin⁻CD34⁺CD43⁺CD45⁻⁺ hematopoietic progenitors (HPs) that emerge after EHT have myelo-lymphoid multilineage potential and can be maintained and expanded in culture. In hPSC differentiation cultures, CD43 (leukosialin) has been found to be the first hematopoietic marker that distinguishes hematopoietic progenitors from endothelium, though some have argued that CD43⁺ hematopoietic progenitors are of the primitive lineage (Sturgeon et al., 2014). Nonetheless, lin⁻CD34⁺CD43⁺CD45⁻⁺ HPs have granulocyte-erythroid-macrophage-megakaryocyte colony forming potential (CFC-GEMM) and T-lymphoid potential, and can generate enucleated erythrocytes with γ- and limited β-globin expression. These cells can be
maintained in culture and expanded with the addition of stem cell factor (SCF), thrombopoietin (TPO), interleukin-3 (IL-3), and interleukin-6 (IL-6) (Choi et al., 2012a; Choi et al., 2009a; Vodyanik et al., 2005; Vodyanik et al., 2006). Other studies have found that the addition of Flt-3 ligand (FLT3L), erythropoietin (EPO), interleukin-11 (IL-11), and endothelial growth factors epithelial growth factor (EGF), insulin-like growth factor 1 (IGF-I), and insulin-like growth factor 2 (IGF-II) can also promote HP maintenance and growth in serum-free media (Ditadi et al., 2015; Sturgeon et al., 2014).

The findings of these studies have facilitated in the development of a completely chemically defined differentiation platform (addressed in Chapter 2). However, while the HPs generated from hPSCs in these systems resemble definitive-type hematopoietic cells, we still do not detect long-term engraftment in immunocompromised mice. Comparative gene expression profiles reveal some abnormalities in hPSC-derived HPs compared to de novo umbilical cord-derived LT-dHSCs. One of the aberrantly regulated signaling pathways of interest is the NOTCH signaling pathway (McKinney-Freeman et al., 2012; Seita and Weissman, 2010). While NOTCH signaling has been shown to be uniquely necessary for the definitive wave of hematopoiesis and not the primitive wave in vivo, there have been conflicting reports on the role of NOTCH signaling during HE specification, EHT, and HP.
1.7 The Role of NOTCH Signaling Pathway during Hemato-endothelial Development

The NOTCH signaling pathway has been extensively studied and found to be a key player of “lateral inhibition,” in which a group of equivalent precursors can adopt different cell fates (Greenwald and Rubin, 1992). It is a highly evolutionarily conserved signaling pathway that has been shown to be critical during embryonic development and adult homeostasis (Andersson et al., 2011). The NOTCH receptor, of which there are 4 in humans and mice (Notch1-4), is a heterodimeric transmembrane cell surface receptor encoded by a single mRNA with the extra-cellular domain (Notch:ECD) consisting of EGF-like repeats linked to the intra-cellular domain (Notch:ICD) consisting of seven ankyrin-like repeats, a nuclear localization signal (NLS), and a transactivation domain. They are linked by disulfide bonds on the Lin/Notch Repeat (LNR) domain on the extra-cellular surface that prevents ligand-independent activation. The NOTCH signaling cascade begins when the NOTCH receptor on the “signal-receiving” cell binds to 1 of 5 of its ligands (DLL1-2, DLL4, and Jag1-2) that is expressed on an adjacent “signal-sending” cell (Bigas et al., 2012; Bigas and Espinosa, 2012; Butko et al., 2016). The binding of the NOTCH ligand physically “tugs” at the receptor and induces a change in the Notch receptors’ conformation which reveals the cleavage site of the ADAM secretase. After ADAM secretase cleaves the Notch:ECD, the entire Notch ligand-Notch:ECD complex is endocytosed into the signal-sending cell. The cleavage by ADAM secretase on the Notch:ECD then recruits γ-secretase to cleave the Notch:ICD and releases it from the cell surface. The freed Notch:ICD is then translocated from the cell membrane of the signal-receiving cell into its nucleus, where it displaces a co-repressor and binds to the RBPjk transcription factor complex (Fortini, 2009; Helbig and Amsen, 2015). This initiates the transcriptional cascade of NOTCH
target genes such as the HES-family, HEY-family, the NOTCH ligand DLL4, cMYC, GATA2, and RUNX1 genes (Ayllon et al., 2015; Bigas et al., 2013; Burns et al., 2005; dela Paz and D'Amore, 2009; Gerhardt et al., 2014; Guiu et al., 2013; Kennedy et al., 2012b; Lizama et al., 2015; Nakagawa et al., 2006; Richard et al., 2013; Robert-Moreno et al., 2005; Robert-Moreno et al., 2008).

While the canonical NOTCH signaling pathway seems straightforward, deciphering the specific role of NOTCH signaling during hemato-endothelial development has been challenging (Bigas and Espinosa, 2012; Butko et al., 2016; Ohishi et al., 2003). The NOTCH signaling pathway is involved in a wide range of developmental programs in all three germ layers (Andersson et al., 2011; Helbig and Amsen, 2015). Aberrant NOTCH signaling caused by mutations in NOTCH pathway genes are associated with a wide range of human diseases including spine defects, skeletal growth disorder, multiple types of leukemia (T-cell acute lymphoblastic leukemia and acute myeloid leukemia), other types of cancer, and has even been implicated in schizophrenia (Andersson et al., 2011). Furthermore, NOTCH signaling is involved in different stages throughout hemato-endothelial development, from arterial specification (Hong et al., 2006; You et al., 2005) to T-lymphocyte development (Laurenti et al., 2013; Stier et al., 2002; Varnum-Finney et al., 2003). Additionally, NOTCH signaling does not follow a traditional “cascade” of signal amplification such as PI3K and MAPK signaling pathways, giving NOTCH activation a stoichiometric relationship between signal input and activation levels which in turn changes the cellular response depending on signal strength (Andersson et al., 2011). Finally, while NOTCH signaling can be inhibited with small molecules such as γ- and ADAM-secretase inhibitors, NOTCH activation is uniquely challenging, as the surface receptor requires the physical “tug” to initiate the signaling cascade. Thus, a few studies have used ectopically
expressed Notch:ICD to activate the signaling pathway, but, as mentioned previously, the
different strengths of NOTCH activation can change the cellular response.

What has been largely accepted from multiple transgenic mouse studies is that the
primitive wave of hematopoiesis is NOTCH-independent (Bigas et al., 2013; Kennedy et al.,
2012b; Nakagawa et al., 2006; Qiu et al., 2008; Sturgeon et al., 2014) while the definitive wave
of hematopoiesis is NOTCH-dependent (Bigas et al., 2012; Bigas and Espinosa, 2012; Kumano
et al., 2003). However, determining what role NOTCH signaling has on each stage of definitive
hematopoiesis, HE specification, EHT, and HP proliferation, has been a challenge.

Transgenic Notch1−/−, but not Notch2−/−, mice are embryonic lethal with major defects
found in the vasculature and a complete absence of intra-embryonic hematopoiesis, but have
functional extra-embryonic yolk sac hematopoiesis (Kumano et al., 2003). Multiple other
knockout mouse and conditional knockout mouse studies have shown that NOTCH activation
during vasculogenesis is necessary for the arterial specification of endothelial cells, which can be
distinguished by the expression of Notch1, EphrinB2, and DLL4 on arterial vascular
endothelium (dela Paz and D'Amore, 2009; Lawson et al., 2002; Zhong et al., 2001). DLL4−/−,
RBPjk−/− and Mindbomb−/− mice are all embryonic lethal, with severe impairment in arterial
vasculogenesis and a failure to develop the dorsal aorta (Kumano et al., 2003; Robert-Moreno et
al., 2005; Robert-Moreno et al., 2008). Without the proper development of the DA, these mice
also lack intra-embryonic hematopoiesis, suggesting that HE that undergo EHT and mature into
LT-dHSCs undergo a Notch-dependent arterial endothelial intermediate. One study has found
that ablation of COUP-TFII expression in venous endothelium alone can increase levels of
NOTCH signaling, leading to an increase in arterial characteristics and generating hematopoietic
clusters in otherwise non-hemogenic sites (You et al., 2005). A recent study investigating the
transcriptome at the single-cell level has found that Type-I pre-HSCs express genes associated with arterial endothelium (Zhou et al., 2016), while another recent study has shown that all endothelial cells in the DA, including hemogenic endothelium, express EphrinB2, and EphrinB2/* mice lack intra-embryonic hematopoiesis (Chen et al., 2016).

However, a few studies have argued that HE and arterial endothelial cells are distinct lineages of endothelial cells, and that the absence of intra-embryonic hematopoiesis in NOTCH signaling-deficient mice is due to the absence of the normal development of the DA which harbors the HE. A transgenic mouse study utilizing the Runx1+23-eGFP enhancer reporter found that eGFP* HE already lose endothelial identity and begin to express genes associated with hematopoietic commitment, including Gata2, Runx1, GFI1, and SCL before EHT begins (Swiers et al., 2013). Human PSC differentiation studies showed evidence that DLL4* endothelial cells are lineage restricted to endothelium and hemogenic potential is restricted to DLL4* endothelium (Ayllon et al., 2015; Cristofaro et al., 2013). Furthermore, hemogenic endothelium and arterial endothelium have been shown to be from distinct progeny, bifurcated by the upregulation of CD184 (CXCR4) and DLL4 on non-hemogenic arterial vascular endothelium (Ditadi et al., 2015).

The role of NOTCH signaling on EHT is even more obscure. Most studies agree that NOTCH is required to upregulate the hematopoietic transcription factors Runx1 and Gata2 in HE for successful EHT (Bigas et al., 2013; Guiu et al., 2013; Hadland et al., 2004; Kennedy et al., 2012b; Mestas and Hughes, 2004; Nakagawa et al., 2006; Qiu et al., 2008; Richard et al., 2013; Robert-Moreno et al., 2008). However, a few mouse studies suggest, that while Notch1 activation is required for HE specification, continuous NOTCH signaling maintains endothelial identity and inhibits EHT. (Clarke et al., 2013; Jang et al., 2015; Lizama et al., 2015; Tang et al.,
In one hPSC study, transgenic hESCs that ectopically expressed SOX17 via ERT-tamoxifen control maintained endothelial identity and were inhibited from EHT with continuous SOX17 expression (Nakajima-Takagi et al., 2013). Another study showed that while NOTCH signaling is required for RUNX1 upregulation, downregulation of NOTCH signaling is required for successful EHT (Richard et al., 2013). In addition, a few AGM-explant studies have also shown an increase in hematopoietic activity when treated with the \( \gamma \)-secretase inhibitor DAPT (Hadland et al., 2004; Lizama et al., 2015).

In contrast, other studies suggest that NOTCH signaling is necessary for EHT. While Jag1\(^{-/-}\) mice had normal arterial development, they had significantly reduced hematopoietic clusters in the DA (Robert-Moreno et al., 2008). In explant culture, while HE isolated from the murine AGM and co-cultured on AGM-derived AKT-expressing endothelial cells generated HSCs with engraftment potential, treatment with DAPT significantly decreased EHT and inhibited the specification of LT-dHSCs (Hadland et al., 2015). Furthermore, hPSC-derived HE have also been shown to decrease EHT when treated with DAPT (Ditadi et al., 2015).

There are a few murine studies that address the temporal- and dose-dependent complications of NOTCH signaling and HSC specification. In one experiment, when Notch-dependent genes Hes1 and Hes5 were knocked out of transgenic mice, Gata2 expression was prolonged and the AGM was found to increase EHT. However, there was a significant decrease in detectable LT-dHSCs with engraftment potential. NOTCH signaling was found to upregulate both Gata2 and Hes1/5, but subsequent repression of Gata2 by Hes1/5 was necessary to generate LT-dHSCs from murine AGM explants (Guiu et al., 2013). In another study, Jag1\(^{-/-}\) mice were found to have decreased EHT in the DA similar to that observed in DLL4\(^{-/-}\) mice, but unlike DLL4\(^{-/-}\) mice, had normal vasculature and arterial development (Robert-Moreno et al., 2008).
Later, the same group used two different NOTCH activation mice with varying degrees of NOTCH sensitivity to show that the level of NOTCH activation was much higher in arterial endothelium compared to LT-dHSCs (Gama-Norton et al., 2015). These findings were consistent with the notion that cell fate is sensitive to NOTCH activation timing and levels, as well as with previous reports that DLL4 is a potent activator of NOTCH signaling while Jag1 is a weak activator of NOTCH signaling.

The role of NOTCH signaling on LT-dHSC maintenance is also contentious. Previous reports suggest that the bone marrow niche express Notch ligands and the activation of Notch is necessary for HSC maintenance. Indeed, in vitro assays that activate NOTCH signaling can stimulate self-renewal and maintain long-term multilineage engraftment potential of both mouse LT-dHSCs (Varnum-Finney et al., 2003; Varnum-Finney et al., 1998) and human LT-dHSCs (Karanu et al., 2001; Ohishi et al., 2002; Shojaei et al., 2005; Suzuki et al., 2006). Others have reported increased proliferation and decreased differentiation of mouse LT-dHSCs with the ectopic expression of Notch:ICD (Carlesso et al., 1999; Stier et al., 2002) or its downstream target, Hes1 (Kunisato et al., 2003; Yu et al., 2006).

Others have shown, however, that Notch signaling is dispensable for normal LT-dHSC maintenance in the bone marrow niche and is only required for lymphoid differentiation (Mancini et al., 2005). Previous reports using an inducible RBPjk knockout mouse showed normal HSC maintenance in the bone marrow and long-term engraftment after transplantation, albeit decreased T-lymphocyte development in the thymus (Han et al., 2002). More recently, Maillard et al showed that LT-dHSCs expressing the dominant-negative form of Mastermind-like (DNMAML, MAML), a cofactor for the Notch:ICD transcription complex, had similar engraftment potential as normal LT-dHSCs. While normal HSCs did increase the expression of
NOTCH downstream target genes when cultured on DLL1-expressing OP9 stromal cells, they did not have a competitive advantage during engraftment when injected with DNMAML-expressing LT-dHSCs (Maillard et al., 2008). Finally, in a recent study, reporter mice expressing a destabilized form of GFP (dGFP) driven by the Hes1-promoter revealed high Hes1-dGFP expression in Type-I pre-HSCs, but decreasing Hes1-dGFP expression in Type-II pre-HSCs. Inhibition of Notch signaling with DAPT inhibited the maturation of Type-I pre-HSCs into definitive HSCs, but engraftable definitive HSCs were still detected from DAPT-treated Type-II pre-HSCs. In addition, maintaining NOTCH activation in Type-I pre-HSCs through DLL1-expressing OP9 stromal cells revealed a decrease in LT-dHSCs that have engraftment potential, suggesting that a specific level of NOTCH activation is necessary for Type-I to Type-II pre-HSC maturation, but NOTCH signaling is dispensable thereafter (Souilhol et al., 2016b).

The NOTCH signaling pathway is known to play a critical role in the development of the definitive wave of hematopoiesis and HSC specification. However, to date, the specific role of NOTCH signaling during HE specification, EHT, and HSC maintenance is still unclear. NOTCH signaling is complex, with different timing and dosage of NOTCH activation having different effects on the same cell. While the NOTCH signaling pathway is largely conserved across all vertebrates, subtle species-specific differences may contribute to the challenges of generating hPSC-derived LT-dHSCs in vitro without a defined platform on which we can manipulate levels of Notch activation during hemato-endothelial differentiation.
Herein, we take advantage of a chemically defined differentiation system to isolate and investigate the role of the NOTCH signaling pathway during hemato-endothelial development. This is the first study we are aware of that shows the effect of both activating and inhibiting NOTCH signaling in a stage-specific manner during hPSC differentiation. We demonstrate that NOTCH activation facilitates EHT, HP expansion, and is required for definitive-type hematopoiesis. Crucially, we identify a transient population of DLL4⁺ HE with arterial-specific gene upregulation that requires increased NOTCH signaling for EHT and gives rise to definitive-type HPs.
CHAPTER 2: TENASCIN C PROMOTES EFFICIENT DEFINITIVE HEMATOPOIETIC DEVELOPMENT FROM HUMAN PLURIPOTENT STEM CELLS
2.1 Abstract

Recent identification of hemogenic endothelium (HE) in human pluripotent stem cell (hPSC) cultures presents opportunities to investigate signaling pathways essential for blood development from endothelium and provides an exploratory platform for de novo generation of hematopoietic stem cells (HSCs). However, the use of poorly defined human or animal components limits the utility of the current differentiation systems for study of specific growth factors required for HE induction and for manufacture of clinical grade therapeutic blood cells. Here, we identified a chemically defined conditions required to produce HE from hPSCs growing in E8 medium, and showed that Tenascin C (TenC), a protein associated with HSC niches, strongly promotes HE and definitive hematopoiesis in this system. Human PSCs differentiated in chemically defined conditions undergo similar stages of development as we described in hPSCs cocultured on OP9 feeders, including the formation of VE-Cadherin^+CD73^ CD235a/CD43^ HE and hematopoietic progenitors with myeloid and T-lymphoid potentials.
2.2 Introduction

In the embryo, hemogenic endothelium (HE) was identified as an immediate direct precursor of hematopoietic progenitors and hematopoietic stem cells (HSCs) (Bertrand et al., 2010; Boisset et al., 2010; Jaffredo et al., 2000; Kissa and Herbomel, 2010; Zovein et al., 2008). Thus, the ability to produce HE from human pluripotent stem cells (hPSCs) is considered a critical step to advance de novo generation of blood progenitors and stem cells. Recent identification and characterization of HE in hPSC cultures by our lab and others, has developed a platform for investigating pathways controlling HE formation and subsequent HSC specification. (Choi et al., 2012c; Kennedy et al., 2012a; Rafii et al., 2012) However, the use of xenogeneic or allogeneic feeder cells, poorly defined serum and matrix proteins, or proprietary medium and supplements with undisclosed chemical composition limits the utility of the current differentiation systems to study essential factors required for HE development and specification. Here we identified a set of factors and matrix proteins capable of supporting hematopoietic differentiation after plating hPSCs from a single-cell suspension in a completely chemically defined medium that is free of serum components and xenogeneic proteins. Importantly, we showed the critical role of the HSC niche matrix component, Tenascin C (TenC), in supporting the development of hemato-endothelial and T-lymphoid cells from hPSCs.

Our prior studies identified distinct stages of hemato-endothelial development following hPSC differentiation in coculture with OP9 (Figure 2-1) (Choi et al., 2012c; Slukvin, 2013a; Vodyanik et al., 2005; Vodyanik et al., 2006; Vodyanik et al., 2010). Plating hPSCs onto OP9 stromal cells induces the formation of primitive streak and mesodermal cells which can be detected based on the expression of apelin receptor (APLNR) (Vodyanik et al., 2010). Because
this marker is also expressed in lineage-committed progenitors at post-mesodermal stages, we demarcated the mesoderm stage based on the absence of Endothelial (CD31, VE-cadherin), Endothelial/Mesenchymal (CD73, CD105) and Hematopoietic (CD43, CD45) cell-surface markers, i.e. by \(\text{EMH}^\text{lin}^-\) phenotype. The early \(\text{EMH}^\text{lin}^-\text{APLNR}^+\) that appear in OP9 coculture on day 2 of differentiation have features of primitive posterior mesoderm (PM), i.e. they acquire expression of the primitive streak genes, \(T\) and \(\text{MIXL}1\), and lateral plate mesoderm genes, FOXF1 and GATA2, and display A\(+\)P\(+\)PDGFR\(\alpha^+\)KDR\(^+\) phenotype (hereafter referred to as A\(^-\)P\(^+\) cells). These cells possess mesenchymoangioblast (MB) potentials, i.e. the potential to form colonies with the capacity to differentiate into mesenchymal stem cells (MSC) and endothelial cells. On day 3 of differentiation, A\(^-\)P\(^+\) cells acquire blast (BL)-CFC or hemangioblast (HB) potential (Vodyanik et al., 2010). Both MB and HB potentials can be detected using colony-forming assay in serum-free clonogenic medium supplemented with FGF2 (Vodyanik et al., 2010). With advanced maturation, \(\text{EMH}^\text{lin}^-\text{APLNR}^+\) mesodermal cells lose BL-CFC activity, upregulate KDR, and downregulate PDGFR\(\alpha\) i.e. acquire the hematovascular mesodermal precursor (HVMP) phenotype, \(\text{EMH}^\text{lin}^-\text{KDR}^{\text{hi}}\text{APLNR}^+\text{PDGFR}^\alpha^{\text{lo/c}}\) (hereafter referred to as K\(^{\text{hi}}\) cells) . K\(^{\text{hi}}\) HVMP cells downregulate the primitive streak genes \(T\) and \(\text{MIXL}1\), and upregulate genes associated with lateral plate and hematovascular mesoderm development, \(\text{FOXF}1\), \(\text{ETV}2\) and \(\text{GATA}2\). K\(^{\text{hi}}\) HVMPs are highly enriched in cells with the potential to form hemato-endothelial clusters on OP9 (Choi et al., 2012c). The endothelial stage of development is defined by the expression of the endothelium-specific marker, VE-cadherin (VEC). The first endothelial cells that coexpress VEC and CD31 emerge from K\(^{\text{hi}}\) mesodermal cells by day 4 of differentiation. The emerging VEC\(^+\) cells represent a heterogeneous population that include CD235a/CD43 CD73\(^+\) non-hemogenic endothelial progenitors (non-HEPs), and CD235a/CD43\(^-\)
CD73– hemogenic endothelial progenitors (HEPs) (Choi et al., 2012c). HEPs lack hematopoietic CFC potential, but acquire it after coculture with stromal cells. The hematopoietic stage of development is defined by the expression of the hematopoietic-specific marker CD43 (Choi et al., 2012c; Vodyanik et al., 2006). The first CD43+ cells emerge within the VEC+ cells on day 4-5 of differentiation. These cells express low levels of CD43 (CD43lo) and coexpress CD235a, but lack CD41a expression, i.e. have the phenotype, VEC+CD43lo235a+41a-. Because these cells have the capacity to form hematopoietic colonies in the presence of FGF2 and hematopoietic cytokines, as well as form a monolayer of endothelial cells on fibronectin, we designated them as angiogenic hematopoietic progenitors (AHPs). The first CD41a+ cell appears within the CD235a+ population. The CD235a+CD41a+ cells are highly enriched in erythro-megakaryocytic progenitors and lack endothelial potential. The progenitors with broad myelolymphoid potential and lin–CD34+CD43+CD45– phenotype can be detected in hPSC cultures shortly after the emergence of CD235a+CD41a+ cells. Acquisition of CD45 expression by lin– cells is associated with progressive myeloid commitment (Vodyanik et al., 2006). In the present work, we demonstrated that the TenC-based chemically defined system is able to obtain all mesodermal and endothelial transitional stages and hematopoietic progenitors with pan-myeloid and lymphoid potentials that we observed using our serum- and OP9 feeder-based differentiation system (Choi et al., 2012c; Vodyanik et al., 2005; Vodyanik et al., 2006; Vodyanik et al., 2010). Because the new differentiation system utilizes hPSCs growing in completely chemically defined xenogene-free Essential 8 (E8) medium on vitronectin (VTN), (Chen et al., 2011a) it opens the opportunity to produce clinical-grade endothelial and myelolymphoid progenitors from hPSCs for therapeutic purposes.
2.3 Results

IMDM/F12 based media significantly improves differentiation efficiency of hPSCs into hematopoietic lineage from single cell suspension in 2D culture

Previously, our lab developed a hPSC differentiation protocol for the efficient generation of hematopoietic progenitors using a coculture method on the mouse stromal cell line, OP9 (Vodyanik et al., 2005; Vodyanik and Slukvin, 2007a). Although the OP9 system supports efficient generation of HE and multilineage hematopoietic progenitors (Figure 2-1), this system is very sensitive to variations in serum quality, stromal cell maintenance, and size of hPSC colonies and clumps used for differentiation (Choi et al., 2011; Vodyanik and Slukvin, 2007a). Forming embryoid bodies (EBs) is another commonly used approach for inducing HE and hematopoietic progenitors from hPSCs (Kennedy et al., 2012a; Ng et al., 2005; Wang et al., 2004). However, EB methods often rely on serum or undefined media and also have significant drawbacks, such as asynchronous differentiation, high variability, and dependence on initial clump size. Additionally, inconsistency in the quality of hPSCs caused by variations in albumin batches used for hPSC maintenance may introduce variations in efficiency of blood development.
Figure 2-1: Schematic diagram of hematopoietic differentiation

Main cell subsets observed in prior differentiation studies using coculture with OP9 feeders and current chemically defined cultures are shown. PM is primitive posterior mesoderm; HVMP is hematovascular mesodermal precursor; HEP is hemogenic endothelial progenitors; AHP is angiogenic hematopoietic progenitors; non-HEP is non-hemogenic endothelial progenitors, HP is hematopoietic progenitors; EMkP is erythromegakaryocytic progenitors. EMHlin− is the lack of expression of Endothelial (CD31, VE-cadherin), Endothelial/Mesenchymal (CD73, CD105) and Hematopoietic (CD43, CD45) markers. lin− is the lack of expression of markers associated with hematopoietic lineages.
To overcome these limitations we set out to characterize chemically defined media and matrix proteins capable of supporting hemato-endothelial differentiation without serum from a single cell suspension of H1 hESCs maintained in the completely defined xenogene-free system using E8 medium on vitronectin (VTN) (Chen et al., 2011b). First, we plated hESCs as single cells and allowed them to attach over 24 hours in E8 media supplemented with 10uM Rho kinase inhibitor on Matrigel (MTG), VTN, or Collagen IV (ColIV) in normoxia. Then, the media was changed to either basal growth factor-free mTeSR1, E8 (DF4S), E8 with an IMDM-base (I4S), or E8 with an IMDM/F12 base (IF4S) supplemented with human recombinant BMP4, FGF2, and VEGF factors which are commonly used to induce blood formation from hPSCs (Pick et al., 2007; Salvagiotto et al., 2011). After 4 days of differentiation cell cultures were evaluated for the presence of CD31<sup>+</sup> cells which coexpress KDR and VE-cadherin (VEC) and are highly enriched in hemato-endothelial progenitors (Choi et al., 2012c). Flow cytometric analysis showed that cells differentiated on ColIV-coated plates in IF6S differentiated most efficiently into CD31<sup>+</sup> hemato-endothelial precursors (Figure 2-2).
Figure 2-2: Generation of D4 HEP in cultures using different basal media and matrix protein

mTeSR1 is mTeSR1 without FGF2 and TGFβ. DF4S is DMEM/F12-based media with 4 supplements: 64mg/L L-asorbic Acid 2-Phosphate Mg²⁺ salt, 14µg/L sodium selenite, 10.6ng/L Holo-Transferrin, and 20mg/L Insulin. I4S is DF4S with IMDM-based media instead of DMEM/F12-based media, but with the 4 previously mentioned supplements. IF4S is DF4S with IMDM/F12-based media instead of DMEM/F12-based media, but with the 4 previously mentioned supplements. VTN is vitonectin matrix; MTG is Matrigel substrate; ColIV is Collagen IV matrix. Flow cytometry plots show percent of CD31⁺ endothelial precursors of day 4 cells differentiated in each media supplemented with 50ng/ml FGF2, BMP4 and VEGF in hypoxia.
Later, we found that the addition of polyvinylalcohol, NEAA, Glutamax, chemically defined lipid concentrate, and monothioglycerol increased cell viability and differentiation efficiency (data not shown). The subsequent basal media is referred to as IF9S (IMDM/F12 plus 9 supplements; see table S1 for complete composition of medium). These results demonstrated that the selected media and supplements made it possible to obtain hemato-endothelial cells in a chemically defined, xenogene-free conditions on ColIV matrix from hPSCs maintained in E8 media.

*Analysis of molecular signatures of hematopoiesis-supporting stromal cell lines identified Tenasin C as an extracellular matrix that is uniquely expressed in OP9 feeders with high hematopoiesis-inducing potential*

Previously, we showed that OP9 is superior to S17 and MS5 stromal cell lines in inducing hematopoietic differentiation (Vodyanik et al., 2005). It was also found that day 8 overgrown OP9 cultures are superior to day 4 semi-confluent OP9 cultures in inducing hematopoietic-CFCs, including multipotential GEMM-CFCs. Since the confluency of the stromal cells affect differentiation efficiency, this led us to believe that there is an extracellular matrix influencing hemato-endothelial differentiation. In order to find the matrix protein(s) critical for hematopoiesis-supporting activity of OP9, we performed molecular profiling of S17 and MS5 stromal cell lines with low hematopoiesis-inducing potential. In addition, we compared overgrown OP9 (day 8) with semi-confluent OP9 (day 4) monolayers. Transcriptome analysis revealed 21 genes showing at least three-fold higher expression in day 8 overgrown OP9 cells as compared to all other stromal cells (Figure 2-3 A). These included genes encoding *Ptn*
(pleiotrophin), a secreted regulator of HSC expansion and regeneration (Himburg et al., 2010b), Rsvo3 (R-spondin 3), an important regulator of Wnt signaling and angioblast development (Kazanskaya et al., 2008), and an extracellular matrix protein Postn (periostin) which is required for B lymphopoiesis (Siewe et al., 2011). Interestingly, one the most highly upregulated gene in overconfluent OP9 was Tnc (Tenascin C) (Figure 2-3 B). TenC is expressed by mesenchymal cells underlying hematopoietic clusters in the Aorta-Gonado-Mesonephros (AGM) region and is required for intraembryonic and postnatal hematopoiesis (Marshall et al., 1999; Nakamura-Ishizu et al., 2012; Ohta et al., 1998). It is also expressed in the bone marrow stem cell niche (Nakamura-Ishizu et al., 2012). Because of these unique properties, we tested whether TenC could support hematopoietic differentiation more effectively than CollIV.
Figure 2-3: Comparing different mouse stromal cell lines supporting hematopoiesis of hPSCs

A) Venn diagram revealing the number of genes differentially expressed between each stromal cell line. B) Heat map of 21 genes uniquely upregulated in over-confluent (day 8; d8) OP9 stromal cell lines as compare to all other stromal cell lines (S17, MS5, and semi-confluent OP9 (day 4; d4)). Tenascin-C (Tnc) is one of the top differentially overexpressed genes in overgrown OP9 cells.
Tenascin C facilitates the development of mesoderm and hemato-endothelial precursors in chemically defined cultures following stage-specific treatment of FGF2, BMP4, Activin A, LiCl, VEGF, and hematopoietic cytokines.

Using OP9 coculture system we revealed major steps of hemato-endothelial development from hPSCs (see introduction and Figure 1). In order to reproduce the hemato-endothelial program observed in OP9 coculture, we identified the optimal combinations of morphogens for mesoderm induction and hemato-endothelial specification, and found the specific growth factors required for the step-wise progression of hPSC differentiation towards HE and blood cells in chemically-defined conditions on ColIV and TenC (Figure 2-1).

During embryonic development, BMP4, Wnt, and TGFβ/Nodal/Activin A signaling have been found to be critical to initiate primitive streak formation and subsequent mesoderm development (Gadue et al., 2005; Keller, 2005). It has been shown that the activation of these signaling pathways is essential to induce the expression of brachyury (T) and KDR (Flk-1, VEGFR2), and initiate mesodermal commitment of mouse and human PSCs (Cerdan et al., 2012; Kennedy et al., 2007; Nostro et al., 2008; Pearson et al., 2008; Pick et al., 2007; Salvagiotto et al., 2011). We have found that high concentrations of BMP4 (50ng/ml) combined with low concentrations of Activin A (15ng/ml) and a supplement of LiCl (2mM) consistently induced expression of the mesodermal surface markers APLNR, KDR, and PDGFRα after 2 days of culture of singularized hESCs on ColIV or TenC, as described above. However, these conditions poorly supported cell survival and required the addition of FGF2 and a hypoxic conditions (5% O2, 5% CO2) to improve cell viability and output of mesodermal cells. Day 2 mesodermal cells differentiated in these conditions expressed PDGFRα and APLNR surface markers, i.e. became
A\(^+\)P\(^+\) cells and displayed MB colony-forming potential (Figure 2-4 A,C), similar to A\(^+\)P\(^+\)
mesodermal cells obtained from day 2 of hPSCs differentiated in OP9 coculture (Vodyanik et al.,
2010). After 2 days of differentiation, we found that only FGF2 and VEGF are sufficient for
A\(^+\)P\(^+\) mesoderm to acquire HB potential on day 3 of differentiation. Day 3 A\(^+\)P\(^+\) cells generated
in chemically defined conditions similar to their counterpart generated in OP9 coculture
expressed \(T\) and \(MIXL1\) primitive streak genes at high level, and \(FOXF1\) and \(GATA2\) lateral plate
mesoderm genes (Figure 2-4 C). The pattern of development was similar in cells cultured on
ColIV and TenC, however the latter produced significantly higher A\(^+\)P\(^+\) cells, and MB and HB
colonies (Figures 2-4 A, 3B, 3D).

We also found that only FGF2 and VEGF are sufficient to advance mesoderm
specification toward a hematovascular fate signified by the increase of KDR and decrease of
PDGFR\(\alpha\) expression on day 4 of differentiation (Figure 2-4 E and Figure 2-5). Similar to K\(^{hi}\)
HVMP cells isolated from hPSC/OP9 cocultures, K\(^{hi}\)CD31\(^-\) (K\(^{hi}\)) cells obtained in chemically
defined conditions downregulated \(T\) and \(MIXL1\) primitive streak genes and upregulated the genes
\(FOXF1, ETV2,\) and \(GATA2\) which are associated with lateral plate and hematovascular
mesoderm development (Figure 2-4 C). Although day 4 differentiated cells lost HB potential
(Figure 2-4 D), K\(^{hi}\) cells were capable of forming hemato-endothelial clusters when sorted and
plated onto OP9 (Figure 2-4 G). In contrast, K\(^{lo}\) (K\(^{lo}\)) cells only formed endothelial clusters
with almost no hemogenic activity (Figure 2-4 G). This is also consistent with differentiation in
OP9 coculture (Choi et al., 2012c). The percentage of K\(^{hi}\) HVMP cells was consistently higher in
TenC cultures (Figure 2-4 F).
Figure 2-4: Mesodermal development of hESCs in defined conditions on ColIV and TenC

Cultures differentiated on ColIV vs TenC for 2, 3, and 4 days in chemically defined conditions. A) Flow cytometry plots and B) graphs comparing percentage of A⁺P⁺ primitive mesodermal population on days 2 and 3. C) Expression of mesoderm lineage genes measured by qPCR and
normalized to RPL13A comparing day 3 P⁺ cells and day 4 Kʰᵢ cells. D) Comparison of MB/HB-colony forming potential of day 2, day 3, and day 4 cultures. E) Flow cytometry plots and F) graphs comparing percentage of KDRʰᵢCD31⁻ (Kʰᵢ) HVMP, CD31⁺, and KDRʰ₀CD31⁻ (Kʰ₀) populations on day 4 of differentiation. G) Hematopoietic and endothelial potentials of Kʰᵢ and Kʰ₀ cells isolated from day 4 differentiated cells after coculture on OP9 for 7 days. Upper panels show flow cytometry of TRA-1-85⁺ gated human cells and lower panels show immunofluorescence staining of cells from OP9 cocultures with Kʰᵢ and Kʰ₀ cells. B), C), D), and G) bars are mean ± SE from at least 3 experiments (*p<0.01). Scale bar represents 100 um. Fluorescence images were captured on Olympus IX71 microscope at 10x magnification using Q!MAGING RetigaExi Fast 1394 and acquired with QCapture software. VE-Cadherin was visualized using a secondary antibody conjugated to DyLight488 and CD43 was visualized using a secondary antibody conjugated to DyLight 594. Images were adjusted and cropped in Adobe Photoshop.

**Figure 2-5: Expression levels of PDGFRα on D3 and D4 KDR⁺ population**

Flow cytometry plot showing the expression of PDGFRα by gated day 3 KDR⁺ cells and by day 4 gated KDRʰᵢ (Kʰᵢ) cells.
Because formation of HVMPs in hPSC/OP9 coculture is closely followed by the development of HE and blood progenitors, we supplemented our cultures with SCF, TPO, IL-6, and IL-3 hematopoietic cytokines in addition to VEGF and FGF2 starting from day 4 of differentiation. Although we noticed that the continuous treatment of cultures with FGF2 and VEGF was sufficient for induction of endothelial progenitors and hematopoietic specification, addition of hematopoietic cytokines was essential to increase the output of these cells in chemically defined cultures. On day 5 of differentiation in these conditions, the previously identified 3 major subsets of the VEC^+ populations (Choi et al., 2012c) emerged: VEC^-CD235a^-CD43^-CD73^+ (non-HEPs), VEC^+CD235a/^CD43^-CD73^- (HEPs), and VEC^+CD43/^CD235a^- (AHPs) (Figure 2-6 A, B). When these subsets were sorted and plated into endothelial conditions, all of them formed a monolayer of VE-cadherin expressing cells with capacity to uptake AcLDL and form vascular tubes in the tube formation assay, consistent with OP9 coculture (Figure 2-6 C). However, hematopoietic CFC-potential was mostly restricted to the VEC^-CD43/^CD235a^- cells (Figure 2-6 D). Importantly, similar to our previous finding with day 5 VEC^- subsets generated in coculture with OP9, the hematopoietic CFC-potential of VEC^-CD43/^CD235a^- cells was detected only in serum-free medium in the presence of FGF2 in addition to hematopoietic cytokines (Figure 2-6 D, Figure 2-7). This indicates that VEC^-CD43/^CD235a^- cells are essentially similar to the AHPs identified in hPSC/OP9 coculture (Choi et al., 2012c). We previously defined HEP as VEC^+CD43^-CD73^- cells lacking hematopoietic CFC-potential, but capable of acquiring it after culture on OP9. To determine whether VEC^+CD43^-CD73^- cells generated in completely chemically defined conditions are similar to HEPs produced in OP9 cocultures, we sorted the day 5 VEC^+ subpopulations and cultured them on OP9 as previously described (Choi et al., 2012c). In these conditions, the HEPs
formed both endothelial and hematopoietic cells with a large number of HE-clusters, while AHPs formed predominantly hematopoietic cells with few endothelial cells and hemato-endothelial clusters. VEC$^+$/CD43$^-$CD73$^+$ cells formed exclusively endothelial clusters, consistent with the non-HEP phenotype (Figure 2-6 C). Cultures differentiated on TenC had a larger population of total CD31$^+$ and VEC$^+$ cells, thereby increasing the population of HEPs, non-HEPs, and AHPs compared to cultures differentiated on ColIV (Figure 2-6 A-B and Figure 2-7).
Figure 2-6: Subsets of D5 VEC$^+$ cells from hESCs in defined conditions on ColIV and TenC

A) Flow cytometric analysis demonstrates major subsets of VEC$^+$ progenitors generated after 5 days of hESC culture in chemically defined conditions on ColIV and TenC. Lower dot plots show VEC$^+$ gated cells. B) Percentages of VEC$^+$ cells and subsets generated in ColIV and TenC cultures. Error bars are mean ± SE from at least 3 experiments (*p<0.01). C) Endothelial and hematopoietic potential of day 5 VEC$^+$ subsets. Progenitor subsets sorted and cultured in either endothelial conditions with subsequent tube formation assay, or on OP9 with immunofluorescent and flow cytometry results after 7 days. Dot plots show expression of VEC and CD43 within TRA-1-85$^+$ gated human cells. Scale bars, 100 μM. D) CFC potential of isolated VEC$^+$ subset in
serum-free clonogenic medium containing hematopoietic cytokines with or without FGF2. Error bars are mean ± SE from 3 experiments (*p<0.01). Scale bar represents 100 um. Fluorescence images were captured on Olympus IX71 microscope at 10x and 20x magnifications using QIMAGING RetigaExi Fast 1394 and acquired with QCapture software. VE-Cadherin was visualized using a secondary antibody conjugated to DyLight488 and CD43 was visualized using a secondary antibody conjugated to DyLight 594. Bright-field images were captured on AMG EVOS XL Core at 10x magnification. Images’ contrast and levels were adjusted, and cropped, in Adobe Photoshop.

Figure 2-7: CFC potential of D5 VEC⁺ subsets in serum-free clonogenic medium containing hematopoietic cytokines without FGF2.

AHP is VEC⁺CD43/CD235⁻CD73⁻ angiogenic hematopoietic progenitors, HEP is VEC⁺CD43/CD235a/CD73⁻ hemogenic endothelial progenitors, non-HEP VEC⁺CD235a/CD43⁻ CD73⁺ non-hemogenic endothelial progenitors.
When numerous floating round hematopoietic cells became visible in cultures on day 6, the hypoxic conditions were not necessary to sustain hematopoietic development. Therefore, from day 6 of differentiation, the cultures were transferred to a normoxic incubator (20% O₂, 5% CO₂). By day 8 of differentiation, the cultures continued to develop and expand the CD43⁺ hematopoietic cells, which composed of CD235a⁺CD41a⁺ cells enriched in erythro-megakaryocytic progenitors and CD235a/CD41a⁻CD43⁺CD45⁻/+ cells which expressed CD34 but lacked other lineage markers (lin-). Consistent with cells differentiated on OP9, hematopoietic colony forming potential was limited to the CD43⁺ subpopulations. CD43⁺ hematopoietic progenitors were generated in significantly higher numbers on TenC compared to ColIV (Figure 2-8 A, B and Figure 2-9). In addition, the GEMM-CFC potential of cultures on TenC was significantly greater than cultures on ColIV (Figure 2-8 D).
Figure 2-8: Subsets of D8 CD43+ cells from hESCs in chemically conditions on ColIV and TenC

A) Flow cytometry analysis shows major subsets of CD43+ cells generated in cultures on ColIV and TenC. Lower dot plots show CD43+ gated cells. B) Cultures on TenC differentiate into CD43+ cells more, statistically significant across at least 3 experiments (*p<0.01). C) Hematopoietic-CFC potential in serum-containing media is limited to the CD43+ subpopulations. D) Cultures differentiated on TenC have more CFC potential than cultures differentiated on ColIV, statistically significant across 3 experiments (*p<0.01).
Figure 2-9: Total number of cells generated from D-1 to D9 on ColIV and TenC.

The numbers of CD31\(^+\) and CD43\(^+\) cells were calculated based on total number of cells times the percentage of positive cells based on flow cytometry.
Tenascin C is superior to Collagen IV for supporting hematopoietic differentiation from a variety of hPSC lines

Although the differentiation protocol was initially developed using H1 hESCs, we found that the chemically defined conditions described here also supports the formation of HE and blood progenitors from other hESC line (H9) and hiPSCs generated from fibroblasts or bone marrow mononuclear cells (Figure 2-10). Previously, we demonstrated that hiPSC obtained through reprogramming of bone marrow mononuclear cells (BM) hiPSCs differentiate less efficiently into the blood cells on OP9 feeders compared to fibroblast-derived (FB) hiPSCs (Hu et al., 2011). These findings have been reproduced when we differentiated BM and FB iPSCs on ColIV. However, differentiation on TenC restored hematopoietic differentiation potential of BM hiPSCs to the level seen with hESCs and FB hiPSCs (Figure 2-10), thereby confirming that TenC is superior over ColIV in promoting hematopoietic differentiation from hPSCs.
Figure 2-10: Differentiation of other hPSC lines in defined conditions

Dot plots show the percentage of CD43\(^+\) cells and their subsets of DF19-9-7T human fibroblast iPSC line, IISH2i-BM9 human bone marrow-derived iPSC line, and H9 human ESC line differentiated for 8 days on either ColIV or TenC.
Tenascin C uniquely supports specification of T lymphoid progenitors from hPSCs

To find out whether our culture system supports the establishment of the definitive hematopoietic program from hPSCs, we analyzed T cell potentials of blood cells generated in our system as an indicator for definitive hematopoiesis (Kennedy et al., 2012a). When we collected CD43+ floating cells from day 9 differentiated cultures and replated them onto OP9 expressing DLL-4 (OP9-DLL4), CD7+CD5+ lymphoid progenitors began to emerge by week 2 of coculture. By week 3, CD4+CD8+ double positive T-cells arose (Figure 2-11 A). Interestingly, CD43+ cells generated on both CollIV and TenC had a capacity to generate CD5+CD7+ lymphoid progenitors, though CD43+ cells generated on CollIV had significantly lower potential. However, progression towards CD4+CD8+ T lymphoid cells was consistently observed only from CD43+ cells generated on TenC, and not from CollIV cultures (Figure 2-11 B). To confirm T cell development, we analyzed the genomic DNA of the hematopoietic cells from OP9-DLL4 cultures for the presence of TCR rearrangements. This analysis demonstrated the presence of multiple PCR products of random V-J and D-J rearrangements at the beta-locus and V-J rearrangements at the gamma-locus indicative of a polyclonal T lineage repertoire (Figure 2-11 C,D). Overall, these findings signify that the extracellular matrix protein, TenC, is essential for supporting the generation of hematopoietic cells with myeloid and lymphoid potential from hPSCs in chemically defined conditions. However, we failed to obtain engraftment following transplantation of TenC differentiated cells in immunocompromised mice (data not shown), which suggests that additional maturation signals are required to activate the self-renewal program in hemato-endothelial progenitors generated in our system.
Figure 2-11: T cell potential of D9 hematopoietic cells from hESC cultures differentiated on ColIV and TenC

A) Flow cytometry analysis and B) graphs of cells collected from ColIV or TenC conditions after culture on OP9DLL4 for 3 weeks. Statistically significant across at least 3 experiments (*p<0.01). C) and D) Analysis for T cell receptor rearrangement by genomic PCR. H1 T-Cells are T-cells derived from differentiating H1 hESCs on TenC. PB control is Peripheral Blood positive control; H1 control is undifferentiated H1 hESC (negative control).
2.4 Discussion

During the last decade, significant progress has been made in hematopoietic differentiation from hPSCs. Multiple protocols for hematopoietic differentiation have been developed and made it possible to routinely produce blood cells for experimentation. However, generation of HSCs with long-term reconstitution potential from hPSCs remains a significant challenge. Hematopoietic cells and HSCs arise from a specific subset of endothelium (HE) in the embryo (Bertrand et al., 2010; Boisset et al., 2010; Jaffredo et al., 2000; Kissa and Herbomel, 2010; Zovein et al., 2008). Therefore, the ability to interrogate the signaling pathways that induce HE specification and endothelial-to-hematopoietic transition in a completely chemically defined environment is essential in order to identify the factors required for HSC specification. Although original protocols for hematopoietic differentiation have employed xenogeneic feeder-cells and/or serum, several serum- and feeder-free systems for hematopoietic differentiation have been recently described (Salvagiotto et al., 2011; Smith et al., 2013; Wang et al., 2012a). However these protocols still require serum components (albumin) and it remains unclear whether these protocols reproduce the distinct waves of hematopoiesis, including the generation of HE with definitive lymphomyeloid potential, observed in the original differentiation systems. Recently, Kennedy et al (Kennedy et al., 2012a) have developed a feeder- and stroma-free condition for EB-based hematopoietic differentiation in a proprietary medium with undisclosed nutrient supplement from hPSCs expanded on MEFs. These conditions reproduced primitive and definitive waves of hematopoiesis and generated HE with T lymphoid potential. Here we developed for the first time, a protocol that enables the efficient production of blood cells in completely chemically defined conditions, free of serum and xenogeneic proteins from a single
cell suspension of hPSCs maintained in chemically defined E8 medium (Chen et al., 2011a). Our protocol eliminates variability associated with animal- or human-sourced albumins, xenogenic matrices, clump size variation and asynchronous differentiation observed in EB systems, and reproduces typical waves of hematopoiesis, including the formation of HE and definitive hematopoietic progenitors, observed in hPSCs differentiated on OP9. Importantly, based on molecular profiling of OP9 and stromal cell lines with different hematopoiesis-inducing activity, we found that TenC matrix protein, which is uniquely expressed in OP9 with robust hematopoiesis-inducing potential, strongly promotes hemato-endothelial and T lymphoid development from hPSCs. TenC is a disulfide-linked hexameric glycoprotein that is mainly expressed during embryonic development. Although TenC mostly disappears in adult organisms, its expression is upregulated during wound repair, neovascularization, neoplasia (Hsia and Schwarzbauer, 2005), and during limb regeneration (Stewart et al., 2013). TenC is found in adult bone marrow where it is expressed predominantly in the endosteal region (Klein et al., 1993; Soini et al., 1993)

Tenascin-C supports proliferation of bone marrow hematopoietic cells (Seiffert et al., 1998) and erythropoiesis (Seki et al., 2006). TenC-deficient mice had lower bone marrow CFC potential (Ohta et al., 1998), failed to reconstitute hematopoiesis after bone marrow ablation, and showed reduced ability to support engraftment of wild type HSCs (Nakamura-Ishizu et al., 2012). In addition, TenC is expressed in thymus and play important roles in T-cell development as evidenced by decreased T-lymphoid progenitors in the thymus and increased proportion of T-cells in the bone marrow of TenC-deficient mice. Interestingly, high levels of TenC expression was also detected in human and chicken aorta-gonad-mesonephros (AGM) region (Anstrom and Tucker, 1996; Marshall et al., 1999), the site where the first HSCs emerge, and in hematopoietic sites of the human fetal liver (Papadopoulos et al., 2004). Because TenC expression is highly
enriched in the subaortic mesenchyme right underneath of hematopoietic clusters, it was suggested that TenC plays a pivotal role in HSC development during embryogenesis (Marshall et al., 1999). TenC is also involved in the regulation of angiogenesis and cardiac endothelial progenitors (Ballard et al., 2006). Our studies demonstrated the superior properties of TenC in promoting hemato-endothelial development from hPSCs. The positive effect of TenC was obvious at all stages of differentiation, including the enhancement of hematovascular mesoderm, HE, and CD43+ hematopoietic progenitors. Importantly, TenC was able to support development of definitive hematopoietic cells with T lymphoid potential, while we were not able to obtain such cells in cultures on ColIV. The Tenascin-C molecule is composed of amino-terminal oligomerization region followed by heptad repeats, EGF-like and fibronectin type III repeats, and a fibrinogen globe (Hsia and Schwarzbauer, 2005). The function of these domains is poorly understood. It is believed that effect and interaction of TenC with cells requires the integrated action of multiple domains (Fischer et al., 1997), although several unique mitogenic domains capable of inducing the proliferation of hematopoietic cells were identified within this molecule (Seiffert et al., 1998). Several signaling mechanisms implicated in cell interaction with TenC have been identified, including the suppression of fibronectin-activated focal adhesion kinase signaling, Rho-mediated kinase signaling, and stimulation of Wnt signaling pathways (reviewed in(Orend, 2005)). Further studies aimed to identify the mechanism of TenC signaling on hPSCs and their hematopoietic derivatives would be of value to understand the role of this matrix protein during development.

In summary, the findings provided here identify TenC matrix protein, and completely chemically defined conditions free of serum/serum components and animal proteins, capable of supporting the scalable production of HE and definitive blood cells from hPSCs. This
differentiation system opens the opportunity for precise interrogation of signaling molecules implicated in hematopoietic differentiation and provides the platform to produce cGMP-grade blood cells for clinical application.

2.5 Methods

Human Pluripotent Stem Cell Maintenance

Human pluripotent stem cells, WA01 (H1), WA09 (H9) embryonic stem cells (hESCs), DF19-9-7T human fibroblast iPSC line, and IISH2i-BM9 bone marrow-derived iPSC line (WiCell, Madison WI) were maintained on vitronectin or matrigel in E8 media supplemented with FGF2 and TGFβ (Peprotech). Cells were passaged when they reached 80% confluency using 0.5 mM EDTA in PBS. The cells were maintained in normoxic conditions with 5% CO2.

Human Pluripotent Stem Cell Differentiation

Single cell suspensions of hPSCs were obtained by treating the hPSC cultures at 80% confluency with 1x TrypLE (Life Technologies). Single cells were plated at an optimized density ranging from 5000 cells/cm² to 15,000 cells/cm² depending on the cell line onto 6-well plates coated with 0.5 μg/cm² of Collagen IV (Sigma-Aldrich) or 0.5μg/cm² Tenascin C (Millipore) in E8 media supplemented with 10μM Rho Kinase inhibitor (Tocris Y-27632). After 24 hours (day 0), the media was changed to IF9S media supplemented (See Table 2-2 for the complete composition of the medium) with 50ng/ml BMP4 (Peprotech), 15ng/ml Activin A (Peprotech), 50ng/ml FGF2 (Miltenyi Biotech), 2mM LiCl (Sigma), and on occasion, 1μM Rho Kinase inhibitor to increase cell viability. On day 2, the media was changed to IF9S media supplemented with 50ng/ml FGF2 and 50ng/ml VEGF. On day 4, the media was changed to IF9S media
supplemented with 50ng/ml FGF2, VEGF, TPO, SCF, IL-6, and 10ng/ml IL-3. On day 6, additional IF9S media supplemented with the same 6 factors were added to the cultures without aspirating the old media. IF9S (IMDM/F12 with 9 supplements) was made in-house with the following: 50% IMDM 50% F12 (Life Technologies) supplemented with 64mg/L L-asorbic Acid 2-Phosphate Mg2+ salt (Sigma-Aldrich), 40 ul/L monothioglycerol (Sigma-Aldrich), 8.4µg/L additional sodium selenite (Sigma-Aldrich), 10mg/L polyvinyl alcohol (Sigma-Aldrich), 1x glutamax (Life Technologies), 1x non-essential amino acids (Life Technologies), 0.1x chemically defined lipid concentrate (Life Technologies), 10.6mg/L Holo-Transferrin (Sigma-Aldrich), and 20mg/L Insulin (Sigma-Aldrich). Differentiation was conducted in hypoxic condition from day 0 to day 5, and transferred to normoxic condition from day 6 to day 9 (Figure 2-1). The 1x TrypLE was used to dissociate and collect cells for analysis.

**Mesenchymo- (MB) and Hemangioblast (HB) Assay and Hematopoietic CFC assay**

MB and HB were detected as described (Vodyanik et al., 2010). Hematopoietic CFC were detected using serum-containing H4436 Methocult (Stem Cell Technologies) or serum-free H4236 Methocult with added FGF2 (20ng/ml), SCF (20ng/mL), IL3 (10ng/mL), IL6 (10ng/mL), and EPO (2U/mL) as described (Choi et al., 2012c).

**Assessment of Hemato-endothelial Potential of Differentiated hPSCs**

Sorted day 4 or day 5 cultures were plated on a confluent layer of OP9 cells in α-MEM (Gibco) supplemented with 10% FBS (Hyclone) supplemented with 100 µM MTG, 50µg/ml ascorbic acid, 50ng/ml SCF, TPO, IL-6, and 10ng/ml IL-3 at a density of 5,000 cells/well of a 6
well plate as described. (Choi et al., 2012c) Cultures were analyzed 4 to 7 days later by immunofluorescent staining or by flow cytometry (Choi et al., 2012c).

*T-cell Differentiation of Day 9 cultures*

The OP9 cell line expressing human DLL4 (OP9-DLL4) was established by using lentivirus expressing human DLL4 under EF1a promoter. After hPSCs differentiation for 9 days, the floating CD43^+^ cells were collected, strained through a 70 μm cell strainer (BD Biosciences), resuspended in T-cell differentiation media consisting of α-MEM (Gibco) supplemented with 20% FBS (Hyclone), IL7 (5 ng/ml), FLT3L (5ng/ml) and SCF (10ng/ml), and cultured on an OP9-DLL4. After 4 days, the cells were harvested using collagenase IV (Gibco) solution (1mg/ml in DMEM/F12, Gibco) and 1x TrypLE (Life Technologies), and passaged onto a fresh layer of OP9-DLL4. After 3 days, the cells were passaged again. Subsequent passages were conducted every 7 days up to 4 weeks, after which the floating cells were collected for flow analysis and genomic DNA extraction for TCR rearrangement assay.

*Statistical Analysis*

Statistical analysis was performed using Microsoft Excel. Data obtained from multiple experiments were reported as the mean +/- SE. Two-tailed Student t test were used to compare 2 groups. Differences were considered significant when P<0.01.

*Flow Cytometry and FACS sorting*

Flow Cytometry was conducted using the using a FACSCalibur flow cytometer (BD) and following antibodies: CD31-FITC (clone WM59), CD34-FITC (8G12), CD41a-FITC/APC
(clone HIP8), CD43-FITC/PE/ APC (clone 1G10), CD45-APC (clone HI30), CD73-FITC/PE
(clone AD2), CD144-FITC/PE/AlexaFluor647 (clone 55-7H1), CD235a-FITC/PE/ APC (clone
GA-R2), KDR-PE/AlexaFluor647 (clone 89106), PDGFRα-PE (clone aR1) (BD Biosciences),
TRA-1-85-FITC/PE (clone TRA-1-85), and APLNR-APC (clone 72133) (R&D Systems).
Sorting was conducted on a FACS Aria (BD), as described previously (Vodyanik and Slukvin,
2007c).

qPCR Assay

Cells were differentiated for 3 and 4 days and sorted on a FACS Aria for the populations
of interest from each day. RNA was collected using quick-RNA MiniPrep (Zymo Research) and
quantified on a NanoDrop (GE Healthcare). Equal amounts of RNA was used for cDNA
synthesis using SuperScript III First-Strand Synthesis System (Life Technologies). qPCR was
conducted using Platinum SYBR Green qPCR SuperMix (Life Technologies). The primer
sequences were obtained from previous publications (Choi et al., 2012a; Vodyanik et al., 2005;
Vodyanik et al., 2010) and can be found in Table 2-1. The reactions were run on a Mastercycler
RealPlex Thermal Cycler (Eppendorf) and the expression levels were calculated by minimal
cycle threshold values (Ct) normalized to the reference expression of RPL13a. The qPCR
products were run on an agarose gel and stained with ethidium bromide to confirm specificity of
the primers.
Table 2-1. qPCR primer sequences

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TCR Rearrangement Assay

Genomic DNA was isolated using quick-gDNA MiniPrep (Zymo Research). TCRb and TCRg clonality was detected using a PCR amplification kit (Invivoscribe) and AmpliTaq Gold DNA polymerase (Applied Biosystems) as previously described (Hu et al., 2011). The PCR products were analysed using heteroduplex analysis on a 6% polyacrylamide gel stained with ethidium bromide.

Microarray analysis of mouse stromal cell lines

A mouse bone marrow stromal cell line, OP9, was obtained from Dr. Toru Nakano (Research Institute for Microbial Diseases, Osaka University, Japan), S17 was obtained from Dr. Kenneth Dorshkind (University of California, Los Angeles) and MS-5 was obtained from the German Tissue Culture Collection. Stromal cell lines were cultured as previously described (Vodyanik et al., 2005). DNA-free RNA was isolated using RiboPure RNA kit and treated with DNase using TURBO DNAfree reagents (Ambion). All samples were processed at the Gene Expression Center of the Biotechnology Center at the University of Wisconsin, Madison. To analyze the total stromal cell lines, we used standard arrays containing 60-mer probes manufactured by NimbleGen Systems (Madison, WI), which contains ~385,000 60-mer probes, covering approximately 36,000 Mus musculus genome transcripts (HG17, NCBI Build 35).
Microarrays were hybridized and processed following previously published protocols (Yu et al., 2007). Gene expression raw data were extracted using NimbleScan software v2.1. Considering that the signal distribution of the RNA sample is distinct from that of the gDNA sample, the signal intensities from RNA channels in all eight arrays were normalized with Robust Multiple-chip Analysis (RMA) algorithm (Irizarry et al., 2003). Separately, the same normalization procedure was performed on those from the mouse gDNA samples. For a given gene, the median-adjusted ratio between its normalized intensity from the RNA channel and that from the gDNA channel was then calculated as follows: \( \text{Ratio} = \frac{\text{intensity from RNA channel}}{\text{intensity from gDNA channel} + \text{median intensity of all genes from the gDNA channel}} \). To find which genes were uniquely upregulated in overgrown OP9 cells, first we first selected genes that showed at least a 3 fold higher expression in overgrown OP9 cells versus semi-confluent OP9 cells, overgrown OP9 cells versus MS5 stromal cells and overgrown OP9 cells versus S17 stromal cells. Then, we selected the genes that were common in all three comparison groups.
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<th>Unit/L</th>
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CHAPTER 3: NOTCH SIGNALING REGULATES THE FORMATION OF ARTERIAL-TYPE DEFINITIVE HEMOGENIC ENDOTHELIUM AND ENDOTHELIAL-TO-HEMATOPOIETIC-TRANSITION FROM HUMAN PLURIPOTENT STEM CELLS
3.1 Abstract

NOTCH signaling is required for the formation of hematopoietic stem cells (HSCs) from embryonic endothelium in the aorta-gonad-mesonephros (AGM) region. Here, using a chemically defined human pluripotent stem cell (hPSC) differentiation system combined with the use of DLL1-Fc and the small molecule DAPT to manipulate NOTCH, we discovered that NOTCH activation leads to formation of arterial type CD144⁺CD43⁻CD73⁻DLL4⁺RUNX1+23-GFP⁺ hemogenic endothelium with NOTCH-dependent capacity to undergo EHT and formation hematopoietic progenitors with broad lympho-myeloid and definitive erythroid potentials. In addition, we demonstrated that NOTCH signaling promotes post-EHT expansion of hematopoietic progenitors. These findings supports the model in which NOTCH signaling is essential for specification of definitive hematopoiesis through triggering arterial specification of hemogenic endothelial progenitors and post-EHT amplification of blood progeny. The ability to produce arterial-type definitive HE, that is the proper precursor for HSC formation in the embryo, aids in in vitro approaches to instruct HSCs fate from hPSCs.
3.2 Introduction

Generating autologous hematopoietic stem cells (HSCs) from induced pluripotent stem cells (iPSCs) that can be precisely genetically modified with designer endonucleases, and subsequently clonally selected, represents a promising approach for novel patient-specific gene therapies. Although multiple studies were able to generate hematopoietic progenitors (HPs) with a HSC phenotype from pluripotent stem cells (PSCs), these cells failed to produce multilineage engraftment reviewed (Rowe et al., 2016; Slukvin, 2016b). Thus, identifying key cellular and molecular programs required for the proper HSC specification in vitro is essential to overcome the current roadblocks of emulating proper HSC specification in vitro.

During development in vivo, HSCs emerge by budding from hemogenic endothelium (HE) lining arterial vessels, primarily from the ventral wall of the dorsal aorta (Bertrand et al., 2010; Dzierzak and Speck, 2008; Medvinsky et al., 2011). NOTCH signaling is essential for arterial specification and generation of HSCs (Bigas et al., 2012; Bigas and Espinosa, 2012; Kumano et al., 2003). Notch1-/-, Dll4-/- and Rbpjk-/- mice, which are embryonic lethal, have severe impairment in arterial vasculogenesis, fail to develop the dorsal artery (Kumano et al., 2003; Robert-Moreno et al., 2005; Robert-Moreno et al., 2008), and lack intra-embryonic hematopoiesis. In contrast, the primitive extraembryonic wave of hematopoiesis does not depend on NOTCH signaling (Bigas et al., 2013; Kumano et al., 2003; Nakagawa et al., 2006; Robert-Moreno et al., 2005). Notch signaling regulates the expression of Runx1, Gata2, Hes1, Hes5 and Hey2 genes important for HSC formation (Ayllon et al., 2015; Bigas et al., 2013; Burns et al., 2005; Gerhardt et al., 2014; Guiu et al., 2013; Kennedy et al., 2012b; Lizama et al., 2015; Nakagawa et al., 2006; Nottingham et al., 2007; Richard et al., 2013; Robert-Moreno et al., 2008), and is required cell-autonomously for HSC formation (Hadland et al., 2015). In addition,
NOTCH activation supports expansion and maintenance of HSCs emerging in the aorta-gonad-mesonephros (AGM) region (Hadland et al., 2015) as well fetal liver and cord blood HSCs (Ohishi et al., 2002; Shojaei et al., 2005). The lack of venous contribution to HSCs along with the shared requirements of Notch, VEGF, and Hedgehog signaling for both arterial fate acquisition and HSC development (Burns et al., 2009; Gering and Patient, 2005; Kim et al., 2013; Lawson et al., 2001; Lawson et al., 2002), led to hypothesis that arterial specification could be critical prerequisite to HSC formation. However, it remains unclear whether the acquisition of arterial identity per se is required for HE to became HSC or arterial specification creates the permissive environment for HSC specification from a distinct endothelial lineage. It also remains controversial whether NOTCH signaling is required for HE to undergo transition into the blood (Hadland et al., 2004; Hadland et al., 2015; Lizama et al., 2015; Nakajima-Takagi et al., 2013; Robert-Moreno et al., 2008).

Previously, we demonstrated that HE represents a distinct lineage of endothelial cells, which can be separated from non-HE by lack of CD73 expression, and that hematopoietic potential is already specified at the HE stage (Choi et al., 2012a; Elcheva et al., 2014; Slukvin, 2016b). To define the role of NOTCH signaling during HE specification, diversification, and blood formation, we employed a chemically defined hPSC differentiation system combined with the use of DLL1-Fc and the small molecule DAPT to manipulate NOTCH signaling following the emergence of the well-defined CD144⁺CD43⁻CD73⁻ population of HE during endothelial-to-hematopoietic transition (EHT). We found that NOTCH activation promotes EHT from CD144⁺CD43⁻CD73⁻ HE and post-EHT expansion of blood cells along with induction of a transient arterial-type CD144⁺CD43⁺CD73⁻DLL4⁺ HE (AHE) that expresses arterial markers and increased levels of genes associated with definitive hematopoiesis, MYB, RUNX1c, and GATA2.
Using a transgenic reporter WA01 hESC line in which RUNX1+23 enhancer mediates eGFP expression, we found that only DLL4⁺, and not DLL4⁻ HE cells, demonstrated enhancer activity that is typically found in HE at sites of definitive hematopoiesis in mouse and zebra fish embryos (Nottingham et al., 2007; Swiers et al., 2013; Tamplin et al., 2015). Hematopoiesis from CD144⁺CD43⁻CD73⁻DLL4⁺ AHE required stroma and was strictly dependent on NOTCH activation. In contrast, NOTCH modulation has limited effect on EHT from the HE fraction that remains DLL4⁻ following NOTCH activation, indicating that definitive hematopoietic activity segregates to AHE. Together, these studies revealed that NOTCH-mediated induction of A HE is an important prerequisite for establishing the definitive hematopoietic program from hPSCs.
3.3 Results

*Immobilized DLL1-Fc Increases NOTCH Signaling in Hemogenic Endothelial Cells and Increases Hematopoietic Activity*

In order to determine the direct effect of NOTCH signaling on hemato-endothelial differentiation from hPSCs, we utilized a modified version of the serum- and feeder-free differentiation system described previously (Uenishi et al., 2014) where we identified developmental stage equivalencies to *in vivo* development that can be identified by cell-surface antigens and functional assays on specific days of differentiation; Day 2-3 APLNR<sup>+</sup>PDGFRα<sup>+</sup> Primitive Mesoderm (D2 or D3 PM), Day 4 KDR<sup>hi</sup>PDGFRα<sup>lo/c</sup>CD31<sup>−</sup> Hematovascular Mesoderm Precursors (D4 HVMP), Day 4 and 5 CD144<sup>+</sup>CD43<sup>−</sup>CD73<sup>−</sup> Hemogenic Endothelial Cells (D4 or D5 HE), and Day 8 CD34<sup>+</sup>CD43<sup>+</sup> Hematopoietic Progenitors (D8 HP) (Choi et al., 2012b). During differentiation, we found that the NOTCH1 receptor is first highly expressed on D4 HE cells while the NOTCH ligand, DLL4, is first expressed on D5 within the CD144<sup>+</sup> (VE-Cadherin) population (Figure 3-1 A) suggesting that NOTCH signaling in hPSC cultures is established at the time of HE formation.

Following the establishment of optimal conditions for EHT culture in defined feeder- and serum-free conditions (see materials), we isolated D4 HE by simple magnetic enrichment of CD31<sup>+</sup> cells, since at this stage, the CD31<sup>+</sup> population is entirely CD144<sup>−</sup>CD43<sup>−</sup>CD73<sup>−</sup> (Choi et al., 2012b; Uenishi et al., 2014). Isolated D4 HE cells were cultured either in control conditions, with the small molecule γ-secretase inhibitor, DAPT, to inhibit NOTCH signaling, or were plated onto the immobilized NOTCH ligand, DLL1-Fc, to activate NOTCH signaling (Hadland et al.,
2015; Ohishi et al., 2002) (Figure 3-1 B). As we confirmed by western blot analysis of the active form of NOTCH1, NOTCH:ICD, and qPCR analysis of the downstream NOTCH1 target gene, HES1, by qPCR, these respective conditions efficiently inhibited and activated NOTCH signaling (Figure 3-1 C). Kinetic analysis of CD144 (endothelial marker) and CD43 (hematopoietic marker) from D4+1 to D4+4 reveals a significant increase in hematopoiesis in the NOTCH activation condition, and a significant decrease in hematopoiesis in the NOTCH inhibition condition compared to the control condition. These results were consistent with other hESC and hiPSC lines (Figure 3-2 A). In addition, similar results were obtained when D4 HE cells were cultured in serum-containing medium on wild-type OP9 stromal cells or OP9 cells transduced with human DLL4 (OP9-DLL4; Figure 3-2 B). We also observed significant increase in the total hematopoietic cell number, particularly the hematopoietic progenitors in the NOTCH activation condition (Figure 3-1 E, F). The effect of DLL1-Fc on hematopoiesis increased as the concentration of immobilized DLL1-Fc and cell density increased (Figure 3-2 C). Similar results were obtained when day 4 HE cells were cultured in serum-containing medium on wild type or human DLL4-expressing OP9 stromal cells (Figure 3-2 B).
Figure 3-1: NOTCH activation increases hematopoiesis from D4 HE

A) NOTCH1 receptor expression is first detected on D4 CD144+ cells. DLL4 expression is first detected on D5 CD144+ endothelial cells. B) Schematic diagram of experiments. Cells were
differentiated for 4 days on collagen-IV, D4 CD144^+CD43^CD73^- HE cells were purified using CD31-microbeads and plated in 3 different NOTCH conditions. C) Western blot of D4 HE cultured for 24 h (D4+1) in presence of DAPT or DLL1-Fc shows a decrease in the activated cleaved form of NOTCH1 in DAPT treated cells, and an increase in the activated cleaved form of NOTCH1 in cells plated on DLL1-Fc. D) qPCR analysis shows decreased HES1 mRNA expression in D4 HE cultured for 12 hours (D4+0.5) with DAPT, while HES1 mRNA expression is increased in cells plated on DLL1-Fc. Results are mean ± SEM for at least 3 independent experiments. E) Flow cytometry on each day from D4+1 to D4+4 shows decreased CD43^+ HPs in the cultures treated with DAPT, and increased HPs in the cultures plated on DLL1-Fc. F) Total numbers of CD43^+ HPs and in cultures plated on DLL1-Fc. Results are mean ± SEM for at least 3 independent experiments. *p<.05, **p<.01, ***p<.001.
Figure 3-2: The effect of NOTCH ligands on hPSCs

A) NOTCH inhibition and activation effects on D4 HEPs are consistent with other human PSC lines. B) OP9-DLL4 has similar effects as DLL1-Fc, as well as adding DAPT on OP9. C) Measuring the effect of increasing concentrations of DLL1-Fc with increasing cell density of D4 HEPs.
The increase in hematopoiesis due to increased NOTCH signaling can be attributed to three reasons: 1) increased EHT, 2) increased hematopoietic expansion or 3) increased survival post-EHT. First, to determine the effect of NOTCH signaling on EHT, we isolated day 4 HE and cultured them with DAPT for either 1 day during initiation of EHT (from D4 to D4+1), or throughout the entire culture (D4 to D4+4), followed by kinetic analysis of CD43 and CD144 expression on each day of the culture period (Figure 3-3 A). As shown in Figure 3-3 B and 3-3 C, even a 24-hour DAPT treatment from D4 to D4+1 significantly inhibited hematopoiesis on D4+2 through D4+4, although DAPT treatment throughout entire culture (D4 to D4+4) had a more profound decrease in hematopoiesis.

We also stained the purified D4 HE before plating with CellTracer to track cell proliferation. When we analyzed the cells in each of the three NOTCH conditions on D4+1, we found that there was a significant increase in the proportion of CD144⁺CD43⁺ to CD144⁺CD43⁻ cells within the first generation of cells in the NOTCH activation (+DLL1-Fc), condition compared to the NOTCH inhibition condition (+DAPT). This result, in combination with the absence of a second generation on D4+1, suggests that the activation of NOTCH signaling at HE stage increased EHT (Figure 3-3 D, E).

To confirm that NOTCH activation affects EHT, we performed a single cell deposition assay of the D4 HE using the OP9 stromal cells and serum-containing medium which support hemato-endothelial development from single cells. Using a DOX-inducible DLL4 OP9 cell line (OP9-iDLL4) we deposited D4 HE onto 96-well plates of 3 different conditions; OP9-iDLL4
with DAPT without DOX-pretreatment (NOTCH inhibition condition), OP9-iDLL4 with DMSO without DOX-pretreatment (control condition), and OP9-iDLL4 with DMSO with pretreatment of DOX (NOTCH activation condition). We found that D4 HE in the NOTCH inhibition condition had a markedly decreased ratio of hematopoietic:endothelial colonies compared to D4 HE cells in the control condition. In contrast, the D4 HE in the NOTCH activation condition had substantially increased ratio of hematopoietic colonies compared to D4 HE in the NOTCH inhibition condition, and a slight increase compared to D4 HE in the control condition (Figure 3-3 F). Together, these results suggest that NOTCH activation at the HE stage facilitates EHT.
Figure 3-3: Increased NOTCH activation facilitates EHT

A) Schematic diagram of experiments. D4 HE cultured in presence of DAPT for 4 days (D4+4) or 1 day (D4+1), or DMSO (control). CD144⁺ endothelial and CD43⁺ blood cells were analyzed following 4 days of culture. B) Flow cytometric analysis demonstrates that NOTCH activation facilitates EHT as evidenced by the decrease in hematopoietic activity when DAPT is added only
from D4 to D4+1. C) Frequencies of endothelial and blood cells in HE cultures treated with DAPT or DMSO (control). Results are mean ± SEM for at least 3 independent experiments. D) Representative flow cytometric cell proliferation analysis and E) bar graph representing at least 3 independent experiments conducted with CellTracer shows an increase in the first generation (Gen1) CD43^+ cells on D4+1 and a proportional decrease in Gen1 CD144^+ endothelial cells, suggesting that the increase in blood cells is due to an increase in EHT and not just proliferating HPs. Generation gates were determined by concatenating D4 to D4+4 results and utilizing FlowJo’s proliferation assay. F) Single D4 HE cells were FACS-sorted into 96 well plate with OP9, OP9+DAPT, and OP9-DLL4. Colonies were scored based on CD43 and CD144 expression on D4+10 by immunofluorescence and counted by eye. Scale bar represents 100 μm *p<.05, **p<.01, ***p<.001
NOTCH Activation on Post-EHT Hematopoietic Progenitors Increases Proliferation Whilst Maintaining Multilineage Potential and Increasing Definitive Characteristics

Next, we determined whether NOTCH has an effect on HPs directly after EHT. While NOTCH1 expression decreases among the CD144+ endothelial population from D4+1 to D4+4, CD144-CD43+ blood cells increase and maintain expression of NOTCH1 post-transition from D4+2 to D4+4, notably among the CD34+ subpopulation (Figure 3-5 A,B), thereby indicating that emerging blood cells are equipped to respond to NOTCH signaling. Since EHT takes place predominantly during the first two days after HE formation, to assess the effect of NOTCH on post-EHT stage we inhibited NOTCH signaling with DAPT from D4+2 to D4+4 (Figure 3-4 A). These studies revealed that inhibiting NOTCH signaling from D4+2 to D4+4 significantly inhibited hematopoietic expansion (Figure 3-4 B and 4-4 C), thereby suggesting that NOTCH signaling may facilitates proliferation of the HPs post-transition.

To confirm this suggestion, we analyzed the D4 HE from the 3 different NOTCH conditions that had been stained with CellTracer to track cell proliferation on D4+4. Consistent with the previous results, we found that NOTCH inhibition with DAPT significantly decreased the proportion of CD43+ blood cells in later generations compared to the control condition. In contrast, cultures that were exposed to the immobilized DLL1-Fc ligand experienced significantly greater CD43+ cell proliferation compared to the control condition. Considered together, these results show that NOTCH signaling not only facilitates EHT, but also increases hematopoietic expansion (Figure 3-4 D, E). None of the conditions affected apoptosis of blood cells post-transition (Figure 3-6), suggesting that the difference in hematopoiesis from HE
following manipulation of NOTCH signaling is not attributed to the NOTCH effect on cell survival.
Figure 3-4: NOTCH activation increases HP proliferation

A) Schematic diagram of experiments. D4 HE cultured in presence of DAPT for 4 days (D4+4), DMSO (control) or with DAPT added on day 2 of secondary culture (D4+2). CD144⁺ endothelial and CD43⁺ blood cells were analyzed following 4 days of culture (D4+4). B) Flow cytometric analysis demonstrates that NOTCH activation is necessary for hematopoietic
expansion as evidenced by the decrease in hematopoietic progenitors when DAPT is added from D4+2 to D4+4. C) Frequencies of hematopoietic and endothelial cells in HE cultures treated with DAPT. Results are mean ± SEM for at least 3 independent experiments. D) Representative flow cytometric cell proliferation analysis and E) bar graph representing at least 3 independent experiments conducted with CellTracer depicts an increase in Gen4, Gen5, and Gen6 CD43+HPs, thereby suggesting that the increase in hematopoietic cells at this stage is due to an increase in cell division and proliferation. NOTCH activation increases cell proliferation, while NOTCH inhibition has the opposite effect. Generation gates were determined by concatenating D4 to D4+4 results and utilizing FlowJo’s proliferation assay. *p<.05, **p<.01, ***p<.001.
Figure 3-5: Notch1 expression on CD34+ hemato-endothelial populations

A) CD144+CD43- endothelial cells from D4+1 to D4+4 and B) CD144-CD43+ hematopoietic progenitors D4+2 to D4+4.
Figure 3-6: Flow cytometry of Annexin V to determine apoptosis during differentiation

Flow cytometry showing the percent of apoptotic cells via Annexin V staining shows there are no apoptotic cells in the A) endothelial and B) hematopoietic populations, showing evidence that the NOTCH signaling effect is purely on proliferation and not survival.
Increased hematopoietic expansion can coincide with increased differentiation and a loss of multipotency (Miller et al., 2002; Seita and Weissman, 2010). In order to determine how NOTCH affects post-EHT hematopoietic differentiation, we took D4+4 cultures from the 3 different NOTCH conditions and plated them in methocellulose to measure their colony forming potential. The total number of colonies was significantly lower in the DAPT treated NOTCH inhibition condition, while there was no significant change in the total number of colonies between the control condition and the NOTCH activation condition. Critically, however, there was a significant increase in multipotent GEMM-CFCs and GM-CFCs, as well as in E-CFC among the hematopoietic progenitor cells from the NOTCH activation condition compared to the control (Figure 3-8 A). These results suggest that NOTCH activation increases expansion of hematopoietic cells while simultaneously maintaining their multilineage potential.

Next, we determined whether increased NOTCH activation increases definitive-type hematopoiesis. Previously, the Runx1+23 enhancer was found to be active in all hematopoietic progenitors, including yolk sac blood islands. However, at pre-EHT HE stage Runx1+23 expression is uniquely active at sites of definitive hematopoiesis, including the para-aortic splanchnopleura, AGM region, vitelline and umbilical arteries (Bee et al., 2009; Ng et al., 2010; Nottingham et al., 2007; Swiers et al., 2013; Tamplin et al., 2015). We generated a hESC reporter line with the Runx1+23 enhancer reporter driving eGFP expression knocked into the AAVS1 locus (Figure 3-7). We differentiated the Runx1+23 cell line, purified the D4 HE cells, and plated them in each of the 3 NOTCH conditions. Critically, there was significantly higher eGFP expression from D4+1 to D4+4 that emerge from the CD144+ population in the NOTCH activation condition compared to the control. In contrast, cells treated with DAPT (NOTCH inhibition) had less eGFP expression compared to the control (Figure 3-8 B).
T cell potential is another hallmark of definitive hematopoiesis (Kennedy et al., 2012a). Comparative analysis of T-cell potential of the D4+4 CD43+ cells from DAPT, DLL1-Fc and control conditions revealed that HPs from the NOTCH inhibition condition had no T-cell potential while HPs from the NOTCH activation condition had significantly increased T-cell potential (Figure 3-8 C). In a separate assay, we collected the floating HPs on D4+4 and continued culture them in a modified erythrocyte expansion condition (Dias et al., 2011). After 10 days, we collected the cells and isolated the mRNA to analyze their globin expression. We found that erythrocytes generated from HPs from the NOTCH activation condition have significantly increased ratios of adult-type $\beta$-globin expression to embryonic $\varepsilon$-globin and fetal $\gamma$-globin expression and the ratio of adult-type $\alpha$-globin expression to embryonic $\zeta$-globin expression, when compared to the erythrocytes generated from HPs from both the NOTCH inhibition condition and the control condition (Figure 3-8 D). Overall, these findings suggest that NOTCH signaling not only increases HP expansion, but that NOTCH activation is also required for definitive hematopoietic stem/progenitor cell specification.
Figure 3-7: Generation of RUNX1+23-eGFP reporter WA01 hESC line

A) Schematic diagram of the construct used for the targeting of RUNX1+23-eGFP reporter into AAVS locus. Donor plasmid was integrated into the cleavage location of the Zinc Finger-Nuclease pair.

B) Southern blot analysis of the WA01 cells targeted with the donor plasmid containing RUNX1+23-eGFP construct. Blot shows EcoRV digested genomic DNA hybridized with DIC-labeled 5’ internal probe 1 (wt=no band, targeted=8.1kb) and 3’ external probe 2 (wt=5.4kb, targeted=8.8kb). Filled arrow = wild type; Asterisk = targeted.

C) D5 flow of 3 different RUNX1+23 reporter hESC lines reveals that all eGFP+ cells are DLL4+CD73-. 
Figure 3-8: NOTCH activation at HE stage increases definitive hematopoiesis

A) Frequencies of hematopoietic progenitor collected form D4 HE after 4 day of differentiation (D4+4 cells) with DAPT or in presence DLL1-Fc. Increase in multipotential GEMM and GM colonies in the DLL1-Fc culture condition suggests that Notch activation increases proliferation and the most immature progenitor maintenance. Results are mean ± SEM for at least 3
independent experiments. B) Flow cytometric analysis of Runx1+23-eGFP transgene expression in D4 HE cultured with DAPT or on DLL1-Fc. There is significantly more Runx1+23 enhancer activity among the cultures plated on DLL1-Fc and a significant decrease in the DAPT treated cultures. C) T cell potential of HP collected after 4 days of culture D4 HE in presence of DAPT or DLL1-Fc. Bars are mean ± SEM for at least 3 independent experiments. D) Ratio of $\alpha/\zeta$, $\beta/\gamma$ and $\beta/\epsilon$ globin chain expression in erythroid cultures generated from D4 HE in presence of DAPT or DLL1-Fc. Results are mean ± SEM for at least 3 independent experiments. *p<.05, **p<.01, ***p<.001
NOTCH Activation of Day 4 HE Increases a Transient Population of DLL4+ HE Cells with Arterial Identity

Previously, we identified CD73 expression to demark the loss of hemogenic potential within the D5 CD144+ endothelial population (Choi et al., 2012a). More recently, DLL4 upregulation among endothelial cells were also found to be associated with decreased of hemogenic potential of endothelium in serum- and stroma-free conditions (Ditadi et al., 2015). As we demonstrated above, D4 HE cells were lacking expression of arterial marker, DLL4. However, when we analyze CD73 and DLL4 expression within the D4+1 and D4+2 CD144+ populations in each of the three NOTCH conditions, we found a significant increase in a unique transient population of CD73’DLL4+ endothelial cells in the NOTCH activation condition, and a delayed upregulation of CD73 expression on DLL4+ endothelial cells compared to the NOTCH inhibition and control conditions (Figure 3-9 A, B). In addition, when we analyze the CD144+ population of the Runx1+23 cell line on D4+1, we found that all of the eGFP+ cells are from within the CD144’CD73’DLL4+ population (Figure 3-9 C and Figure 3-7). Since DLL4 is expressed by HE underlying intaraortic hematopoietic clusters in the AGM (Richard et al., 2013), our results suggest that the DLL4+ population may resemble arterial-type definitive HE found in the developing aorta of the embryo.

To corroborate this hypothesis, we evaluated the expression of arterial, venous and definitive hematopoietic markers by real-time qPCR analysis of FACSorted D4 CD144+CD43-CD73’ HE that are DLL4+ by default (D4 HE) and D5 CD144+ endothelial subpopulations CD144+CD43’CD73’DLL4+ (D5 HE:DLL4+), CD144+CD43’CD73’DLL4- (D5 HE:DLL4-), CD144’CD43’CD73’DLL4+ (D5 nonHE:DLL4+), CD144+CD43’CD73’DLL4+ (D5
nonHE:DLL4⁺), and CD144⁺CD43⁺CD73⁻DLL4⁻ (D5 nonHE:DLL4⁻), (Figure 3-9 D). This analysis reveals that the D5 HE:DLL4⁺ and nonHE:DLL4⁺ populations have increased expression of \textit{NOTCH1}, DLL4, \textit{EFNB2}, \textit{HEY2}, \textit{SOX17}, and \textit{CXCR4} genes associated with arterial endothelium, and a decreased expression of \textit{NR2F2} associated with venous endothelium when compared to D5 HE:DLL4⁻ and nonHE:DLL4⁻ populations. In contrast, D5 HE:DLL4⁻ demonstrated an increased expression of \textit{NR2F2} venous marker. Interestingly, genes associated with definitive hematopoiesis, \textit{MYB} and \textit{GATA2}, were expressed significantly higher in the D5 HE:DLL4⁺ population compared to the D5 HE:DLL4⁻ population and D5 nonHE DLL4⁺ populations (Figure 3-9 E). We also revealed that emerging D4 HE cells that at this stage are lacking DLL4 expression were different from D5 HE:DLL4⁻ and D5 HE:DLL4⁺ cells. D4 HE did not express significant levels of arterial and venous markers, but retained expression of \textit{HAND1}, which is expressed in extraembryonic and lateral plate mesoderm (Barnes et al., 2010), suggesting that D4 HE may represent immature hemogenic endothelial cells.
Figure 3-9: NOTCH activation induces formation of arterial type HE cells

A) Flow cytometric analysis of DLL4 and CD73 expression following culture of D4 HE for 1 or 2 days in presence of DAPT or DLL1-Fc. Notch activation on D4 HE specifically increases
CD144⁺CD73⁻DLL4⁺ population. (B) Frequencies of DLL4⁺ cells in hemogenic (CD73⁻) and non-hemogenic fractions of endothelium following 1 and 2 days cultures of D4 HE in presence of DAPT or DLL1-Fc. Results are mean ± SEM for at least 3 independent experiments. C) Flow cytometric analysis of Runx1+23 enhancer activity following 1 day culture of D4 HE in presence of DAPT or DLL1-Fc. Runx1+23 enhancer activity is limited to CD144⁺CD73⁻DLL4⁺ population. D) Schematic diagram of FACS sorting of endothelial populations formed on D5 of differentiation. E) qPCR analysis of arterial (NOTCH1, DLL4, EPHB4, HEY2, SOX17, CXCR4), venous (NR2F2), hematopoietic (MYB, GATA2) and mesodermal (HAND1) genes in D5 endothelial subpopulations.
Definitive-type Hematopoietic Progenitors Emerge from Arterial-Type Hemogenic Endothelium
Upon NOTCH Activation

To determine the hematopoietic potential of endothelium with arterial identity, we continued differentiation of hPSCs to D5 and then sorted the D5 CD144⁺CD43⁻CD73⁻DLL4⁻ (HE:DLL4⁻) and D5 CD144⁺CD43⁻CD73⁻DLL4⁺ (HE:DLL4⁺) endothelial subpopulations (Figure 3-10 A). While we did not detect any CD43⁺ blood cells from D5 HE:DLL4⁺ on D5+4 in serum- and feeder-free conditions with or without DLL1-Fc (data not shown), these cells produced blood when plated on OP9-DLL4 (Figure 3-10 B). In contrast, D5 HE:DLL4⁻ cells undergo EHT and develop HPs on D5+4 on both OP9 and OP9-DLL4. However, unlike previously when we used D4 HE cells before they bifurcate into D5 HE:DLL4⁺ and HE:DLL4⁻, there was no significant difference in blood production between the D5 HE:DLL4⁻ cells plated on OP9 versus OP9-DLL4 (Figure 3-10 B, C). The results were consistent across different iPSC lines as well (Figure 3-11). In addition, DAPT treatment from D5 to D5+2, D5+2 to D5+4, and from D5 to D5+4 significantly inhibited hematopoietic activity of D5 HE:DLL4⁺ population, while DAPT treatment of HE:DLL4⁻ cultures had no effect on hematopoietic activity (Figure 3-10 D), suggesting that hematopoiesis from D5 HE:DLL4⁺, in contrast to D5 HE:DLL4⁻, is NOTCH-dependent.

Next, we determined whether the HPs from each of the D5 HE subsets have differential definitive hematopoietic potential. When the HPs from the D5 HE subpopulations were plated in methocellulose colony forming medium, the HPs which emerged from the HE:DLL4⁺ subpopulation cultured on OP9-DLL4 had increased in colony forming cells, particularly of
GEMM-CFCs compared to the HPs from D5 HE:DLL4\(^+\) on OP9 and OP9-DLL4 (Figure 3-10 E).

When we collected the floating HPs derived from D5 HE:DLL4\(^+\) on OP9 and OP9-DLL4, and HPs derived from HE:DLL4\(^+\) on OP9-DLL4, and continued to grow them in the aforementioned erythrocyte expansion and maturation culture (Dias et al., 2011), we found that erythrocytes generated from HPs derived from the HE:DLL4\(^+\) on OP9-DLL4 have significantly increased ratios of $\beta$-globin expression to $\varepsilon$-globin and $\gamma$-globin expression, and an increased ratio of $\alpha$-globin expression to $\zeta$-globin expression, when compared to the erythrocytes generated from HPs derived from D5 HE:DLL4\(^-\) on OP9 and OP9-DLL4 (Figure 3-10 F).

We also conducted a limiting dilution assay (LDA) for lymphoid potential, and found that 1 in 14 HPs derived from D5 HE:DLL4\(^+\) on OP9-DLL4 have T-cell potential, while 1 in 44 HPs derived from HE:DLL4\(^-\) on OP9-DLL4 have T-cell potential. Crucially, HPs derived from D5 HE:DLL4\(^-\) on OP9 and D5 HE:DLL4\(^-\) on OP9 with DAPT had only 1 in 10,706 and 1 in 10,895 had T-cell potential, respectively (Figure 3-10 G), thereby suggesting that D5 HE:DLL4\(^+\) phenotype enriches for HE that can produce HPs with T-lymphoid potential.
Figure 3-10: Arterial-type HE undergo EHT under high NOTCH activation and produce definitive-type HPs

A) Schematic diagram of subsequent experiments. D5 CD144^CD43^CD73^ were sorted based on DLL4 expression (D5 HE:DLL4^+/−) using FACS and cultured on either OP9 or OP9-DLL4 for...
4 days (D5+4). B) and C) Flow cytometric analysis of CD43⁺ hematopoietic and CD144⁺ endothelial cells following culture of D5 HE:DLL4⁺ and D5 HE:DLL4⁻ on either OP9 or OP9-DLL4. Bars in C) are mean ± SEM for at least 3 independent experiments. D) The effect of NOTCH inhibition with DAPT on blood production from D5 DLL4⁺ and DLL4⁻ HE. No significant differences were found when HE:DLL4⁻ cells were treated with DAPT. Results are mean ± SEM for at least 3 independent experiments. *p<.05, **p<.01, ***p<.001 E) CFC potential of hematopoietic cells generated from D5 DLL4⁺ and DLL4⁻ HE following 5 days culture on OP9-DLL4. Results are mean ± SEM for at least 3 independent experiments. CFC-GEMMs are significantly increased in DLL4⁺ cultures on OP9-DLL4. F) Ratio of α/ζ, β/γ and β/ε globin chain expression in erythroid cultures generated form hematopoietic cells collected from D5 DLL4⁺ and DLL4⁻ HE cultured on OP9-DLL4 (D5+4 cells). Results are mean ± SEM for at least 3 independent experiments. *p<.05, **p<.01, ***p<.001. G) Limiting dilution assay to determine the frequency of T cell progenitors within the D5+5 HPs generated from HE:DLL4⁻ on OP9, HE:DLL4⁻ on OP9-DLL4, and HE:DLL4⁺ on OP9-DLL4. p<0.01.

See also Figure S5.
Figure 3-11: D5 HE subsets derived from hiPSC lines have the same response to OP9 and OP9-DLL4

D5+4 flow plots of D5 sorted HE:DLL4- and HE:DLL4+ cultured on OP9 or OP9-DLL4 for 4 days reveal hemogenic activity from HE:DLL4+ cells only when cultured on OP9-DLL4.
In order to determine whether there are any molecular differences between HPs derived from HE:DLL4+ and HE:DLL4- cells, we performed RNA-seq analysis of CD235a/CD41a-CD34-CD43+CD45+ cells generated from these two different hemogenic endothelial cells following tertiary culture on either OP9 or OP9-DLL4 (Figure 3-12 A). As a basis for the analysis, genes that were differentially expressed in a 3-way Bayesian model involving HPs from HE:DLL4- on OP9 (condition 1), HE:DLL4- on OP9-DLL4 (condition 2) and HE:DLL4+ on OP9-DLL4 (condition 3) were used with focus specifically on genes upregulated in HE:DLL4+ vs. HE:DLL4- derived HPs obtained from OP9-DLL4 cocultures. Among 131 differentially expressed genes in this category, we identified two cell surface markers of HSCs in AGM, ACE and TEK (Hsu et al., 2000; Jokubaitis et al., 2008), and the following nine transcription factors: MECOM, GFI1b and ERG, essential for AGM and fetal liver hematopoiesis (Goyama et al., 2008; Sato et al., 2008; Taoudi et al., 2011; Thambyrajah et al., 2016); ARID5B, BCOR, and KDM6B, control lymphoid development (Beguelin et al., 2016; Lahoud et al., 2001; Manna et al., 2015); ZNF93, highly expressed in T cells (Bellefroid et al., 1993); and RUNX1T1 and HOXB8, regulate expansion of blood progenitors (Basecke et al., 2005; Redecke et al., 2013) (Figure 3-12 B). Using the known transcription-target relationships obtained by combining largely complementary data from HTRIdb (Bovolenta et al., 2012) and CellNet (Cahan et al., 2014), 163 regulatory interactions involving 110 transcription factors upstream of the nine differentially expressed transcription factor-encoding genes were pulled to construct a regulatory network in HPs derived from HE:DLL4+ cells on OP9-DLL4 (Figure 3-12 C). The database-derived structure of the network has been confirmed by our RNA-Seq data: transcription factors that are active according to our regulon analysis (red nodes) are apparently responsible for the upregulation of mRNA level of the target genes (large nodes). Three out of nine target genes
(MECOM, RUNX1T1, GFI1B) have also an evidence of their protein-level activity (reddish color on the graph) detected as enrichment of their known targets among the differentially expressed genes. Interestingly, GATA2, SOX17, SOX18, MYB, PBX1, PRDM14, DACH1, KLF4, HOXA5, HOXA7 and NOTCH1 were identified as upstream regulators of these genes, thereby suggesting that the molecular program in HPs derived from the arterial-type HE:DLL4⁺ is driven by transcriptional regulators implicated in definitive hematopoiesis.

Together, these results imply that arterial-type CD144⁺CD43⁻CD73⁻DLL4⁺ HE represents the precursor of definitive NOTCH-dependent hematopoiesis with broad lympho-myeloid and definitive erythroid potential, while the CD144⁺CD43⁻CD73⁻DLL4⁻ phenotype is associated with emerging immature HE endothelium (D4) or HE that has primitive NOTCH-independent hematopoietic potential (D5).
Figure 3-12. HPs derived from DLL4⁺ HE activate definitive hematopoietic program

A) Experimental strategy for generating and characterizing HE:Dll4⁺/- Derived HPs. D4 HE cells where cultured on DLL1-Fc for 24h, followed by purification of D4+1 HE:Dll4⁺ and HE:Dll4⁻ and subsequent culture on OP9 or OP9-DLL4. Five days later (D4+1+5), CD34⁺CD43⁺CD45⁺CD235α/41α⁻ population was FACSorted from each condition and RNA was extracted for RNA-seq. B) A heatmap of differentially expressed transcription factor genes in HPs derived from indicated cell populations. The expression is shown as a log ratio of gene expression relative to HPs generated from HE:Dll4⁻ cells on OP9-DLL4. C) Transcriptional regulatory network reconstructed with the nine transcription factor-encoding genes (the nodes with incoming interactions) differentially expressed in HPs derived from HE:Dll4⁺. Size of the
nodes represents relative abundance of mRNA of the respective gene, while color density represents enrichment of known targets of that transcription factor (regulon members) among the differentially expressed genes. Network visualization was performed using Cytoscape ver. 3.4.0 (Shannon et al., 2003).
3.4 Discussion

Our findings support the model that NOTCH signaling is essential for specification of definitive lympho-myeloid hematopoiesis by eliciting arterial specification of HE from hPSCs. We demonstrated that NOTCH activation promotes formation of transient CD144+CD43-CD73- DLL4+ HE population with high expression of arterial genes and active Runx1+23 enhancer that mark arterial type HE in AGM, umbilical and vitelline arteries (Bee et al., 2009; Ng et al., 2010; Richard et al., 2013; Swiers et al., 2013; Tamplin et al., 2015). Although CD144+CD43-CD73-DLL4+ AHE have lower hemogenic capacity compared to DLL4- HE, the hematopoietic potential of AHE is strictly NOTCH dependent. AHE is specified from CD144+CD43-CD73-DLL4- immature HE cells emerging on D4 of differentiation in a NOTCH-dependent manner following acquisition of an arterial CD144+CD43-CD73-DLL4+ phenotype, while CD144+CD43-CD73-DLL4- HE cells that failed to undergo arterial specification on day 5 of differentiation retained mostly primitive hematopoietic potential and were minimally affected by NOTCH activation (Figure 3-13). Demonstrating that definitive hematopoietic potential is highly enriched in arterial type HE is in concordance with in vivo studies that demonstrated the link between arterial specification and lymphoid cell and HSC development in the yolk sac and embryo proper (de Bruijn et al., 2000b; Gordon-Keylock et al., 2013; North et al., 1999; Rybtsov et al., 2016; Yzaguirre and Speck, 2016).

Previous studies have found that NOTCH pathways are active during hematopoietic differentiation of mouse and human PSCs. The transient NOTCH activation increased the generation of CD45+ hematopoietic progenitors (Ayllon et al., 2015; Jang et al., 2015; Kim et al., 2013; Lee et al., 2013) and NOTCH inhibition with DAPT decreases the percentage of CD45+ cells in cultures of hPSC-derived CD34+ progenitors (Ditadi et al., 2015). NOTCH activation in
hPSC cultures is predominantly mediated through the NOTCH ligand, DLL4, expressed by endothelial cells (Ayllon et al., 2015). However, whether NOTCH signaling affects HE specification, EHT per se or the fitness (survival and expansion) of HPs at post-EHT stage remains unknown. In the present study, we provided evidence that NOTCH has multiple effects on hematopoiesis from HE. First, we demonstrated that NOTCH signaling is important for the specification of arterial-type HE: DLL4⁺ and the promotion of EHT from these cells. In addition, NOTCH activation also potentiates the expansion of the most immature multipotential HPs post-EHT, consistent with its effect on AGM- and cord blood-derived HSPCs (Hadland et al., 2015; Ohishi et al., 2002; Shojaei et al., 2005). Thus, proper assessment of the effect of NOTCH on hematopoiesis and applying NOTCH-dependence as a criterion to distinguish definitive and primitive hematopoiesis from hPSCs requires careful experimental design, thereby allowing for the separation of the effect of NOTCH signaling on AHE specification, EHT and HP amplification.

Overall, our studies indicate that regulation of NOTCH signaling would be important to mimic the arterial HE, definitive lympho-myeloid hematopoiesis and HSC specification in hPSC culture. Nevertheless, we failed to achieve engraftment from NOTCH-activated cultures in our pilot studies (data not shown). As previous studies have shown, the timing (Guiu et al., 2013) and strength (Gama-Norton et al., 2015) of NOTCH signaling is tightly regulated during EHT and post-EHT. In mouse embryo, NOTCH signaling is especially critical for pre-HSC type I (VE-Cad⁺CD45⁻CD41⁻CD43⁻) development and their transition into pre-HSC type II (Ve-Cad⁺CD45⁺CD41⁺CD43⁺) in the AGM, however later stages of HSC development are less dependent on NOTCH (Souilhol et al., 2016a). Thus, NOTCH signaling may need to be fine-tuned in hPSC cultures to establish long-term engrafting HSCs. It is also likely that acquisition of
arterial features by HE is a necessary but not a sufficient prerequisite for HSC formation from hPSCs. In yolk sac, arterial vessels in contrast to venous vessels and capillaries produce lymphoid cells (Yzaguirre and Speck, 2016), yet direct association of arterial specification with HSC commitment at extraembryonic sites have not been shown. Several signaling pathways, including those uncoupled from aortic specification such as HOXA (Dou et al., 2016; Ng et al., 2016), TGF-β (Monteiro et al., 2016a), retinoic acid (Chanda et al., 2013; Dou et al., 2016; Ghiaur et al., 2013), inflammation (He and Liu, 2016; He et al., 2015; Li et al., 2014), hormone (Heo et al., 2015; Kim et al., 2016), and blood flow induced shear stress (Kim et al., 2015; North et al., 2009), have all been shown to serve roles during HSC development and may be affected in in vitro generated cells. The investigation of the cross-talk among all of these different signaling pathways and manipulating them together is probably necessary to recapitulate embryonic definitive hematopoietic development and generate long-term engrafting HSCs in vitro.
The most immature hPSC-derived CD144⁺CD43⁻CD73⁻ HE cells expressing NOTCH1 but lacking arterial and venous identity arise on day 4 of differentiation. NOTCH activation induces specification of arterial-type CD73⁻ HE and CD73⁺ non-HE that are DLL4⁺, first detectable on day 5 of differentiation. DLL4⁺ HE cells upregulate arterial markers, but also express hematopoietic genes. Subsequently, arterial-type HE:DLL4⁺ are NOTCH-dependent and produce hematopoietic progenitors that have definitive-type characteristics. Day 4 HE cells that are not DLL4⁺ by day 5 of differentiation undergo EHT independent of NOTCH activation and produce NOTCH-independent hematopoietic progenitors with primitive potential.
3.5 Material and Methods

Human Pluripotent Stem Cell Maintenance and Differentiation

Human pluripotent stem cells, WA01 hESC line, DF19-9-7T fibroblast-hiPSC line, IISH2i-BM9 bone marrow-iPSC line, and IISH3i-CB6 cord blood-iPSC line, were maintained and passaged in chemically defined conditions using vitronectin and E8 media, as previously described (Chen et al., 2011a). The human PSCs were differentiated into hematoendothelial lineages using a modified protocol previously described (Uenishi et al., 2014). On Day -1, hPSCs were singularized and plated on collagen IV-coated plates (0.5 µg/cm²) at a cell density of 7,500 cells/cm² in E8 media supplemented with 10uM Rock inhibitor (Y-27632, Cayman Chemicals). On Day 0, the media was changed to IF9S media supplemented with BMP4, FGF2 (50 ng/ml), Activin A (15 ng/ml, Peprotech), LiCl (2mM, Sigma), and ROCK inhibitor (0.5 uM, Cayman Chemicals) and cultured in hypoxia (5% O₂, 5% CO₂). On day 2, the media was changed to IF9S media supplemented with FGF2, VEGF (50 ng/ml, Peprotech), and 2.5 uM TGF β inhibitor (SB-431542, Cayman Chemicals). On day 4, cell cultures were singularized and stained with anti-CD31 microbeads (Miltenyi) for 15 minutes. Cells were washed and CD31+ HE were purified using MACS LS columns (Miltenyi). Purified CD31+ HE were then plated at a density of 20,000 to 30,000 cells/cm² on collagen IV-coated plates (1ug/cm²) that were either co-coated with IgG-Fc fragments or DLL1-Fc (made in-house), in IF9S media supplemented with FGF2, VEGF, EGF, IGF-I, IGF-II, TPO, IL-6 (50 ng/ml), SCF (20 ng/ml), IL-3, FLT3L (10 ng/ml, Peprotech), and ROCK inhibitor (5 uM, Cayman Chemicals), and where specified, DMSO (1:1000, Fisher Scientific) or DAPT (10 µM, Cayman Chemicals), and cultured in normoxia (20% O₂, 5% CO₂). A sample of the purified cells was analyzed by flow cytometry, and experiments were continued only if the purity of the HE was over 95% CD144+. On Day
4+1, the media was replaced with fresh media containing the same supplements without ROCK inhibitor. On day 4+3, extra media with the same supplements was added to the culture.

**OP9 Maintenance and Co-culture**

OP9, OP9-DLL4, and the inducible OP9-iDLL4 (made in-house) cell lines were maintained in αMEM with 20% FBS (GE) on gelatin-coated plates in normoxia as previously described (Vodyanik and Slukvin, 2007c). Using TrypLE (Thermo), OP9 were passaged at a 1:8 ratio every 3-4 days when they were 80% confluent. One day before co-culture with differentiated human HE cells, OP9 lines were treated with mitomycin C (1 mg/ml) for 2 hours and then plated at a density of 12,500 cells/cm² as previously described (Zhang et al., 2005). D4 HE cells or D5 CD144+ subsets were plated onto OP9 lines at a density between 1000 to 2000 cells/cm² in media containing αMEM with 10% FBS (GE), TPO, SCF, IL-6 (50 ng/ml), IL-3, and FLT3L (10 ng/ml). Media was changed after 24 hours, and extra media added 2 days later. Experiments conducted with DAPT were treated with 20 µM, while corresponding control conditions had DMSO added at a 1:500 dilution.

**Generation of DLL4 DOX-inducible OP9-iDLL4**

Human DLL4 gene fragment was amplified by PCR from a vector previously used to establish the OP9-DLL4 cell line. The DLL4 gene fragment was subsequently cloned into a pSIN-pTRE-DLL4-P2A-eGFP||EF1a-M2rtTA-IRES-Puro vector made in house. OP9 cells were then transduced by lentiviral vector containing the pSIN construct. 5 days later the infected OP9 cells were treated with Puromycin for 2 weeks during expansion. Samples of the OP9-iDLL4
cells were treated with doxycycline for 24 hours, then DLL4 and eGFP expression were confirmed by flow cytometry.

**Single-Cell Deposition Assay for Endothelial-to-Hematopoietic Transition**

One day before single-cell deposition, the OP9-iDLL4 cell line was treated with mitomycin C as described above, and passaged into 96-well plates at a density of 12,500 cells/cm². OP9-iDLL4 used for the NOTCH activation condition was incubated with doxycycline for 24 hours after passaging into 96-well plates. On the day of single-cell sorting, OP9-iDLL4 media was changed to αMEM with 10% FBS (GE), TPO, SCF, IL-6 (50 ng/ml), IL-3, FLT3L (10 ng/ml), and DMSO (1:500) for the control, and NOTCH activation conditions, or DAPT (20 µM) for the NOTCH inhibition condition. Day 4 differentiated human pluripotent stem cells were singularized, stained for CD309-PE and CD144-APC (Miltenyi Biotech), and was single-cell sorted into individual wells of the 96-well plates using a FACS Aria II. One day after sorting, the media was changed to fresh media without DMSO or DAPT, and extra media was added every 3 days. Seven days later, the plates were fixed and stained for immunofluorescent staining with anti-CD144 (rabbit, eBioscience) and anti-CD43 (mouse, BD Biosciences) primary antibodies and anti-rabbit AlexaFluor488 and anti-mouse AlexaFluor594 secondary antibodies (Jackson Immunology) in order to score the hematopoietic/endothelial colonies.

**CellTracer Proliferation Assay**

D4 CD31⁺ HE cells were incubated in PBS with CellTracer (1 µg/ml, Thermo) for 20 minutes at 37°C. After washing, the cells were plated on collagen IV-coated plates with either
Fc-IgG or DLL1-Fc and the modified Day 4 media, as described above, at a higher density of 30,000 to 40,000 cells/cm² due to toxicity from the CellTracer. Aliquots of the purified cells were analyzed by flow cytometry to determine the purity of the MACS cells and establish the Generation 0 peak for the proliferation assay. Secondary cultures were collected every day after plating for flow cytometry analysis, and calibration beads were used to generate compatible CellTracer results. After D4+4, FlowJo Analysis software was used to concatenate the data from each day and the proliferation platform was used to calculate the number of cell divisions that each cell underwent. Those peaks were re-applied to individual sets of data to determine the percentage of each generation within the hematoendothelial populations.

**T-Cell Differentiation and T-cell Limiting Dilution Assay**

Total D4+4 cultures were singularized, strained, and cultured in T-cell differentiation conditions on OP9-DLL4 for 3 weeks as previously described (Kennedy et al., 2012b; Uenishi et al., 2014; Vizcardo et al., 2013). For D5+4 cultures, only the floating hematopoietic cells were collected and cultured in T-cell differentiation conditions. Limiting Dilution Assays were conducted with the floating cells collected from D5+4 cultures (HE:Dll4⁻ on OP9+DAPT, OP9+DMSO, and OP9-DLL4, and HE:Dll4⁺ on OP9-DLL4). Row A of a 96-well plate received 500 cells/well, and each subsequent row afterwards had half the previous row (Row B contained 250, Row C contained 125… Row H contained 3-4 cells). The wells were scored 2 weeks later by eye and flow-cytometry for CD5⁺CD7⁺ containing cells. Positive threshold was set at 167 CD5⁺CD7⁺ cells/well. Extreme limiting dilution analysis was conducted using the previously established algorithm (Hu and Smyth, 2009).
**Red Blood Cell Differentiation and Maturation of D4+4 Cultures**

In order to assess the definitive erythropoietic potential of hematopoietic progenitor cells, we adopted our previously describe red blood cell differentiation protocol (Dias et al., 2011) to become chemically defined and feeder- and serum-free. Floating cells were collected, washed, and plated back into their respective cultures for D4+5 cells, or plated onto collagen IV-coated plates for D5+4 cells, with IF9S supplemented with dexamethasone (10 µM), EPO (2 U/ml), SCF, FLT3L, TPO, IL-6 (100 ng/ml), and IL-3 (10 ng/ml). Extra media with the same supplements was added 2 days later. An additional 2 days later, the cultures were treated with half-media changes every 2 days with IF9S supplemented with dexamethasone (10 µM), SCF (100 ng/ml), and EPO (2 U/ml). The floating cells were collected 10 days later to analyze by flow cytometry and RNA isolated for qPCR analysis.

**Generating Runx1+23 Enhancer Reporter cell line**

Runx1+23 enhancer fragment (Tamplin et al., 2015) was amplified by PCR and subsequently cloned into the AAVS-SA-2A-PURO vector (gift from Gadue Lab, The Children’s Hospital of Philadelphia). Human ESCs were transfected with zinc-finger nuclease vectors and later puromycin-resistant individual cells were clonally expanded and on-targeted clones were selected, as previously described (Jung et al., 2016).

**Southern Blot**

Southern Blot (SB) analysis was performed by DIG-labeling hybridization (Roche). Briefly, 10 µg genomic DNA was digested using a EcoRV restriction enzyme for overnight, separated on a 0.7% agarose gel for 6 hours, transferred to a nylon membrane (Amersham), and
incubated with DIG-labeling probes. The external probe is a DIG-labeled 600 nucleotide fragment that binds to the EcoRV-digested fragment of the 5’ external region. The internal probe is a DIG-labeled 700 nucleotide fragment that binds to the EcoRV-digested fragment of the eGFP region.

Hematopoietic Colony Forming Unit Assay

Hematopoietic colony forming unit assay was conducted in serum-containing H4436 Methocult (Stem Cell Technologies) as previously described (Choi et al., 2012b; Uenishi et al., 2014; Vodyanik and Slukvin, 2007b).

Flow Cytometry and FACS-sorting

Flow Cytometry was conducted using the MACSQuant 10 (Miltenyi Biotech). FACS-sorting was conducted on a FACS Aria II (BD) as previously described (Choi et al., 2012b; Uenishi et al., 2014; Vodyanik and Slukvin, 2007b).

qPCR Analysis

Cells were differentiated for the respective days and sorted on a FACS Aria II. RNA was collected using RNA MiniPrep Plus (Invitrogen) and quantified on a NanoDrop (GE Healthcare). Equal amounts of RNA were used for cDNA synthesis using SuperScript III First-Strand Synthesis System (Life Technologies). qPCR was conducted using Platinum SYBR Green qPCR SuperMix (Life Technologies). The reactions were run on a Mastercycler RealPlex Thermal Cycler (Eppendorf) and the expression levels were calculated by minimal cycle threshold values (Ct) normalized to the reference expression of RPL13a. The qPCR products were run on an
agarose gel and stained with ethidium bromide to confirm specificity of the primers. Primer sequences can be found in Supplementary Table 3.

**RNA-Seq data processing and analysis**

Total RNA was isolated from the CD235a/CD41α/CD34⁺/CD45⁺ derived from HE:DLL4⁺ and HE:DLL4⁻ cells using the RNeasy mini Plus Kit (Qiagen). RNA purity and integrity was evaluated by capillary electrophoresis on the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). One hundred nanograms of total RNA was used to prepare sequencing libraries using the TruSeq RNA Sample Preparation kit (Illumina, San Diego, CA). Final cDNA libraries were quantitated with the Qubit Fluorometer (Life Technologies, Carlsbad, CA) and multiplexed with eighteen total indexed libraries per lane. Sequencing was performed using the HiSeq 3000 (Illumina, San Diego, CA) with a single read of 64 bp and index read of 7 bp.

Base-calling and demultiplexing were completed with the Illumina Genome Analyzer Casava Software, version 1.8.2. Following quality assessment and filtering for adapter molecules and other sequencing artifacts, the remaining sequencing reads were aligned to transcript sequences corresponding to hg19 human genome annotation. Bowtie v 1.1.2 was used, allowing two mismatches in a 25 bp seed, and excluding reads with more than 200 alignments (Langmead et al., 2009). RSEM v 1.3.0 was used to estimate isoform or gene relative expression levels in units of “transcripts per million” (tpm), as well as posterior mean estimate of the “expected counts” (the non-normalized absolute number of reads assigned by RSEM to each isoform/gene) (Li and Dewey, 2011; Li et al., 2010). R statistical environment (R core team, 2014) was used at all of the stages of downstream data analysis. The entire set of libraries was pre-normalized as a pool using median normalization routine from EBSeq package (Leng et al., 2013). EBSeq with
10 iterations was applied to call for differential expression. The EBSeq’s default procedure of filtering low-expressed genes was suppressed by setting the $Q_{trmCut}$ parameter to zero. Genes with assigned value of Posterior Probability of Differential Expression above 0.95 were preliminary selected. Subsequently, only genes demonstrating the Critical Coefficient (Moskvin et al., 2014) value above 1.5 were retained as differentially expressed.

**Statistical Analysis**

Statistical analysis was performed in PRISM software. Data obtained from multiple experiments were reported as mean+/- standard error. Where appropriate, either a 1-way ANOVA or 2-way ANOVA were utilized with a Bonferroni post-hoc test. Differences were considered significant when *$p<0.05$, **$p<0.01$, or ***$p<0.001$.**
### Table 3-1. Antibodies Used in This Study

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**Table 3-2. Fluorescent Reagents Used in This Study**

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Table 3-3. Primers used for qRT-PCR in This Study

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CHAPTER 4: CONCLUSION AND FUTURE DIRECTION
4.1 Accomplishments and Advancements

We have successfully developed a completely defined, feeder- and embryoid body-free two-dimensional platform for human pluripotent stem cell (hPSC) differentiation into hematopoietic lineages. We produced a chemically defined basal media based on IMDM:F12 that supports efficient mesodermal induction, hemogenic endothelial (HE) specification, endothelial-to-hematopoietic transition (EHT), and hematopoietic progenitor (HP) expansion with the addition of stage-specific growth factors, cytokines, and small molecules. Initially, we used collagen IV (ColIV) as the extracellular matrix (ECM) on which the hPSCs differentiate into hematopoietic progenitors. Then, we have identified that Tenascin C (TenC) is a critical ECM component for the specification of definitive-type hematopoietic progenitors (Uenishi et al., 2014).

Utilizing our defined and directed differentiation platform, we isolated the role of the NOTCH signaling pathway during each stage of HE specification, EHT, and HP expansion and maturation. We identified a transient CD144⁺CD43-CD73-RUNX1+23-eGFP⁺NOTCH1 hiDLL4⁺ hemogenic endothelial population (HE:DLL4⁺) that have arterial endothelial characteristics, which arise from a more immature CD144⁺CD31⁺CD43-RUNX1+23-eGFP-NOTCH1⁺DLL4⁻ hemogenic endothelial progenitor (HEP) population that have neither hematopoietic, arterial, or venous characteristics. Increased NOTCH activation is required for HEPs to gain the HE:DLL4⁺ phenotype, whereas low levels of NOTCH activation increased the CD144⁺CD43-CD73-RUNX1+23-eGFP-NOTCH1 loDLL4⁻ hemogenic endothelial (HE:DLL4⁻) fraction and CD144⁺CD43-CD73⁺DLL4⁺ non-hemogenic endothelium (non-HE). The HE:DLL4⁻ population was found to undergo EHT even in the presence of DAPT, a NOTCH inhibitor, and give rise to
hematopoietic progeny more closely resembling the primitive wave of hematopoiesis. In contrast, the HE:DLL4+ population required high levels of NOTCH activation to undergo EHT, and while the number of hematopoietic progeny was significantly less than from the HE:DLL4- population, the HPs that emerged from HE:DLL4+ had increased definitive-type hematopoietic potential, including increased GEMM-CFCs, T-lymphoid potential, and increased α- and β-globin expressing erythrocytes. In addition, consistent with studies using de novo hematopoietic stem cells (HSCs) from cord blood, maintaining hPSC-derived HPs on DLL1-Fc ligands showed increased proliferation while preserving their multilineage potential and progenitor-like state. Taken together, we have refined our model of human hematopoietic development and are one step closer to generating long-term engrafting definitive hematopoietic stem cells from hPSCs.

We have utilized our defined and directed differentiation platform to investigate the effects of TenC as a basement ECM and the specific roles of NOTCH signaling during hematoendothelial development. The advantages of this system extend to other signaling pathways as well. Mammalian hematopoietic development has been shown to involve hedgehog (HH), retinoic acid (RA), inflammation, hormone, and blood flow-induced shear stress signaling pathways. We have already utilized our differentiation platform to study these signaling pathways in other studies and have identified specific stages at which these pathways affect hemato-endothelial differentiation from hPSCs.

The defined nature of our differentiation platform was developed with future clinical applications in mind. The use of a completely defined, animal product-free system is readily GMP-certifiable, which allows for smoother translation of hPSC-derived cellular products from research to clinical applications. While the hematopoietic progenitors generated from this platform do not currently engraft and populate immunocompromised mice and thus have limited
therapeutic potential, the initial stages of the procedure to induce primitive mesoderm differentiation is now being used in part to generate mesenchymal stem cells from HLA homozygous donor-derived iPSCs and is currently in clinical phase I trials in the UK to treat steroid-resistant graft-versus-host disease (A study of CYP-001 for the Treatment of Steroid-Resistant Acute Graft Versus Host Disease, NCT02923375).

Despite decades of hPSC research and increased understanding of the complex systems of mammalian hematopoietic development, the ability to generate LT-dHSCs continues to elude researchers. We have identified specific signaling pathways and transcription factors necessary to differentiate hPSCs into the unique population of HE that gives rise to HPs with definitive-type hematopoiesis. In addition, researchers have developed methods of generating definitive erythrocytes (Dias et al., 2011), platelet-producing megakaryocytes (Takayama et al., 2008), functional macrophages, granulocytes (Choi et al., 2011; Choi et al., 2009b), natural killer cells (Hermanson et al., 2016; Knorr and Kaufman, 2010; Ni et al., 2011; Woll et al., 2009), genetically engineered CAR-T cells (Themeli et al., 2013), and even B-cells (Vodyanik et al., 2005) from hPSCs-derived hematopoietic progenitors. Unfortunately, when injected into immunocompromised mice, these HPs still do not reconstitute a human immune system.
4.2 Current Theories on the Limited Engraftment Potential of hPSC-derived Hematopoietic Progenitor Cells

Hematopoietic stem cell development is complex. To date, we have not been successful in generating LT-dHSCs from human and mouse PSCs (without ectopic expression of specific transcription factors). While many researchers are trying to elucidate the reasons for the failures, a few theories exist that shape the approach taken to overcome this limitation.

The fact that mammalian hematopoietic development involves two independent waves creates a complexity unique to this organ system. In the embryo, anatomical location can be used to distinguish the primitive and definitive waves of hematopoiesis. *In vitro*, however, directed differentiation in a culture dish has limits. We must rely on cell surface markers to identify the population of cells we are generating in culture, yet specific markers still do not exist to distinguish primitive versus definitive hematopoietic progeny (Ditadi et al., 2017). Multilineage myeloid potential, lymphoid potential and globin expression in erythroid differentiation are currently used to identify definite-type hematopoiesis from hPSC cultures (Ditadi et al., 2015; Kennedy et al., 2012b; Sturgeon et al., 2014; Uenishi et al., 2014). However, recent studies have revealed that a subset of hemogenic endothelium in the AGM and yolk sac of developing embryos generates lymphoid-lineage restricted hematopoietic progenitors (Boiers et al., 2013; Yoshimoto et al., 2011; Yoshimoto et al., 2012). In addition, a separate wave of erythro-myeloid progenitors has been identified during development that emerges before LT-dHSCs, but generate erythrocytes expressing fetal globins associated with early definitive hematopoiesis (Chen et al., 2011c; McGrath et al., 2015; McGrath et al., 2011). These findings reconcile the observation that the AGM of murine embryos at E10.5 contain over 600 budding hematopoietic cells but only 2
to 3 LT-dHSCs are detectable (Bigas and Waskow, 2016; Kumaravelu et al., 2002; Taoudi et al., 2008; Zhou et al., 2016). While our arterial-type HE cells produce HPs resembling definitive-type hematopoiesis, it is possible that they are the lineage-specific HP cells.

Along with this theory, RNA-sequencing data and direct reprogramming studies have shown that PSC-derived HPs do not have the exact same molecular signatures as *de novo* LT-dHSCs from cord blood (McKinney-Freeman et al., 2012). Hence, mouse PSC-derived HPs have only been able to engraft with limited lineage reconstitution when HoxB4 was ectopically expressed (Kyba et al., 2002). In addition, Doulatov et al has found that reprogramming hPSC-derived myeloid lineage cells back to an HSC-state can produce engraftable HPs (Doulatov et al., 2013). These findings suggest that during hPSC differentiation, not all of the signaling cues required for LT-dHSC specification are present in the culture. The complexity of combining all of the signaling cues that have been identified to play a role in HSC specification renders recapitulating the in vivo environment difficult. In addition, while our study has addressed the aberrant NOTCH activation between *de novo* LT-dHSCs and hPSC-derived HPs, other studies suggest that perfecting the specific strength and level of NOTCH activation may be necessary to achieve LT-dHSC specification (Guiu et al., 2013).

Another theory hypothesizes that, while we are able to generate LT-dHSCs in culture, we cannot maintain their stem-like state *in vitro*, and are differentiating beyond the HSC-state into multipotent progenitors (MPPs) that lack long-term engraftment potential. *In vivo* studies reveal that LT-dHSCs migrate to the fetal liver soon after EHT (Ema et al., 1998; Ema and Nakauchi, 2000; Paige et al., 1984; Rebel et al., 1996b). While the mouse and human AGM have been shown to produce 2-3 definitive HSCs per embryo equivalent, soon after they emerge and migrate to the fetal liver, they expand to over 50 LT-dHSCs per embryo equivalent. The stem
cell niche in the fetal liver may have unique signaling cues which promote LT-dHSC expansion without exhaustion that have yet to be elucidated (Chou and Lodish, 2010; Khan et al., 2016; Zhang et al., 2006; Zhang and Lodish, 2004). While much research has focused on the stem cell niche in the adult bone marrow, reproducing that microenvironment would theoretically only promote quiescence and limit the expansion of definitive HSCs. Thus, the ability to generate large quantities of LT-dHSCs from hPSCs may rely on identifying the signaling cues present in the fetal liver HSC-niche, reproducing that in culture, and being able to isolate pre-HSCs out of the culture that promoted EHT and plating them into culture conditions that mimic the fetal liver microenvironment.

### 4.3 Future Direction

Our ability to generate LT-dHSCs from hPSCs will depend on furthering our understanding of *in vivo* development and reproducing them *in vitro*. Advancing technology in live imaging, single-cell RNA sequencing, and high-throughput screening has already contributed to our knowledge of the complex developmental pathways of mammalian hematopoiesis and LT-dHSC specification. In the future, investigating the cross-talk between all of the different signaling pathways involved and manipulating them in concert will be critical in recapitulating *de novo* LT-dHSC specification. In addition, our pursuit for increasing HP differentiation efficiency from hPSCs has overlooked the possibility that limiting the frequency of EHT is necessary for LT-dHSC specification, as has been shown with NOTCH-dependent GATA2 expression control (Guiu et al., 2013). Finally, continuously modifying our current
culture condition to recreate the AGM, fetal liver, and bone marrow microenvironment will be necessary to generate, expand, and maintain LT-dHSCs from hPSCs.

We have come a long way from the first discovery of the extra-embryonic primitive wave of hematopoiesis and the intra-embryonic origins of the definitive wave of hematopoiesis. The clinical applications of blood transfusion and hematopoietic stem cell transplant have revolutionized disease treatment. The future of patient-specific personalized therapies and regenerative medicine lies in our ability to further our understanding of human hematopoiesis and in recapitulating the developmental process \textit{in vitro}. 
APPENDIX A: A HUMAN VE-CADHERIN-TDTOMATO AND CD43-EGFP DUAL REPORTER CELL LINE FOR STUDYING ENDOTHELIAL-TO-HEMATOPOIETIC TRANSITION
A.1 Abstract

Human embryonic stem cell (hESC) line WA01 was genetically modified using zinc-finger nucleases and the PiggyBac/transposon system to introduce a fluorescence reporter for VEC (tdTomato) and CD43 (eGFP). Phenotypic and functional assays for pluripotency revealed the modified hESC reporter lines remained normal. When the cells were differentiated into hemato-endothelial lineages, either by directed differentiation or direct reprogramming, flow cytometric and fluorescence microscopy showed that VEC+ endothelial cells express tdTomato and CD43+ hematopoietic progenitors express eGFP.
A.2 Resource Details

Endothelial-to-hematopoietic transition (EHT) is a unique process during developmental process that gives rise to blood cells, including hematopoietic stem cells (HSCs). Modeling EHT in a culture in vitro is essential for identifying the molecular program involved in HSC specification. To track EHT from during human embryonic stem cells (hESCs) differentiation into hemato-endothelial lineages, we made a VE-cadherin-tDTomato/CD43-eGFP (VEC-tDTomato/CD43-eGFP) dual reporter fluorescent cell line based on the WA01 hESC line. VE-cadherin (CDH5, CD144) is the one of the most specific marker of endothelial cells (Breier et al., 1996; Lampugnani et al., 1992; Vittet et al., 1997), while CD43 (leukosialin/SPN) is a pan-hematopoietic marker (Remold-O'Donnell et al., 1987) that is expressed by all hematopoietic progenitors during hPSC differentiation cultures (Choi et al., 2011; Vodyanik et al., 2006) and HSCs in the embryo and adult bone marrow (Inlay et al., 2014; Moore et al., 1994; Rybtsou et al., 2014). A construct containing the CD43 promoter driving eGFP expression (Tiyaboonchai et al., 2014) was targeted to the AAVS1 locus by ZFN nuclease (Fig. A-1 A). After integrating the CD43-eGFP construct to the AAVS1 locus, we isolated and selected specific clones that had correct integration of the construct into the AAVS1 locus without random integration as determined by southern blot (Fig. A-1 B). We subcloned the -3394/+39 VE-cadherin promoter followed by the tdTomato into the PiggyBac vector and co-electroporated the construct with transposons into the selected CD43-eGFP reporter cell clones (Fig. A-1 A). After isolating and selecting individual VEC-tDTomato/CD43-eGFP dual reporter clones, we confirmed the identity of the transgenic cell line by Short Tandem Repeat (STR) analysis (Table A-1). Individual clones were assayed for pluripotent phenotype by measuring the expression of transcriptional factors,
OCT4, SOX2, and NANOG. The dual reporter (DR) cell line retained expression of pluripotency markers at the level similar to wild type H1 hESCs (Fig. A-1 C). Pluripotent potential was measured using embryoid body differentiation, and the DR cell line showed capacity to produce all three germ layers following spontaneous differentiation (Fig. A-1 D). Also, we confirmed this dual reporter clones is normal karyotype (Fig. A-1 E).

To determine the specificity and functionality of the DR cell line, we differentiated it for 8 days in chemically defined conditions (Uenishi et al., 2014) and analyzed for the expression of VEC and CD43 with antibodies and their respective fluorescent protein. Typically, VEC⁺ hemogenic endothelial progenitors are first detected on day 4 of differentiation, while CD43⁺ hematopoietic progenitors can be detected from day 5 of differentiation (Fig. A-2 A). Fluorescence microscopy showed tdTomato and eGFP were detected from day 4 and day 5, respectively, and continued through day 8 (Fig. A-2 A, B). Flow cytometry analysis revealed the tdTomato⁺ cells were exclusively detected in the VEC⁺ endothelial population (Fig. A-2 B) while GFP⁺ cells were almost exclusively detected in CD43⁺ hematopoietic cells (Fig. A-2 B). Consistent with the current understanding of hematopoietic development, CD43-GFP⁺ cells arose from VEC-tdTomato⁺ hemogenic endothelial cells from day 5.

Recently we demonstrated that overexpression of ETV2 and GATA2 in undifferentiated hPSCs induces the formation of hemogenic endothelium which undergoes EHT (Elcheva et al., 2014). Following infection of VEC-tdTomato/CD43-GFP dual reporter hESCs with ETV2 and GATA2-expressing lentiviruses, we observed expression of tdTomato⁺ within the endothelial population which subsequently underwent transition to eGFP⁺ blood cells (Fig. A2-C), and we checked flow cytometry between day 4 to 6. We observed that CD43-eGFP blood cells generated from VEC-tdTomato endothelial cells gated tdTomato⁺ cell and increased between day 4 to 6.
after infection of ETV2 and GATA2 lentiviruses (Fig. A-2 D). Overall, these results demonstrate that the dual VEC-tdTomato/CD43-eGFP reporter hESC line allows for easy visualization of EHT and provides a useful tool for assessing molecular factors involved in EHT regulation.
A.3 Materials and Methods

Maintenance and Hematopoietic differentiation of hESCs

H1 (WA01, Wicell, Madison, WI) hESCs were cultured on vitronectin (Stem Cell Technologies), in E8 medium (Chen et al., 2011a). Cells were passaged every 5 days (80% confluency) using 0.5 mM EDTA in PBS. hESCs were differentiated in collagen IV-coated plate as previously described (Uenishi et al., 2014).

Vector construction

ZFN vectors targeting the AAVS1 locus and AAVS1-SA-2A-PURO-CD43 promoter-eGFP vector were generated as previously described (Tiyaboonchai et al., 2014). The VE-Cadherin promoter-tdTomato construct was cloned to Piggybac transposon vector (Transposagen). The VE-Cadherin -3394/+39 promoter region was amplified by PCR from BAC clone.

Gene targeting of the AAVS1 locus and PiggyBac system

Cells were dissociated into single cells by treatment with TrypLE (Life technologies). One million cells were resuspended in 100 µl reagent (1 X 10^6 cells) of Amaxa human stem cell nucleofector kit 2 (Lonza) with 1 µg ZFN-left and right plasmid each and 10 µg AAVS targeting plasmid. For targeting PiggyBac, 0.5 µg transposase plasmid and 5 µg transposon plasmid were electroporated using program A-13 according to manufacture protocol (Amaxa). The electroporated cells were resuspended with E8 (Stem Cell Technologies) culture medium and rock inhibitor (10 µM, Tocris Y-27632) and then they were plated and cultured with E8 growth
medium in 6-well plate. Puromycin and Zeocin selection (0.5 µg/ml, Life technologies) was started 3 days after electroporation. After 10 days, surviving colonies were singularized and sorted to 96 well plate as single cells, from which they were expanded individually.

**Southern blot**

Southern blot (SB) analysis was performed, as described in the protocol included in the DIG-labeling hybridization (Roche). Briefly, 10 µg genomic DNA was digested using restriction enzymes for O/N, separated on a 0.7% agarose gel, transferred to a nylon membrane (Amersham) with DIG-labeling probes. The external probe 1 was 600 base pair ApaLI fragment in 5’ external region, and internal probe1 was 600 base pair EcoRV fragment in internal region of AAVS-CD43 promoter-GFP.

**Embryonic body (EB) formation**

Cell harvested and cultured in ultra-low attachment well (Corning) to induce EB formation. EBs were cultured for 21 days with E6 media. Medium was changed every 2-3 days. EB were harvested at 14 days and 21 days and extracted RNA and analysis by RT-PCR with marker of three germ layers (Table 2).

**Flow cytometry**

Cells were dissociated into single cells by treatment with 1x Tryple. Cells analyzed using MACSQuant 10 (Miltenyi Biotech). Antibodies used included CD43-APC-Vio770 (Miltenyi Biotech) and VEC-VioBlue (Miltenyi Biotech). Intracellular staining to determine OCT4 (BD),
NANOG (BD), and SOX2 (BD) expression was performed using FIX & PERM cell permeabilization reagents (BD).

**Time-Lapse Microscopy**

Endothelial to hematopoietic transition (EHT) was monitored by time-lapse microscopy using fluorescent optics. H1ESCs line expressing VE-Cadherin-Td tomato and CD43-GFP dual reporter were transduced with GATA2+ETV2 lentiviral constructs and cultured for 4 days until endothelial cluster were formed (Elcheva et al., 2014). Time-lapse confocal imaging was performed over two days to capture blood formation. Time lapse movies were recorded using Nikon Eclipse Ti-E configured with an A1R confocal system, motorized stage (Nikon Instruments Inc. Melville, NY), and Tokai-Hit Stage Top Incubator (Tokai Hit CO., Ltd., Shizuoka-ken, Japan) at 37°C and 5% CO2. Images were acquired using Nikon Elements (NIS – element C) imaging software for every 5 min with CFI Plan Fluor DLL 10X NA 0.5 WD 2.1MM objective (Nikon Instruments Inc. Melville, NY). The time-lapse serial images were converted to Quick-time movies (.mov) and analyzed using ImageJ software (NIMH, Bethesda, MD).
Figure A-1: Characterization of VEC-tdTomato/CD43-eGFP dual reporter H1 hESCs.

A) Schematic of the constructs used for targeting of CD43-eGFP reporter into AAVS locus and VEC-tdTomato reporter by PiggyBac system. B) Southern blot analysis of ApaL1 or EcoRV
digested genomic DNA of CD43-eGFP cell. Asterisk, wild type; filled triangle, targeted; arrow, off-targeting. C) Flow analysis of intracellular staining. Expression of transcriptional factors, OCT4, SOX2, and NANOG, by dual reporter and wild type of hESCs. D) Differentiation of dual reporter cells to three germ layers. Expression of three germ layers markers AFP (endoderm), PAX6 (ectoderm), ETV2 and T (mesoderm), and GAPDH (internal control) in day 0, day 14 and 21 EBs of dual reporter cell (DR) and wild type of hESC analyzed by RT-PCR. E) Normal Karyotype (46, XY) of dual reporter clone.
Figure A-2: Differentiation of dual reporter cells into three germ layers and hematoendothelial cells.

Kinetic analysis of CD43-eGFP and VEC-tdTomato expressed during hematopoiesis by fluorescence microscopy A) and flow cytometry B). Cells were differentiated and checked for
eGFP and tdTomato fluorescence everyday from day 3 to day 8. VEC-reporter cell expressed tdTomato and CD43-reporter cell expressed eGFP. Scale bar is 300 µM. Gray histogram is gated on the CD43−VEC− population, green histogram is gated on the CD43+ population and represents eGFP expression, and red histogram is gated on the VEC+ population and represents tdTomato expression. C) GATA2+ETV2 induced cells with endothelial morphology can give rise to blood cells. Transition of VEC expressing cuboidal cells (endothelial cells) into round hematopoietic cells is associated with the loss of VEC expression (red) and acquisition of CD43 expression (green). Column 1- VEC-tdTomato; Column 2- CD43-eGFP; Column 3- VEC- tdTomato and CD43-eGFP merged. D) Cells were treated with GATA2+ETV2 virus and checked for CD43-eGFP and VEC-tdTomato at day 0, 4, 5, and 6 by flow cytometry.
Table A-1. Short Tandem Repeat (STR) profiling of dual reporter with the original cell line

Table 1

<table>
<thead>
<tr>
<th>STR locus</th>
<th>H1 wild type</th>
<th>H1.CD43/CD144 Reporter (DR)</th>
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<tr>
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<td>12, 13</td>
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<td>vWA</td>
<td>X, Y</td>
<td>X, Y</td>
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<tr>
<td>Amelogenin</td>
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<td>12, 13</td>
</tr>
<tr>
<td>Penta_D</td>
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<td>9, 13</td>
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<td>TH01</td>
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Table A-2. Primer sequence for the three germ layers

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<th>Target</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>PAX6</td>
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<td>CGTTGGACACGTTTTTGATTG</td>
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<td>ETV2</td>
<td>TCTTTGAAGCGGTACCAGAG</td>
<td>GGGACCTCGGTGTTAGTT</td>
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<td>T</td>
<td>GACAATTTGGCAGCCTTG</td>
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<tr>
<td>GAPDH</td>
<td>GAAGGTGAAGGTCGGAGT</td>
<td>GAAGATGGTGATGGGATTC</td>
</tr>
</tbody>
</table>
REFERENCES


de Bruijn, M.F., Speck, N.A., Peeters, M.C., and Dzierzak, E. (2000a). Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. EMBO J 19, 2465-2474.

de Bruijn, M.F., Speck, N.A., Peeters, M.C., and Dzierzak, E. (2000b). Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. The EMBO journal 19, 2465-2474.


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