Modelling Human Developmental Hematopoiesis. Towards in vitro Generation of Human Hematopoietic Stem Cells

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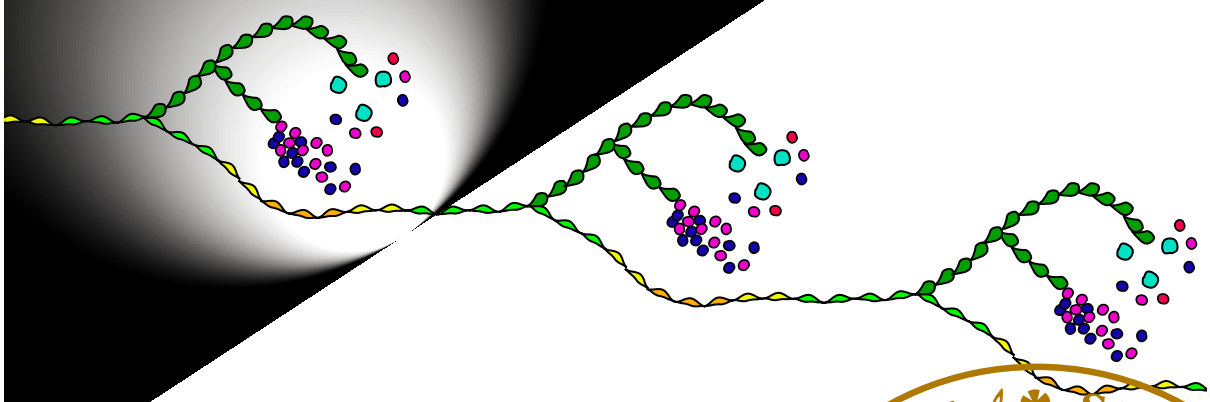
FACULTY OF MEDICINE | LUND UNIVERSITY 2017



Modelling Human Developmental Hematopoiesis

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Towards *in vitro* Generation of Human Hematopoietic Stem Cells

Carolina Guibentif

LUND
UNIVERSITY

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Modelling Human Developmental Hematopoiesis
Towards in vitro Generation of Human Hematopoietic Stem Cells

Abstract

The possibility to manufacture hematopoietic stem cells (HSCs) in the laboratory would provide an indefinite source of cells for patients requiring bone marrow transplantation. Moreover, combined with the progress in gene editing techniques, it would provide a novel platform for gene and cell replacement therapies for a range of currently incurable congenic and acquired disorders. During my PhD, I worked with an optimized protocol for in vitro blood generation from human Pluripotent Stem Cells (hPSCs). It was designed to mimic human hematopoietic development and it allowed us to explore some aspects that could elicit in vitro generation of HSCs. Using single-cell transcriptional analysis, we could explore the gene expression dynamics driving the endothelial-to-hematopoietic transition that occurs during in vitro differentiation. We also used this platform to explore the role of adrenergic signaling in human hematopoietic development, and identified reactive oxygen species as a major hurdle likely impairing the generation of functional HSCs from hPSCs. In this thesis, I present these studies in the context of the development of the hematopoietic system as it occurs during embryonic development, and in the framework of the latest progress in the quest of in vitro generation of HSCs.

Key words: human pluripotent stem cells; embryonic development, endothelial-to-hematopoietic transition; Hematopoiesis

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To my Family

Content

Abbreviations

ADRA – Adrenergic Receptor α
ADRB – Adrenergic Receptor β
AGM – Aorta-Gonad-Mesonephros
EB – Embryoid Body
BMP – Bone Morphogenetic Protein
BMT – Bone Marrow Transplantation
CMP – Common Myeloid Progenitor
CAR – Chimeric Antigen Receptor
CB – Cord Blood
cAMP – cyclic Adenosine Monophosphate
CFU – Colony Forming Unit
DA – Dorsal Aorta
EHT – Endothelial-to-Hematopoietic Transition
EMP – Erythro-Myeloid Progenitor
ESC –Embryonic Stem Cell
ETP – Earliest Thymic Progenitor
FGF – Fibroblast Growth Factor
GMP – Granulocyte Macrophage Progenitor
GVHD – Graft Versus Host Disease
HE – Hemogenic Endothelium
HH – Hedgehog
HLA – Human Leukocyte Antigen
hESC – human Embryonic Stem Cell
hPSC – human Pluripotent Stem Cell
HSC – Hematopoietic Stem Cell
HSPC – Hematopoietic Stem and Progenitor Cell
ICM – Inner Cell Mass
IFN – Interferon
IL – Interleukin
iPSC – induced Pluripotent Stem Cell
LPM – Lateral Plate Mesoderm
MEP – Megakaryocyte-Erythroid Progenitor.
Mk – Megakaryocyte
MLP – Multilymphoid Progenitor
MPP – Multipotent Progenitor
NE – Norepinephrine
NK – Natural Killer
NO – Nitric Oxyde
NT – Notochord
PGE2 – Prostaglandin E2
PS – Primitive Streak
pSP – para-aortic Splanchnopleure
RA – Retinoic Acid
Rho123 – Rhodamine 123
ROS – Reactive Oxygen Species
SDF – Stromal-Derived Factor
TF – Transcription Factor
TH – Tyrosine Hydroxylase
VEGF – Vascular Endothelial Growth Factor
YS – Yolk Sac

List of Publications

Papers included in this thesis

Paper I

Guibentif C., Rönn R.E., Böiers C., Lang S., Saxena S., Soneji S., Enver T., Karlsson G., Woods N.-B. Single-Cell Analysis Identifies Distinct Stages of Human Endothelial-to-Hematopoietic Transition. 2017 Cell Reports; Vol. 19, issue 1, p10–19

Paper II

Guibentif C., Rönn R.E., Ayad Fatah M., Saxena S., Moraghebi R., Parmar M., Leeb-Lundberg L.M.F., Kokaia Z., Lindvall O., Woods N.-B. Human Hematopoietic Cell Emergence during Development is Modulated by Norepinephrine Signaling Independent of β2-Adrenergic Receptor. *Manuscript in preparation*

Paper III

Saxena S., Rönn R.E.*, **Guibentif C.***, Moraghebi R., Woods N-B. Cyclic AMP Signaling through Epac Axis Modulates Human Hemogenic Endothelium and Enhances Hematopoietic Cell Generation. 2016 Stem Cell Reports; Vol. 6, issue 5, p692–703

Paper IV

Rönn R.E., **Guibentif C.**, Saxena S., and Woods N.-B. 2016. Reactive Oxygen Species Impair the Function of CD90+ Hematopoietic Progenitors Generated from Human Pluripotent Stem Cells. 2017 Stem Cells; Vol. 35, issue 1, p197–206

Papers not included in this thesis

Rönn R.E., **Guibentif C.***, Moraghebi R.*, Chaves P., Saxena S, Garcia B., Woods N-B. Retinoic acid regulates hematopoietic development from human pluripotent stem cells. 2015 Stem Cell Reports; Vol. 4, issue 2, p269–281

Sachdeva R., Jönsson M.E., Nelander J., Kirkeby A., **Guibentif C.**, Gentner B., Naldini L., Björklund A., Parmar M., Jakobsson J. Tracking differentiating neural progenitors in pluripotent cultures using microRNA-regulated lentiviral vectors. 2010 Proceedings of the National Academy of Sciences; Vol. 107, no. 25, p11602–11607

Abstract

The possibility to manufacture hematopoietic stem cells (HSCs) in the laboratory would provide an indefinite source of cells for patients requiring bone marrow transplantation. Moreover, combined with the progress in gene editing techniques, it would provide a novel platform for gene and cell replacement therapies for a range of currently incurable congenic and acquired disorders. During my PhD, I worked with an optimized protocol for *in vitro* blood generation from human Pluripotent Stem Cells (hPSCs). It was designed to mimic human hematopoietic development, and allowed us to explore some aspects that could elicit *in vitro* generation of HSCs. Using single-cell transcriptional analysis, we could explore the gene expression dynamics driving the endothelial-to-hematopoietic transition that occurs during *in vitro* differentiation. We also used this platform to explore the role of adrenergic signaling in human hematopoietic development, and identified reactive oxygen species as a major hurdle likely impairing the generation of functional HSCs from hPSCs. In this thesis, I present these studies in the context of the development of the hematopoietic system as it occurs during embryonic development, and in the framework of the latest progress in the quest of *in vitro* generation of HSCs.

Background

1. The quest of making Hematopoietic Stem Cells

a. The Hematopoietic Stem Cell – definition and clinical relevance

The Hematopoietic Stem Cell (HSC) is the clinically relevant component to be transferred to a patient during Bone Marrow Transplantation (BMT). BMT is a life-saving procedure commonly used as part of cancer therapy to replace the hematopoietic system, following intense cytotoxic treatments needed to eradicate malignant cells. BMT is currently also the only curative strategy for a wide range of acquired and inherited conditions, such as thalassemia and bone marrow failure syndromes (Copelan, 2006). In Europe, the use of BMT has been growing significantly over the past decades, the annual rate of transplanted patients having increased approximately 10-fold since 1990. In 2014, more than 36 000 patients were transplanted in Europe (Passweg et al., 2016). This is due to significant investments in creating collection banks, but also to the growing number of BMT indications and the evolving criteria for patient eligibility for this treatment, particularly in hematological malignancies such as leukemias and lymphomas.

The HSC is the best characterized adult stem cell, and BMT is nowadays the only stem cell replacement therapy routinely used in the clinic. The key features conferring the HSC this therapeutic value (as well as the status of a "stem cell") are self-renewal, i.e. the capability of maintaining its own pool for the life-time of an individual, and multipotency, the ability of differentiating into every mature effector cell type of the blood and immune system. This differentiation occurs by sequential lineage commitment stages, through successive intermediate progenitor states with a progressive decrease in lineage potential and self-renewal (Figure 1, lower panel). In the BMT setting, HSCs have therefore the crucial role of homing to the patient's bone marrow, repopulating his/her HSC pool, as well as regenerating all the blood and immune effector cell types.

To maintain homeostasis of the organism, the balance between self-renewal of HSCs and their differentiation into effector cells requires a tight regulation. In steady-state, HSCs reside in the bone marrow surrounded by numerous other cell types including mesenchymal stromal cells, endothelial cells, osteoblasts and sympathetic neurons (reviewed in Sanchez-Aguilera and Mendez-Ferrer, 2016). Together, through their physical properties and the production of signaling molecules, these different cell types provide the HSC a specific environment of extrinsic regulatory factors, the so-called niche (See Figure 1, upper panel). The importance of the niche for HSC regulation and identity is supported by the fact that so far, attempts of HSC *ex vivo* expansion have mostly led to the loss of HSC self-renewal, multipotency and therefore of their repopulating ability (Walasek et al., 2012). In addition, selective depletion of niche components in mouse models was shown to severely compromise HSC homeostasis (Nakamura-Ishizu et al., 2014; Omatsu et al., 2010; Visnjic et al., 2004). Intrinsic HSC gene networks also play an important role in regulation, as evidenced by the identification of specific genomic mutations affecting the balance between HSC self-renewal and differentiation ultimately causing leukemia (reviewed in Corces-Zimmerman and Majeti, 2014). Recent evidence also suggests the existence of inherent cell-autonomous behavior of HSCs in terms of self-renewal status and lineage differentiation propensity (Yu et al., 2016), although further studies are required to identify the mechanisms underlying this behavior.

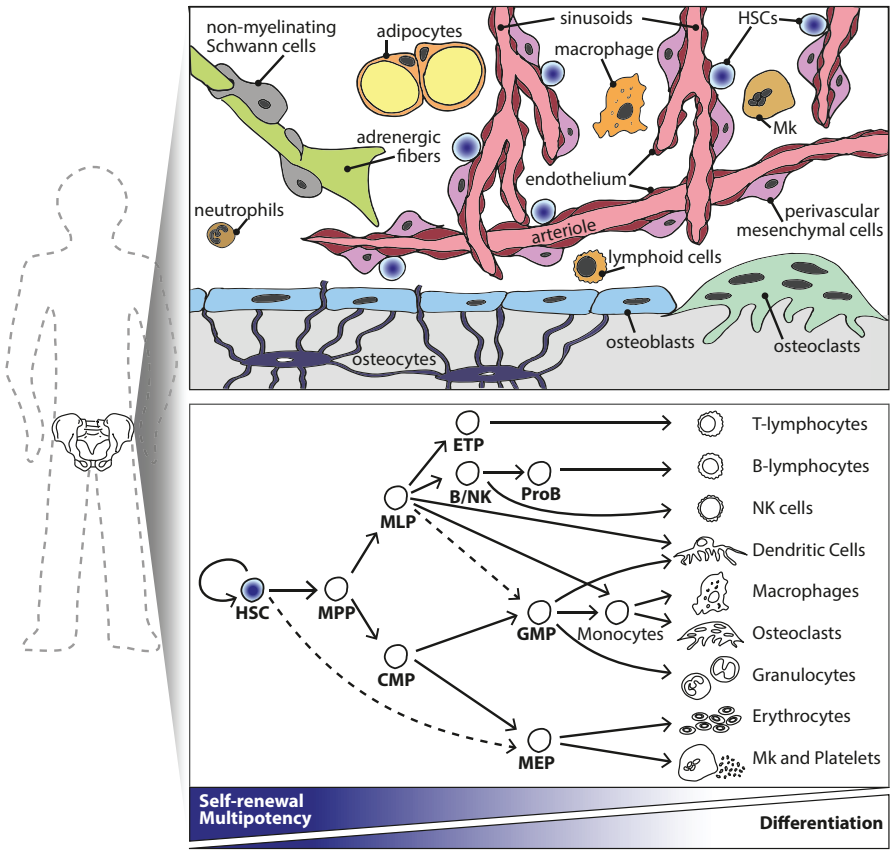

Figure 1: Hematopoietic Stem Cell homeostasis

In the adult, HSCs reside in the bone marrow in multiple anatomical locations, including the hip bone (pictured, typically punctured during the collection of HSCs for BMT), the skull, sternum, ribs, long bone extremities and vertebrae. Within the bone marrow, HSC homeostasis is maintained in part by the niche, a complex environment that includes several structures and cell types (represented in the upper panel, using Sanchez-Aguilera and Mendez-Ferrer, 2017). The latest studies indicate that HSCs are physically associated preferentially in proximity of blood vessels, especially sinusoids (the most abundant type of blood vessels in the bone marrow), although some can be found occasionally close to arterioles (reviewed in Morrison and Scadden, 2014). Several niche cell types have been shown to regulate the HSC through secreted as well as membrane-bound factors.

The HSC has the capacity to differentiate into every effector cell type of the blood and immune system (see lower panel, adapted from Doulatov et al., 2012). This differentiation occurs through successive intermediate progenitor stages with a progressive restriction in self-renewal and multipotency. The precise identity and homogeneity within these intermediate progenitors is still under investigation, the very existence of a CMP cell being currently questioned (dashed lines, see Notta et al., 2016).

HSC: hematopoietic stem cell, Mk: megakaryocytes, MPP: multipotent progenitor, MLP: multilymphoid progenitor, CMP: common myeloid progenitor, ETP: earliest thymic progenitor, NK: natural killer, GMP: granulocyte macrophage progenitor, MEP: megakaryocyte-erythroid progenitor.

b. Currently available sources of HSCs for BMT

Approximately half of the cases of BMT are allogeneic (Passweg et al., 2016), therefore requiring a compatible donor. Allogeneic BMT always carries the risk of graft versus host disease (GVHD), where the immune cells resulting from the transplant attack the patient's own tissues. GVHD is one of the main causes for morbidity following BMT, imposes the need for immunosuppression, and thereby allows for opportunistic infections among other complications. Cord blood (CB) has emerged as a promising alternative source of HSCs with lower risk of GVHD (Gutman et al., 2016), however the number of HSCs in a CB unit is low, one adult patient typically requiring two Human Leukocyte Antigen (HLA)-compatible CB units for transplantation (reviewed in Ballen et al., 2013; Brunstein and Wagner, 2006).

This is why the vast majority of allogeneic BMTs are performed in cases of leukemia and lymphomas, where the risks linked to GVHD are outweighed by the graft-versus-tumor effect associated. In this setting the engrafted immune cells, aside from attacking the patient's own tissues, also kill residual tumor cells remaining from the cytotoxic treatment which would otherwise cause a relapse of the treated malignancy; graft-versus-tumor effect therefore significantly contributes for patient survival (Copelan, 2006). However, in conditions where graft-versus-tumor effect is not required, such as congenital disorders and bone marrow failure syndromes, GVHD still remains a major threat hindering the use of a potentially curative allogeneic BMT.

Furthermore, despite the recent progress in establishing CB banking systems and the growth of bone marrow donor registries worldwide, there is still a shortage in HLA-matched donors for BMT, particularly concerning the case of ethnical minorities (Koh and Chao, 2008). Novel sources of HSCs are therefore required to supply these needs.

In this thesis, we explore the use of human pluripotent stem cells (hPSCs) as a potential unlimited source of HSCs for transplantation.

c. Human Pluripotent Stem Cells as a promising novel source of HSCs

While the above mentioned multipotency defines the ability of a cell to differentiate into all cell types within a given tissue, pluripotency designates the ability of a cell to generate every mature cell type in the whole body. Human embryonic stem

cells (hESCs) are human pluripotent stem cells (hPSCs) isolated from the inner cell mass of a human embryo at the blastocyst stage of development (Thomson et al., 1998; see also Figure 2A). While maintained in specific culture conditions, these cells can be expanded *in vitro* virtually indefinitely while maintaining their properties of pluripotency and self-renewal.

Since 2006 in the mouse (Takahashi and Yamanaka, 2006), and 2007 in the human (Takahashi et al., 2007; Yu et al., 2007), induced Pluripotent Stem Cells (iPSCs) can be generated from mature cell types of adult individuals such as skin fibroblasts, and display the same characteristics of self-renewal and pluripotency as embryonic stem cells. In these seminal publications, pluripotency was induced in mature somatic cells through retrovirus-mediated over-expression of genes coding for the transcription factors (TFs) OCT4, SOX2, KLF4, and cMYC (Figure 2B). During the following years, a better understanding of the underlying mechanisms of reprogramming enabled the design of alternative transcription factor cocktails, safer techniques for their delivery; and an increased variety of starting cell types have been reprogrammed (reviewed in Gonzalez et al., 2011).

Human PSCs can be expanded indefinitely *in vitro*, while maintaining their potential to give rise to any cell type of the human body, including the hematopoietic lineage. In addition, considerable progress has been made to successfully engineer these cells genetically (reviewed in Hendriks et al., 2016). With the theoretical possibility of differentiating hPSCs into HSCs, new avenues for HSC-based therapies can be envisioned. Patient-specific HSCs could be generated for autologous transplantation preventing GVHD, and genetically corrected patient-specific iPSCs could be used as a platform for gene therapy in congenital disorders (Hanna et al., 2007; Raya et al., 2009; Wang et al., 2012b). Moreover, importantly, each donation would represent an unlimited self-renewing source of cells, contributing for overcoming the current shortage in available HSCs for transplantation. Drug screening and disease modeling are also attractive applications of patient-specific HSC generation (Ye et al., 2014; Yung et al., 2013).

Therefore, significant effort has been made during the last decades towards the generation of HSCs from hPSCs. Differentiation of hPSCs directed towards the hematopoietic lineage has been largely successful, given that nearly all mature hematopoietic cell types have been generated from hPSCs [such as dendritic cells (Vodyanik and Slukvin, 2007), erythrocytes (Lapillonne et al., 2010; Ma et al., 2008; Yang et al., 2012), platelets (Nakamura et al., 2014); osteoclasts (Grigoriadis et al., 2010), other mature myeloid cells (Choi et al., 2011); T cells (Kennedy et al., 2012;

Timmermans et al., 2009), B-cells (Carpenter et al., 2011), NK cells (Woll et al., 2005)]. Notably, exciting progress has been made in the last years using human hPSCs for generating "off-the shelf" T and NK-cells expressing chimeric antigen receptors (CAR) for immunotherapy in cancer (Hermanson et al., 2016; Themeli et al., 2013).

Despite the successful differentiation of hPSCs into mature hematopoietic effector cells, generating HSCs capable of reconstituting a patient's bone marrow and immune system has not been achieved. This HSC key property is assessed experimentally through the obtained cells' ability of robustly repopulating an immunocompromised mouse upon transplantation. So far, these transplantation attempts into mouse models resulted in lower levels of chimerism when compared to primary HSCs (obtained from CB or bone marrow), showing lower self-renewal, and they generally produced myeloid-squewed progeny, a sign of compromised multipotency (Lu et al., 2009; Ran et al., 2013; Tian et al., 2006).

Efforts in optimizing the *in vitro* conditions for the generation of HSCs from hPSCs rely for the most part on understanding the events, during embryonic development, that elicit the generation of HSCs *in vivo*. The next section will therefore describe the current understanding of the *in vivo* roadmap from pluripotency to the HSC.

2. From fertilization to the HSC *in vivo*: the developmental model to mimic

After fertilization, the resulting zygote is totipotent, having the potential to give rise to every embryonic as well as supporting extra-embryonic tissues. Sequential cell divisions and the first differentiation event originate a structure called the blastocyst (see Figure 2A). The blastocyst consists of two parts: the trophoblast, later responsible for the formation of the chorion (fetal element of the placenta), and the inner cell mass (ICM) which will give rise to all cell types in the body as well as the yolk sac and the amniotic cavity (reviewed in Surani et al., 2007). This ability of the ICM to originate every cell in the organism is designated as pluripotency. Upon isolation and *in vitro* expansion, these pluripotent cells from the ICM can give rise to the aforementioned embryonic stem cell lines (Thomson et al., 1998). This section will describe the sequence of events occurring in early embryonic development, from the pluripotent stage to hematopoietic specification, which current hPSC differentiation protocols aim to mimic *in vitro* in order to generate de novo HSCs.

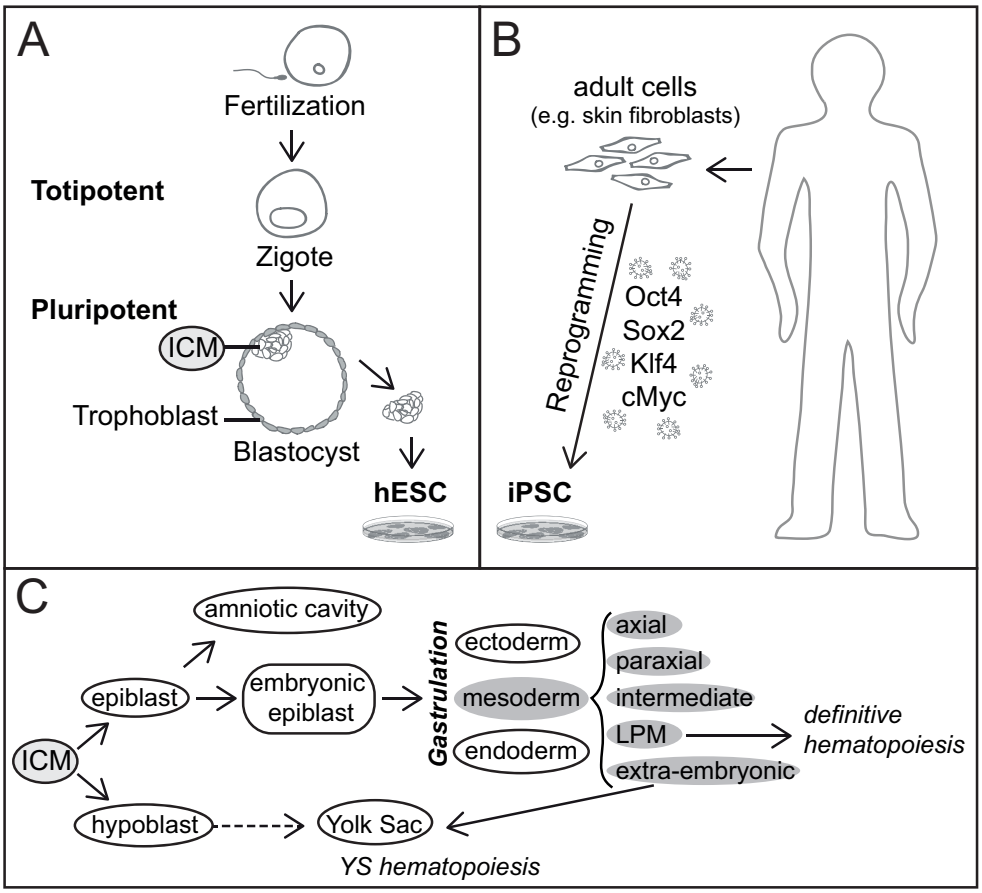

Figure 2: Pluripotent Stem Cells and early embryonic development

A. After fertilization, the resulting cell (the zigote) is totipotent, meaning it has the potential to give rise to the whole embryo as well as to all extra-embryonic structures required for its survival and development. Sequential cell divisions and differentiation generate the blastocyst. Its inner cell mass (ICM) is pluripotent, meaning it can give rise to all cells of the embryo proper. The ICM can be isolated and expanded *in vitro*, resulting in pluripotent human Embryonic Stem Cell lines (hESCs).

B. Induced Pluripotent Stem Cells are also pluripotent, and can be obtained from virtually any mature cell type in the body (such as adult skin fibroblasts). This is achieved through over-expression of a set of transcription factors that will reprogram the terminally differentiated mature cells into a pluripotent stem cell state. The resulting iPSCs can be maintained and expanded *in vitro* in the same conditions as hESCs.

C. Early lineage specification events in embryonic development, from the ICM to blood.

ICM: Inner Cell Mass; YS: Yolk Sac; LPM: Lateral Plate Mesoderm

a. Early Embryonic Development: the road to the first blood cells

During implantation, the cells of the ICM undergo a series of commitment steps forming the hypoblast (also called primitive endoderm), which later gives rise to the Yolk Sac (YS), and the epiblast which later commits into the amniotic cavity and the embryonic epiblast. The embryonic epiblast will originate the final organism (reviewed in Nichols and Smith, 2009). In the embryonic epiblast, the next cell commitment event is the formation of the three primordial germ layers in a process termed gastrulation (Figure 2C).

Gastrulation begins around day 15 of development in the human (E6 in the mouse): starting at a pit in the mid-posterior location of the epiblast disk, the tissue thickens along a line towards the posterior extremity forming the so-called primitive streak (PS). Epiblast cells start then migrating towards the PS and ingress through it forming new layers of tissue below the top epiblast layer. As the cells migrate, they respond to instructive cues in the new environment and in turn become active players shaping their new changing environment. Hence, cells that migrate earlier through the PS, those migrating later, and those not migrating at all through the PS, will be exposed to different signals and will therefore give rise to different germ layers. As gastrulation progresses, the first migrating cells will displace the hypoblast and form the definitive endoderm. The second wave of cells, colonizing the space between the epiblast and the definitive endoderm, forms the mesoderm. Finally, the cells remaining in the epiblast become the ectoderm. Each of these three germ layers resulting from gastrulation will give rise to a different set of tissues. For instance, ectoderm will form the nervous system and the outer layer of the skin, endoderm will give rise to tissues in the gut and lungs, and mesoderm will form muscles, heart, blood vessels and… blood (Schoenwolf and Larsen, 2009).

In the developing embryo, in order to commit to mesoderm, the migrating cells are exposed to signaling cues including BMP, Nodal-smad, and canonical Wnt (reviewed in Arnold and Robertson, 2009). The timing as well as the anterior/posterior site of ingression through the PS will in turn specify mesoderm sub-lineages. The first and most posterior mesoderm migrating cells commit to extra-embryonic mesoderm and will further migrate to extra-embryonic tissues, namely the YS and the chorion/placenta, where they will contribute to vessel formation and blood generation. Subsequently emerge the lateral plate mesoderm (LPM, precursor to the intra-embryonic vascular and hematopoietic systems), intermediate (precursor to the urogenital system), paraxial (precursor to the somites), and axial (precursor to the

notochord) mesoderm (reviewed in Tam and Loebel, 2007). Therefore, during development both vascular endothelium and hematopoietic cells derive from at least two mesoderm sub-lineages: extra-embryonic mesoderm and LPM.

Following gastrulation, neurulation starts taking place around day 18 of human development, where a portion of the ectoderm thickens and forms the neural plate, the precursor of the central nervous system (brain and spinal cord). The edges of the neural plate will form longitudinal neural edges, which by fusing dorsally will close the neural tube. During closure of the neural tube, at gestational day 22 in the human, a population of cells lining the closing gap undergo epithelial-to-mesenchymal transition and delaminate off the dorsal edge of the neural tube. This motile population of cells, designated as the neural crest (also commonly referred to as "the fourth germ layer"), migrate following specific paths throughout the organism and differentiate into a varied repertoire of tissues. The lineage fates of these migrating cells depend on the location they reach, and include the neurons and glia of the peripheral nervous system, skin melanocytes, endocrine cells of the adrenal medulla and thyroid, smooth muscle, craniofacial skeleton, and some tooth components (Schoenwolf and Larsen, 2009). Therefore, in addition to a migratory behavior, the neural crest has the stem cell properties of self-renewal and multipotency. Interestingly, recent lineage tracing studies have strongly indicated that while most of the cells in the HSC bone marrow niche are mesoderm derivatives, both its sympathetic neural components (Katayama et al., 2006) and a subset of mesenchymal stromal cells also derive from the trunk neural crest (Nagoshi et al., 2008). Such interplay between the hematopoietic system and neural crest derivatives also occurs in developmental settings, which will be further elaborated upon in the following sections.

b. Hematopoiesis throughout Development

Due to obvious ethical reasons, the access to human fetuses for studying the emergence of the first HSCs is limited. Numerous vertebrate animal models have been instrumental for shaping the current knowledge on hematopoietic development. These include the frog (*Xenopus laevis*), quail and chick, used in seminal observations, owing to easy access to the fetal tissues developing outside of the mother (Dieterlen-Lievre and Martin, 1981; Kau and Turpen, 1983). With the progress in cloning and the generation of transgenic mouse strains, the murine system emerged as an invaluable mammalian counterpart to human allowing for gene targeting approaches (reviewed in Schmitt et al., 2014). Zebrafish has also emerged as an advantageous vertebrate model system to

use for functional screens thanks to a fast breeding rate and the possibility for easy genetic and chemical perturbations of its development (reviewed in Robertson et al., 2016). Most findings of *in vivo* development described in this section therefore refer to studies made in the mouse and zebrafish.

During development, as the organism size and complexity progresses, needs for a circulation system providing oxygen and nutrient supply, tissue remodeling, waste disposal as well as immunity also evolve. Successive transient hematopoietic systems are therefore formed to support the growing fetus, before culminating in the formation of the first HSCs and the establishment of the final hematopoietic system that will sustain the organism after birth and throughout life (reviewed in Palis, 2016). These distinct functional, spatial and temporal hematopoietic events have been designated as "hematopoietic waves" and have been broadly classified into two categories: the primitive and the definitive waves (Figure 3).

Yolk Sac Primitive Hematopoiesis

The first wave of hematopoiesis generates progenitors producing a limited range of hematopoietic sub-lineages, including large nucleated erythroblasts expressing embryonic globins, and is therefore classified as primitive. It occurs in the extra-embryonic structures (namely in the Yolk Sac (YS) and the allantois) at embryonic day 7.25 (E7.25) in the mouse. These first hematopoietic cells have been shown to emerge from mesodermal intermediate structures, the blood-islands, consisting in clusters of cells capable of giving rise to both endothelium (for the formation of blood vessels) as well as blood, termed hemangioblasts (Haar and Ackerman, 1971). The product of this primitive wave contains limited progenitor potential and consists mainly in mature hematopoietic cells, primarily short-lived primitive erythroblasts, as well as some megakaryocytes and macrophages (Moore and Metcalf, 1970; Palis et al., 1999; Tober et al., 2007).

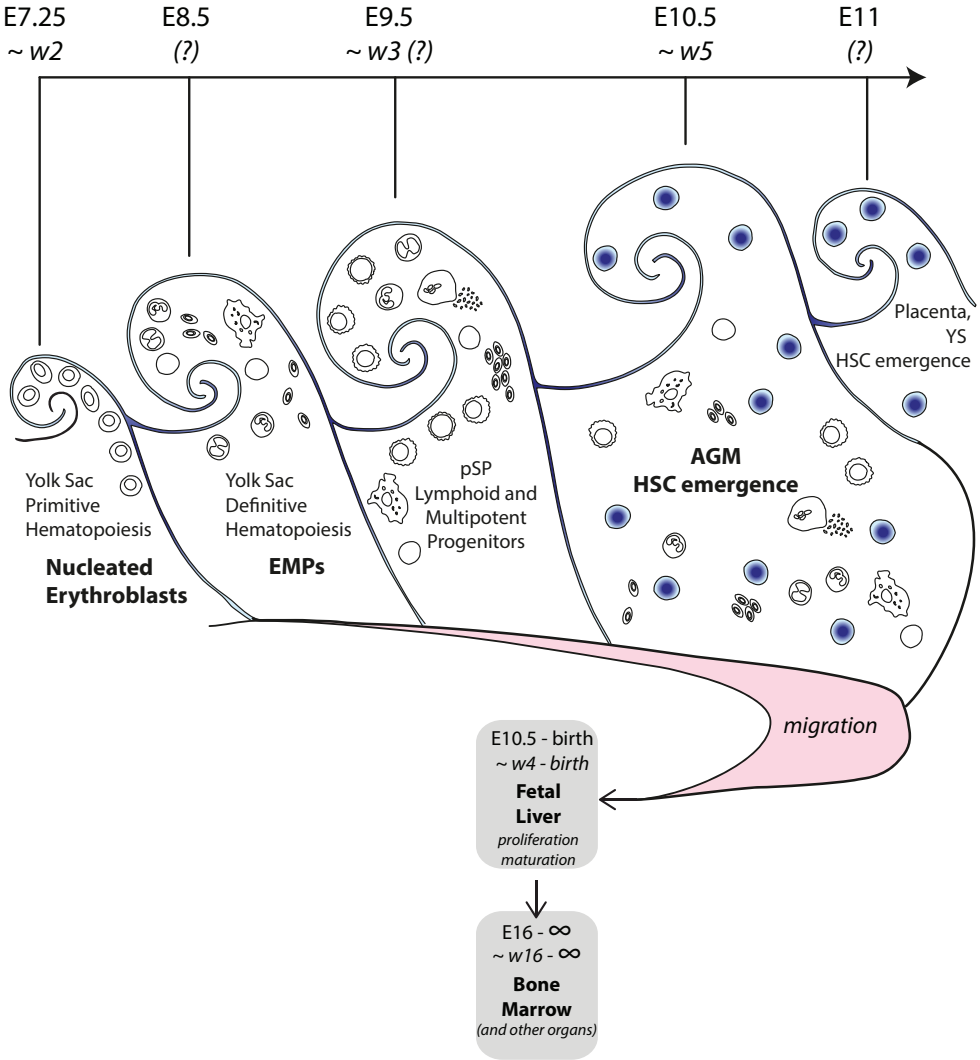

Figure 3: Hematopoietic waves during development

During development, several hematopoietic emergence events have been described, with different lineage potentials and different characteristics, to support survival and growth of the embryo at different stages of development (therefore with different requirements). In both mouse and human, these successive hematopoietic emergence events were shown occur first in the Yolk Sac (YS), and later in the embryo proper in the para-aortic splanchnopleure (pSP), developmental precursor of the aorta-gonad-mesonephros (AGM) region. The timeline at the top shows the time points in the mouse (in days *post-coitum*) and in the human (in italic font, in weeks *post coitum*). After emergence, the hematopoietic cells migrate to the Fetal Liver, the main hematopoietic organ during development, where they undergo further maturation and expansion until around birth. They start colonizing the bone marrow and other hematopoietic niches (such as the thymus and the spleen) during fetal development. The bone marrow will be the main hematopoietic organ after birth and throughout post-natal life.

Definitive erythro-myeloid progenitors emerge in the Yolk Sac

This primitive wave is then followed by a first definitive wave, at E8.5 also in the YS, with the production of erythro-myeloid progenitors (EMPs) with a more comprehensive differentiation potential compared to the primitive wave. Upon release into the circulation and migration to the fetal liver, these EMPs generate enucleated erythroid cells with adult characteristics (smaller in size and expressing mostly adult globins), macrophages [some of which will become tissue resident and persist through adulthood (Gomez Perdiguero et al., 2015)], granulocytes including neutrophils, basophils and eosinophils, and mast cells (McGrath et al., 2015).

Human Yolk Sac hematopoiesis

In the human, blood islands can be observed in the YS as early as day 15 (reviewed in Tavian and Peault, 2005), the YS being main site of hematopoiesis until week 6, when this role is taken over by the fetal liver (Palis and Yoder, 2001). Myeloid and erythroid progenitors can thus be detected in the YS until around week 6, when progenitor activity becomes restricted to the embryo proper (Huyhn et al., 1995; Migliaccio et al., 1986; Palis and Yoder, 2001). As in murine development, human early YS hematopoiesis results mostly in erythroid and myeloid cells. While between week 4 and 5 YS erythroid cells possess exclusively embryonic globins, from week 5 onwards the frequency of fetal globins starts to increase followed by that of adult globins (after 3 months), with embryonic globins becoming undetectable by week 12 (Palis and Yoder, 2001).

Multipotent progenitors emerge prior to HSCs

The fact that these early hematopoietic events give rise essentially to cells of the myeloid lineage led to the belief, for many years, that lymphoid potential would mark the emergence of *bona fide* HSCs. This concept has now been challenged with the detection in the mouse, by several independent groups, of lymphoid and lympho-myeloid multipotent progenitors devoid of long-term adult repopulating potential prior to HSC emergence. These progenitors were detected already from E9.5 in the YS and in the para-aortic Splanchnopleure (pSp, the developmental precursor of the aorta-gonad-mesonephros region (AGM) region; Boiers et al., 2013; Inlay et al., 2014; Kobayashi et al., 2014; Yoshimoto et al., 2011; Yoshimoto et al., 2012). It is however unclear whether these all belong to one same intermediate lympho-myeloid progenitor wave or if they arise independently from each other. Similarly, in the human embryo, hematopoietic progenitors with lympho-myeloid potential were also detected in the P-

Sp (not in the YS) before HSC emergence, around week 3 of gestation (Tavian et al., 2001).

Definitive Hematopoietic Stem Cell emergence

Finally, the first multilineage adult-repopulating HSCs were shown to emerge in the dorsal aorta in the AGM region at E10.5 in the mouse (Muller et al., 1994), around week 4 to 6 in the human (Ivanovs et al., 2011; Tavian et al., 1996). Shortly after emergence of the first HSCs in the AGM, other sites were shown to acquire de novo HSC generation potential, namely other major arteries, the placenta, the YS, and the head (de Bruijn et al., 2000; Gekas et al., 2005; Gordon-Keylock et al., 2013; Kumaravelu et al., 2002; Li et al., 2012; Robin et al., 2009). The notion that the head is a site of hematopoietic cell emergence from endothelium is however still under debate (Iizuka et al., 2016). After emergence, newly formed HSCs migrate through the circulation to the fetal liver (Kumaravelu et al., 2002; Zovein et al., 2008). The fetal liver is a major hematopoietic niche during ontogeny, providing the environment for expansion and maturation of progenitors and more importantly for the expansion of the HSC pool (Ema and Nakauchi, 2000). The HSCs will subsequently colonize the bone marrow, where they ultimately reside after birth, mostly in a quiescent state, during the lifetime of the individual (Cheng et al., 2000; Christensen et al., 2004).

The mechanism underlying the generation of the first HSCs in the AGM region has been given considerable attention, as insights in this process could provide the "recipe" to be applied in the *in vitro* protocols for HSC derivation from hPSCs.

c. Endothelial-to-Hematopoietic transition and the generation of HSCs in the AGM

After identification, in the murine system, of the AGM as containing the first adult-repopulating HSCs during development (Muller et al., 1994), the *ex-vivo* culture of AGM explants confirmed that this is a *de novo* HSC emergence site. However, this did not completely exclude that the AGM could be a site of maturation and expansion of precursors migrated from the YS, as others have suggested (Ghiaur et al., 2008; Lux et al., 2008). After emergence, HSCs subsequently colonize the fetal liver where they undergo further maturation and expansion (Medvinsky and Dzierzak, 1996). Further dissection and sub-culture of AGM explants allowed the identification, within this region, of the ventral side of the dorsal aorta (DA) as a major site of HSC generation, along with other major vessels of the developing fetus including the vitelline and

umbilical arteries (de Bruijn et al., 2000; Taoudi and Medvinsky, 2007). These findings demonstrated a close association between hematopoietic emergence and the developing vasculature. In chick models hematopoietic development had already been associated with the formation of intra-aortic clusters of hematopoietic cell in the ventral wall of the DA (Dieterlen-Lievre and Martin, 1981), and similar structures could also be observed in cultured murine AGM explants, which were shown to contain HSCs (Garcia-Porrero et al., 1995). The close physical association of emerging blood with the endothelial lining of blood vessels, as well as the expression by these hematopoietic cells of a number of endothelial genes, suggested an endothelial origin of hematopoietic cells (Garcia-Porrero et al., 1998). This notion was strengthened by lineage tracing and genetic studies in both chick (Jaffredo et al., 1998) and mouse (Chen et al., 2009; Li et al., 2012; Zovein et al., 2008).

Evidence for an endothelial origin of definitive hematopoiesis in the AGM culminated with live-cell imaging data from several laboratories and numerous models including AGM explants and zebrafish (Bertrand et al., 2010; Boisset et al., 2010; Kissa and Herbomel, 2010; Lam et al., 2010). Together, these studies demonstrated that in the ventral side of the dorsal aorta, a subset of endothelial cells undergo morphological changes, from an adherent, spindle-shaped cell, bending off the endothelial monolayer, and turning into a round cell which eventually divides, detaches and is released into circulation. These morphological changes are coupled with the acquisition of hematopoietic markers and were shown to be Runx1 and Cbfb dependent (Chen et al., 2011; Chen et al., 2009; Kissa and Herbomel, 2010; Lam et al., 2010; Liakhovitskaia et al., 2009). This process of emergence of the first HSCs from an endothelial precursor was coined endothelial-to-hematopoietic transition (EHT). Numerous signaling pathways have been implicated in the mechanism triggering an endothelial cell to undergo EHT (see Table 1 and next section). The notion that a given endothelial cell possesses the intrinsic capacity of undergoing EHT gave rise to the concept of hemogenic endothelium (HE) designating the endothelial cell subset displaying this unique capacity.

d. Molecular mechanisms regulating EHT and HSC generation

The emergence of the first HSCs occurs during a short developmental window at a specific anatomical location (the ventral side of the DA within the AGM region). It is therefore likely the result, at least in part, of a precise conjunction of a large set of

extrinsic signals (listed in Table 1) produced by the surrounding tissues during that specific time-point.

Blood Flow

Blood flow, starting at E8.25 in the mouse and around day 22 in the human prior to emergence of the first HSCs, has been shown to be a major trigger of EHT. Indeed, the Ncx1 knockout mouse model, with impaired cardiac development and absence of heartbeat and a reduced blood flow, presents impaired generation of hematopoietic cells in the embryo proper (Adamo et al., 2009; Lux et al., 2008), with similar effects shown in zebrafish (North et al., 2009; Wang et al., 2011). Further studies suggest that fluid sheer forces induce intracellular calcium release in the endothelial cells of the DA wall, activating the production of Prostaglandin E2 (PGE2), which in turn elicits HSC emergence through downstream cyclic Adenosine Monophosphate (cAMP) signaling (Diaz et al., 2015; Goessling et al., 2009). Previous studies had indicated intracellular nitric oxide synthesis as the signaling mediating Runx1 expression and hematopoietic emergence downstream to sheer stress (Adamo et al., 2009; North et al., 2009). These two separate models can be unified by the fact that cAMP signaling (downstream to PGE2) can activate, through the PKA/PI3K cascade, the intracellular synthesis of nitric oxide in the setting of angiogenesis (Namkoong et al., 2005). This leaves us with the following sequence: sheer stress -> calcium efflux -> PGE2 production -> cAMP synthesis -> PKA/PI3K cascade -> nitric oxide synthesis -> Runx1 expression -> EHT.

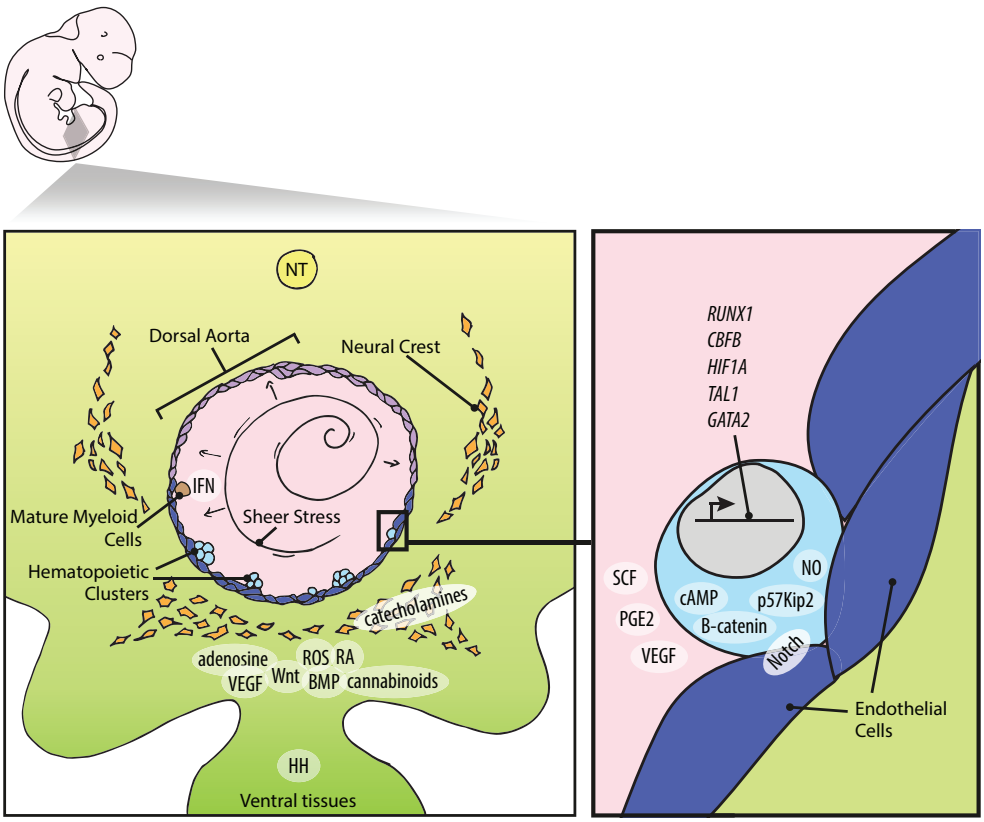

Figure 4: Hematopoietic Cell emergence in the AGM region

The first long-term adult repopulating HSCs emerge at E10.5 in the mouse, around week 5 in the human. A subset of endothelial cells, located mostly in the ventral side of the dorsal aorta undergo endothelial-to-hematopoietic transition (EHT), and acquire a hematopoietic phenotype forming hematopoietic clusters in on the wall of the aorta. This process of emergence was shown to be determined by a combination of several signals produced by the local environment (left panel) as well as cell-intrinsic regulators (right panel). Sheer stress from the circulation, Neural Crest cells in the process of migrating to form the dorsal region of the neural tube, and surrounding tissues were also suggested to secrete signaling factors contributing to hematopoietic emergence. NT: Notochord, IFN: Interferon, VEGF: vascular endothelial growth factor, ROS: Reactive Oxygen Species, RA: Retinoic Acid, BMP: Bone Morphogenic Protein, NO: Nitric Oxyde, cAMP: cyclic AMP

Catecholamines

The narrow developmental window of HSC emergence at day 10.5 also coincides with the migration of the neural crest-derived sympathoadrenal progenitors, from the trunk dorsal neural tube, through the AGM region and the vicinity of the DA (Nagoshi et al., 2008). Around this time-point, these cells accumulate laterally and below the DA, forming the developing sympathetic ganglia and the adrenal anlage, where they mature into tyrosine hydroxylase (TH)-expressing cells (therefore capable of producing catecholamines) (Nagoshi et al., 2008). This led to the hypothesis that this neural crest-derived progeny could also contribute to the regulation of HSC emergence in the AGM, which was confirmed in a Gata3 knockout mouse model (Fitch et al., 2012). The latter study reported that in the absence of Gata3 there is an impairment in the development of neural crest-derived TH-expressing cells in the vicinity of the DA coupled to a reduction in HSC emergence. Given that in this model hematopoietic development could be rescued by providing agonists of adrenergic receptors, it strongly indicated that adrenergic signaling, mediated by catecholamines produced by migrating neural crest progeny, played a role in stimulating HSC emergence (Fitch et al., 2012).

The ventral wall of the DA

Within the DA, the restricted ventral localization of *de novo* HSC emergence has been attributed to two major factors: the presence of signaling cues secreted by tissues on the ventral side of the DA, and the specific intrinsic characteristics of the ventral endothelium related to the developmental dynamics of the DA wall. In the murine system, ventral tissues were indeed shown to promote hematopoietic emergence, through secretion of Hedgehog (HH) from the endoderm-derived gut (Peeters et al., 2009). In addition, studies in the avian embryo propose an explanation for the narrow developmental window of hematopoietic emergence in the DA floor, by showing that shortly after hematopoietic emergence there is a dynamic remodeling of the endothelial wall, with the replacement of ventral DA hemogenic endothelium (of splanchnopleural origin) by non-hemogenic somite-derived endothelial cells (Pouget et al., 2006).

Arterial specification

In Table 1, a subset of extrinsic and intrinsic regulators of HSC emergence share the property of also playing crucial roles in endothelial and arterial specification, particularly VEGF and Notch signaling, as well as the genes *HEY2* and *SCL/ TAL1* (see references in Table 1). This corroborates the close developmental relation between endothelium and blood, and suggests the endothelial arterial molecular state as being

permissive to the onset of a hematopoietic program. The recent studies reporting direct TF-mediated reprogramming of endothelial cells into engrafting HSCs further support this notion (Sandler et al., 2014; Sugimura et al., 2016).

Inflammatory signaling

Interestingly, recent studies have suggested that the earlier waves of hematopoiesis have an impact on the subsequent hematopoietic emergence events. Notably, these reports have demonstrated that inflammatory cytokines produced by the YS hematopoiesis myeloid progeny regulate hematopoietic cell emergence in the AGM through interferon and tumor necrosis factor signaling (Espin-Palazon et al., 2014; Li et al., 2014; Sawamiphak et al., 2014).

Table 1: Regulators implicated in HSC emergence

Regulators implicated in HSC emergence	References	Model organism
Inflammatory signaling	Sawamiphak et al., 2014 IFNg Espin-Palazon et al., 2014 TNFa	zebrafish
	Li et al., 2014 IFN	mouse
Notch	Guiu et al., 2014 Hadland et al., 2015 Jang et al., 2015	mouse
	Monteiro et al., 2016 Burns et al., 2005 Burns et al., 2009 Rowlinson and Gering, 2010 Kim et al., 2013	zebrafish
Blood Flow / shear stress	Adamo et al., 2009 (through NO) Diaz et al., 2015 (through PEG2 -> cAMP -> PKA)	mouse
	North et al., 2009 (through NO) Wang et al., 2011	zebrafish
PGE2	Diaz et al., 2015	mouse
	North et al., 2007 Goessling et al., 2009 Esain2015	zebrafish
Wnt needs to be up for HE specification, down for emergence	Chanda et al., 2013 Ruiz-Herguido et al., 2012 Corrigan 2009	mouse
Adenosine	Jing et al., 2015	zebrafish
mTorC2	Zhou et al., 2016	mouse
RA	Chanda et al., 2013 Goldie et al., 2008	mouse
Catecholamines	Fitch et al., 2012	mouse
Glucose metabolism	Harris et al., 2012 (through ROS -> HIF1a -> Runx1)	zebrafish
TGFb	Monteiro et al., 2016 (downstream from VEGF, upstream from Notch)	zebrafish
SCF	Rybtsov et al., 2014	mouse

Table 1 cont.: Regulators implicated in HSC emergence

Regulators implicated in HSC emergence	References	Model organism
HH	Gering and Patient, 2005; Kim et al., 2013; Rowlinson and Gering, 2010; Souilhol et al., 2016 (SHH produced in dorsal region) Wilkinson et al., 2009	zebrafish
	Peeters et al., 2009 (produced in ventral tissues) Souilhol et al., 2016 (SHH produced in dorsal region)	mouse
VEGF	Rowlinson and Gering, 2010 Burns et al., 2009	zebrafish
FGF	Lee et al., 2014	zebrafish
BMP4	Wilkinson et al., 2009	zebrafish
	Crisan et al., 2015 Souilhol et al., 2016 (inhibitory)	mouse
GPR56	Solaimani Kartalaei et al., 2015	zebrafish
Cannabinoids	Esain et al., 2015 (mostly act in AGM HSC proliferation through PGE2)	zebrafish
Runx1	Yokomizo et al., 2001 Chen et al., 2009	mouse
	Bresciani et al., 2014 Burns et al., 2009 Kissa and Herbomel, 2010 Lam et al., 2010	zebrafish
Cbf-beta	Chen et al., 2011	mouse
Hey2	Rowlinson and Gering, 2010	zebrafish
SCL/Tal1	Rowlinson and Gering, 2010 Kim et al., 2013	zebrafish
	Shivdasani et al., 1995	mouse
SOX17	Nobuhisa et al., 2014 (for maintenance of the emerged HSCs)	mouse
Gata2	Ling et al., 2004 Gao et al., 2013 de Pater et al., 2013	mouse
HIF1a	Harris et al., 2012	zebrafish
	Imanirad et al., 2014	mouse
Tbx16	Burns et al., 2009	zebrafish
Hdac1	Burns et al., 2009	zebrafish
P57Kip2/cdkn1c	Mascarenhas et al., 2009	mouse
IGF2	Mascarenhas et al., 2009	mouse

Table 1 cont.: Regulators implicated in HSC emergence

Regulators implicated in HSC emergence	References	Model organism
Cdca7 downstream from Notch	Guiu et al., 2014	mouse
Foxc2 downstream from Notch	Jang et al., 2015	mouse
Evi1	Konantz et al., 2016	zebrafish
Rac1	Ghiaur et al., 2008 (they don't look at engraftment)	mouse

3. From hPSCs to the HSC *in vitro*: current progress

The existing knowledge of *in vivo* development of HSCs, described in the previous section, indicates that the directed differentiation of hPSCs to an HSC fate requires their development into mesoderm, followed by commitment to hemato-endothelial lineage, and finally hematopoietic specification and maturation (Figure 5A). As discussed earlier, a large range of mature hematopoietic effector cell types have been successfully derived from hPSCs, presumably through these developmental stages. In this section I will address the approaches that have successfully achieved mesoderm and hemato-endothelial commitment of hPSCs, followed by a discussion of the studies reporting the generation of engrafting hematopoietic progenitors (and whether these are *bona fide* HSCs), and finally I will describe the surrogate readouts currently used for assessing hPSC-derived definitive hematopoiesis (Figure 5B).

a. Making blood – the generic protocols

The first papers on hematopoietic induction from of hESCs published in the early 2000s were to a great extent based on the work made in the mouse system, which had been developing since 1985 (Chadwick et al., 2003; Doetschman et al., 1985; Kaufman et al., 2001). It was indeed with murine embryonic stem cells that were established the two main differentiation techniques most commonly used nowadays, both in mouse and human settings: mouse bone marrow stromal cell co-culture and embryoid body (EB) formation (Figure 5C).

Stromal cell co-culture

The importance of the niche environment for adult hematopoiesis suggests that, in a developmental approach of HSC generation, a stromal niche would also be necessary. Indeed, co-culturing ESCs with mitotically inactive mouse bone marrow stromal cells was proven to induce hematopoietic fate through both membrane-bound as well as secreted factors produced by the stroma. This was shown with several stromal lines, including OP9 (Cho et al., 1999; Nakano et al., 1994; Nakayama et al., 2000), S17 (Kaufman et al., 2001) and MS5 (Berthier et al., 1997). However, the identity of the factors in question and the precise mechanisms behind these effects of stromal cells are not yet fully elucidated. Also, one can question whether a clonal culture, containing stromal cells exclusively, accurately simulates an *in vivo* niche that contains, as discussed above (Figure 1), a large variety of cell types in addition to the stromal mesenchymal

fraction. It is also interesting to note that a co-culture consisting of stroma derived from an adult environment can stimulate emergence of blood in a developmental context – some groups have addressed this by testing cells isolated from embryonic and fetal tissues with some improvements in terms of blood output (Ledran et al., 2008). In a clinical translational perspective, a major drawback of stromal co-culture is the fact that these stromal cell lines are derived from animal sources (typically mouse). Also, even if one could use immortalized human stromal lines, the risk of contaminating the hPSC-derived material to be introduced in a patient with these immortalized cells susceptible of tumorigenesis undermines this strategy. Although stromal cell co-culture can be useful in the context of research and discovery, this approach is incompatible with the long-term goal of generating hPSC-derivatives for the clinic.

Embryoid body EB generation

EBs are embryo-like three-dimensional structures obtained by culturing pluripotent stem cell aggregates in suspension (Chadwick et al., 2003; Doetschman et al., 1985; McClanahan et al., 1993). Just as in early embryonic development, this technique relies on cell-cell contact and paracrine signaling within each cell aggregate for proliferation and spontaneous generation of cells belonging to all three primordial germ layers. This approach may better simulate *in vivo* development where blood emerges in the context of other developing tissues, with other hPSC-derived tissues spontaneously providing a niche for hematopoietic development. However, this approach relies on the stochastic nature of EB development, introducing a factor of variability in the obtained results. The combination of EB culture and subsequent plating in adherent cultures – on gelatin (Saeki et al., 2009), or matrigel with OP9 cells (Woods et al., 2011) – was shown to further increase the efficiency of blood generation.

Exogenous addition of complementary factors

Together with the use of stromal co-culture, EB generation or a combination of both, additional factors introduced into the differentiation media are instrumental for promoting blood development and/or for *ex-vivo* hematopoietic cell maintenance. Hence, BMP4 was shown to be critical for inducing mesoderm specification and blood production from pluripotent stem cells (Chadwick et al., 2003; Johansson and Wiles, 1995), acting in synergy with VEGF (Nakayama et al., 2000; Pick et al., 2007; Woods et al., 2011). Fgf2 was also identified as promoting hematopoietic induction (Berthier et al., 1997; Pick et al., 2007). Hematopoietic cytokines were also shown to improve the hematopoietic output in PSC differentiation, namely interleukins (IL) 1, IL3, IL6, IL7,

IL11, Kit-ligand/SCF, G-CSF, erythropoietin, Flt3-ligand, insulin-like growth factor (IGF)1 and thrombopoietin (Biesecker and Emerson, 1993; Bigas et al., 1995; Chicha et al., 2011; Keller et al., 1993; Nisitani et al., 1994; Palacios et al., 1995; Pick et al., 2007; Srivastava et al., 2007; Wang et al., 2012a; Woods et al., 2011); although it is likely that this positive effect has more to do with expansion of obtained progenitors than with improvements in terms of hematopoietic developmental specification.

Consistent with the pathways identified *in vivo* (see Table 1), addition of TGFβ1 and PGE2 was also shown to promote hESC-derived hematopoiesis (Woods et al., 2011). Conversely, Wang et al. (2012) showed that the inhibition of TGFβ signaling with the chemical inhibitor SB431542 promoted the endothelial to hematopoietic transition (Wang et al., 2012a). At this EHT stage, retinoic acid (Yu et al., 2010) and Notch signaling (Yu et al., 2008), were also indicated as being inducers of blood emergence.

In the past years, it has become increasingly recognized that, just as during development, there should be progression in terms of environment and regulation for *in vitro* blood generation. These protocols need to be further optimized by having a step-wise approach when it comes to addition of growth factors and other signaling molecules. Indeed, factors that promote mesoderm development may be detrimental for EHT, or may compromise the functionality of already formed hematopoietic cells.

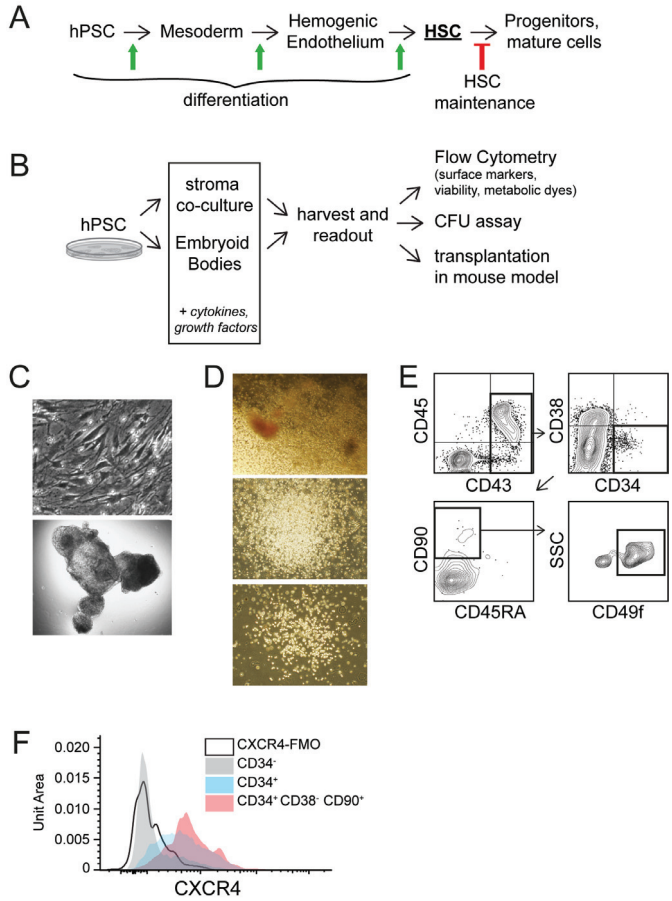

Figure 5: hPSC differentiation to HSCs

A. Main steps in the differentiation of hPSCs towards the HSC. The *in vitro* conditions for indefinite HSC maintenance have not yet been fully characterized. Therefore, in addition to HSC developmental specification, preventing obtained HSCs from differentiating into downstream mature progenitors is an additional challenge.

B. Main strategies for hematopoietic differentiation of hPSCs, and for evaluation of the obtained hematopoietic cells

C. Top: representative picture of stromal cell culture (OP9 cells); bottom: embryoid body after 8 days of cell suspension culture

D. Representative pictures of hematopoietic colonies obtained in CFU assays. Top: GEMM (stands for "granulocyte, erythrocyte, macrophage, megakaryocyte", contains multiple erythro-myeloid lineages); middle: granulocytic colony; bottom: macrophage colony

E. Representative FACS plots for identification of cells with an HSC immunophenotype after 16 days of differentiation

F. Surface CXCR4 expression in hPSC-derived hematopoietic cells. Note that its expression is specific to CD34+ cells, and highest in cells with an HSC immunophenotype.

b. The quest for an hPSC-derived definitive HSC

The current gold-standard readout for assessing the generation of an HSC is its capacity to provide sustainable long-term multilineage engraftment when transplanted in an immunocompromised mouse model. Achieving this goal has been challenging both in murine and in human systems.

The first promising results were achieved with murine ESCs, where overexpression of HoxB4 followed by OP9 co-culture conferred multilineage engraftment to mouse ESC-derived hematopoietic cells in primary and secondary lethally irradiated mice, giving rise to adult beta-globin-expressing erythroid cells (Kyba et al., 2002). Although promising, the reconstitution levels were low (under 50% in primary lethally irradiated recipients) and despite the use of a reversible inducible system, when stopping administration of doxycyclin after transplantation there was no assessment of residual *HoxB4* transgene expression, making it questionable to rule out transgene-dependency of the transplanted cells. Indeed, these promising results were subsequently reproduced in additional independent studies using constitutive *HoxB4* overexpression (Bonde et al., 2008; Chan et al., 2008; Matsumoto et al., 2009; Ohno et al., 2010; Pilat et al., 2005), however no engraftment capacity was reported when *HoxB4* overexpression was effectively transient (Jackson et al., 2012; Tashiro et al., 2012). When tested the human system, *HoxB4* over-expression did not lead to engraftment potential of hPSC-derived cells, nor to adult globin expression in their erythroid progeny, although it seems to stimulate generic hematopoietic development and colony-forming capacity of the resulting cells (Jackson et al., 2016; Lee et al., 2008; Unger et al., 2008; Wang et al., 2005). In the human, engraftment of hPSC-derived hematopoietic cells was achieved with over-expression of *HOXA9*, *ERG*, *RORA*, *SOX4* and *MYB*, reproducibly inducing myelo-erythroid short-term engraftment in immunocompromised mice (Doulatov et al., 2013).

Another strategy for achieving engraftment of the obtained cells was trying to mimic the niche of HSC emergence, by co-culturing the hPSCs with cell lines obtained from sources involved in the ontogeny of blood development. In this context, the use of mouse AGM-derived stromal cells promoted a slight increase in engraftment of hESC-derived blood cells in immunocompromised mice (Ledran et al., 2008). In this study, the transcriptional analysis of the used cell line enabled the identification of positive enhancers of hematopoiesis from hESC, namely TGFβ1 and 3, however these engraftment levels have not been robustly reproduced.

Recently, promising results were also achieved using teratoma formation. In this setting, hPSCs are transplanted subcutaneously (Amabile et al., 2013) or into testes (Suzuki et al., 2013) of immunocompromised mice, and are allowed to grow into a teratoma containing mature cells from all three primordial germ layers. Within this mass of developing tissues, putative hematopoietic stem and progenitor cells (HSPCs) arose and generated progeny, which was detected in the mouse's peripheral blood and hematopoietic organs. Teratoma dissociation and transplantation into secondary recipient mice led to human reconstitution. Given that Suzuki et al. show reconstitution of primary mice at 12 weeks post-transplantation, while Amabile et al. report reconstitution of primary and secondary recipients, this has been considered as long-term reconstitution. Interestingly, Suzuki et al. obtain lymphoid reconstitution, contrarily to the myeloid skewing usually observed in hPSC-derived grafts. However, the obtained chimerism levels are generally low and none of these studies report robust reproducibility of the system. Also, although these results show the possibility of generating engrafting hematopoietic material from hPSCs without requiring transgene over-expression, teratoma formation is still a very undefined system and unlikely to be readily translated into clinical settings.

c. Assessing hematopoietic potential – intermediate readouts

In the absence of engraftment capacity, several *in vitro* assays are used to evaluate the produced hematopoietic cells (Figure 5B). The first line of characterization is the analysis of the cells' surface immunophenotype. For this, at the end of the differentiation protocol the cell cultures are dissociated into single-cell suspension, analyzed by flow-cytometry and assessed for the expression of a set of cell surface markers (Figure 5E). Typically, this starts with the analysis of the pan-hematopoietic markers CD43 and CD45. These are expressed in all postnatal hematopoietic cells (except for mature erythrocytes), including HSCs. During human development, CD43 has been shown to mark the first hematopoietic cells, prior to CD45 (Vodyanik et al., 2006), however this finding was made using in vitro hPSC differentiation, while the analysis of human embryos shows expression of CD45 in all early emerged hematopoietic cells (Ivanovs et al., 2014; Tavian and Peault, 2005). Within the general $CD43^{+}/CD45^{+}$ hematopoietic fraction, CD34 is co-expressed on progenitor cells with proliferation capacity and some degree of multilineage differentiation potential. When singularized and plated in methylcellulose (a semisolid medium that restricts cellular migration), these cells are therefore capable of generating hematopoietic colonies that can be scored by

morphology (Figure 5D). This functional assay for progenitor potential, the colony forming unit (CFU) assay, has however a readout generally restricted to erythro-myeloid lineages.

Long-term engrafting primary HSCs from CB and bone marrow, in addition to being $CD43^{+}CD45^{+}CD34^{+}$, display the following marker combination: $CD38^{-}$ $CD45RA^{-}$ $CD90^{+}/CD49f^{+}$, although in these primary sources this immunophenotype only enriches to approximately 1 in 10 cells being a long-term HSC (Notta et al., 2011). Interestingly, although hPSC-derived hematopoietic cells do not engraft, in our *in vitro* differentiation protocol we do obtain cells possessing this marker combination (Figure 5E).

Long-term engraftment of HSCs and repopulation of the host require the ability of the injected cells to migrate throughout the circulatory system of the host and home to their niche in the bone marrow. This homing ability is in great extent determined by the expression of the receptor CXCR4 on the cell surface of HSPCs. This receptor binds the chemokine stromal-derived factor (SDF)-1, allowing the HSCs to respond to this chemokine and migrate towards its increasing gradient. Hence, the production of SDF-1 by the bone marrow cells guides the HSCs towards the niche and regulates HSC homing and egress from the bone marrow (Kim and Broxmeyer, 1998; Nagasawa et al., 1996; Peled et al., 1999). We and others show the expression of CXCR4 by *in vitro*-produced hematopoietic cells, including the HSC-like fraction (Figure 5F; Tian et al., 2006). Human PSC-derived hematopoietic cells have in fact been reported to be able to home to the bone marrow, having then impaired ability to proliferate and sustain durable engraftment (Ng et al., 2016). In order to rule out homing issues as the cause for lack of engraftment, hPSC-derived cells were also directly introduced into the bone marrow of immunocompromised mice, using intra-femoral injection, in order to assess whether once incorporated directly in the niche they could self-renew and contribute the host's hematopoiesis. In these studies, although transplanted cells survived, they displayed severe proliferation defects compared to their primary counterparts (Wang et al., 2005). Although hematopoietic-specific impairments cannot be ruled out as a hurdle for hPSC-derived HSC generation, a major issue regarding *in vitro* derived hematopoietic cells is their generally lower survival rates and reduced proliferation ability compared to their primary counterparts (from cord blood or bone marrow).

In developmental settings, the key HSC characteristics of self-renewal and multipotency are associated with the last definitive wave of hematopoiesis occurring in the major vessels of the embryo at 10.5 in the mouse, around week 5 in the human. It has therefore been hypothesized that the lack of engrafting cells in hPSC-derived

hematopoietic cultures is due to the fact that current differentiation protocols generate developmentally early hematopoiesis, such as primitive blood or YS definitive EMPs with limited self-renewal and erythro-myeloid restricted hematopoiesis. The fact that the few engraftment data available reports short-term and myeloid-skewed chimerism supports this possibility (reviewed in Slukvin, 2013). In order to assess definitive hematopoiesis, some authors have therefore used lymphoid potential as a definitive hematopoiesis readout. For this, *in vitro* derived CD43/45^{+} CD34^{+} hematopoietic progenitors are co-cultured with Notch ligand-expressing OP9 cell lines, shown to induce lymphoid differentiation, and T-cell commitment was assessed by flow cytometry and functional assays. The type of lymphoid lineages produced and their further functional assessment is often requested as an additional indication that they are equivalent to their *in vivo* counterparts. Such functional assessments include VDJ chain recombination, cytotoxic activity, activation in response to cytokines (Kennedy et al., 2012; Timmermans et al., 2009; Woll et al., 2005).

Another readout of hematopoietic ontogeny is the globin gene expression by the erythroid progeny of the obtained hematopoietic progenitors. During development, different oxygen requirements as well as different levels of oxygen available resulted during evolution in the expression of hemoglobin components harboring different oxygen binding kinetics. Hence, the first erythroid cells produced during the primitive YS hematopoietic wave express mostly embryonic globins, while erythroid cells produced throughout adulthood express adult-type globins. In the human setting and in some non-human primates, an additional intermediate fetal set of globins has been described (reviewed in Sankaran et al., 2010). In contrast to the mouse, where only one globin switch has been described (primitive to definitive erythropoiesis both occurring in the YS), in the human two switches have been shown to occur during development: from embryonic (ε-globin, coded by HBE1) to fetal (γ-globins, coded by HBG1 and HBG2), and from fetal to adult (β-globin, coded by HBB) (reviewed in Sankaran et al., 2010). As these switches occur progressively between week 5 of development and 6 months after birth, at a given developmental time-point more than one set of globins can co-exist [in fact most adults even retain residual expression of fetal globin along with the adult form, in average below 2%, with relative values varying among individuals (Thein et al., 2009)]. When evaluating the ontogeny of *in vitro* produced cells, the ratios of transcript levels such as fetal/embryonic, adult/fetal and adult/embryonic globins expressed by the erythroid progeny are therefore used to determine the developmental stage of the resulting hematopoiesis. Although adult β-globin is occasionally detected in hPSC-derived cells, embryonic and fetal globins are

usually largely prevalent, even compared to CB-derived cells (Kennedy et al., 2012; Ng et al., 2016; Woods et al., 2011). This strongly indicates that *in vitro* hPSC differentiation protocols mostly mimic early stages of hematopoietic development.

Aims of the Thesis

The ultimate objective of the work presented in this thesis is the successful generation of functional hematopoietic stem cells from human pluripotent stem cell sources. Our contribution to the field can be divided in three aims:

- Investigating the gene expression dynamics of *in vitro* human endothelial-to-hematopoietic transition (**Paper I**)
- Assessing the role of adrenergic signaling in human hematopoietic development by looking at the effect of Norepinephrine (**Paper II**) and of one of its secondary messengers, cyclic AMP (**Paper III**) in our differentiation cultures
- Determining the levels of reactive oxygen species during *in vitro* human hematopoietic development and their detrimental effects for the functionality of produced hematopoietic cells (**Paper IV**)

Summary of Results

Paper I

Single-Cell Analysis Identifies Distinct Stages of Human Endothelial-to-Hematopoietic Transition

A novel strategy for molecular dissection of EHT

Live-cell imaging studies including our own have shown that, consistent to what has been reported *in vivo*, endothelial to hematopoietic transition (EHT) is a rare event, challenging to detect in hPSC differentiation cultures. In addition, the lack of specific markers to precisely distinguish hemogenic endothelium from the remaining endothelium, and for isolating the first emerged hematopoietic cells from downstream blood, have hampered a detailed molecular analysis of EHT. In this study we took advantage of single-cell transcriptional analysis to molecularly characterize cells related to EHT in our hPSC-differentiation cultures. We analyzed the broad and heterogeneous $CD34^+$ fraction, that includes $CD34^+CD43^-$ and $CD34^+CD43^+$ cells (enriched for endothelial and hematopoietic cells respectively) using single-cell qRT-PCR for genes involved in endothelium, hematopoiesis, and for genes previously shown to be involved in EHT. Overall, this allowed us to capture a continuum of transcriptional states, from an endothelial signature to a hematopoietic signature as well as putative states in between.

The single $CD34^+$ cells were index-sorted, so that in addition to gene expression analysis, we also obtained the surface immunophenotype of each cell. The cells were analyzed for CD34, CD43, CD90, CD73 and CXCR4, markers relevant for the previously characterized populations HSC-like ($CD43^+CD34^+CD90^+$), hemogenic endothelium ($CD43^-CD34^{hi}CD90^{hi}CD73^-CXCR4^-$), arterial endothelium ($CD43^-CD34^{hi}CD90^{hi}CD73^+CXCR4^+$) and venous endothelium ($CD43^-CD34^{hi}CD90^{hi}CD73^+CXCR4^-$) (Ditadi et al., 2015; Ronn et al., 2015). In addition to these markers, the use of a cell line transduced with an exogenous reporter system expressing GFP under the control of the *WAS* gene regulatory sequences (Munoz et al.,

2012) provided an additional marker (WAS-GFP) for tracking the onset of a hematopoietic program within the analyzed cells.

Molecular identification of a sortable population of cells undergoing EHT

By combining the transcriptional signatures identified and the surface immunophenotype, we could identify several relevant transcriptional states that could be enriched for using surface markers. In particular, we identified a transcriptional cluster of cells located at the interface between the endothelial and the hematopoietic signatures which along with a clear endothelial signature also showed up-regulation, compared to the remaining endothelial groups, of key hematopoietic genes. An additional sort enriching for this transcriptional cluster increased the number of cells identified as undergoing EHT, along with the complexity of this EHT cluster which could then be further sub-divided in three sub-groups. Notably we observed increased expression, throughout these three sub-groups, of a set of hematopoietic genes. This was also correlated with an increase in GFP fluorescence along these EHT cluster sub-groups.

Challenging the current model of hematopoietic development?

In order to assess the lineage potential of this EHT cluster, we sorted cells enriching for this population ($CD34^{+}CD43^{int}CD90^{hi}CD73^{-}$), which we then separated based on GFP expression, and assessed their progeny following 5 days of sub-culture. Interestingly, we observed that while the EHT-GFP^{-} cells gave rise to a broad repertoire of mature hematopoietic cells, the EHT-GFP^{+} gave rise almost exclusively to $CD45^{+}$ cells and no GlycophorinA-expressing progeny. Both these populations consisted in cells that were in undergoing EHT, up-regulating key hematopoietic genes (notably Runx1, a well-known driver if EHT *in vivo*) while still in the process of down-regulating their endothelial program. This suggested to us that during EHT, emerging hematopoietic cells already start restricting their hematopoietic mature sub-lineage potential prior the complete down-regulation of their endothelial program.

Paper II

Hematopoietic Cell Emergence during Human Development is Modulated by Norepinephrine Signaling Independent of β2-Adrenergic Receptor

A fortuitous finding

This project started with the purpose of deriving primary human stromal lines, from the AGM region of human embryos, to be used for co-culturing hPSC in our protocol of *in vitro* blood generation. The rationale was that primary tissues surrounding the dorsal aorta, in the AGM region, would produce signaling molecules that would elicit hematopoietic emergence from hPSCs. We could access two embryos of 42 and 58 days of gestation (shortly after blood emergence), from which we dissected the urogenital ridges as well as the dorsal aorta proper, and upon gentle dissociation the cells were plated in adherent cultures for morphological evaluation and expansion. Interestingly, while most resulting cultures displayed mesenchymal and endothelial morphologies, two of the wells developed structures reminiscent of neurospheres. Immunohistochemistry confirmed that these structures contained cells of the neural lineage, expressing MAP2 (mature neuronal marker) and GFAP (glial marker). The time-point and anatomical location indicated this neural potential to originate from neural crest progenitors, previously shown to migrate in the proximity of the dorsal aorta forming the sympathetic ganglia and the adrenal anlage (Nagoshi et al., 2008; see also Figure 4). This led us to formulate the hypothesis that signals from these structures of the developing peripheral nervous system would secrete factors, thereby also playing a role in AGM hematopoietic emergence.

Norepinephrine addition to hPSC-derived cultures

In order to test this hypothesis, we decided to exogenously add the neurotransmitter most commonly secreted by the peripheral nervous system, Norepinephrine (NE), to our hPSC differentiation cultures and assess whether hematopoietic emergence was affected. We observed a trend towards increased frequencies of hematopoietic cells, strongest for the most immature immunophenotype HSC-like. Functionally, we also noted a higher CFU content in the cultures differentiated in the presence of NE. Together these results suggested to us that hematopoietic emergence can indeed be promoted by NE signaling. At the time, this was confirmed by a publication in the murine setting reporting that, in a mouse model

with impaired neural crest development, HSC generation in the AGM is compromised, and that this phenotype could be reversed with addition of adrenergic agonists (Fitch et al., 2012). The detection of β2-adrenergic receptor (ADRB2) in nascent hematopoietic cells led these authors to suggest it as the mediator of NE signaling, however this was not tested pharmacologically. We then proceeded to the identification of the mechanism behind NE signaling in hPSC-derived blood emergence.

Identifying the adrenergic receptors at play

NE binds and activates the adrenergic receptors, belonging to the G-protein coupled receptor family. There are 9 adrenergic receptors in total, distributed in 3 subtypes: ADRA1 (ADRA1a, ADRA1c, ADRA1d) activate Phospholipase C and intracellular calcium release, ADRB (ADRB1, ADRB2, ADRB3) activate Adenylyl Cyclase and production of intracellular cAMP, while ADRA2 (ADRA2a, ADRA2b, ADRA2c) have an overall inhibitory effect on these signaling pathways. As ADRB2 had been suggested in the above-mentioned publication as the main receptor mediating adrenergic signaling, we started by testing this hypothesis. However, addition of the specific ADRA2 antagonist (ICI118,551) to our hPSC-derived cultures did not have any negative impact on the NE effect, suggesting that this is not the main adrenergic receptor at play. Gene expression analysis and modulation of general ADRB and ADRA2 receptors led to a model where ADRB3 receptor mediates hematopoietic emergence, where a negative feed-back provided by the simultaneous activation of the ADRA2c receptor (and its downstream inhibitory signaling) is also required. Activation of ADRA2a on the emerging HSC-like cells seemed to be required to prevent their downstream differentiation into mature blood cells, thus also contributing for an increase in the frequency of HSC-like cells. These results provided the framework for the next paper, studying cAMP signaling, secondary messenger downstream of ADRB receptors.

Paper III

Cyclic AMP Signaling through Epac Axis Modulates Human Hemogenic Endothelium and Enhances Hematopoietic Cell Generation

The active role of ADRB receptors mediating NE signaling suggest that their downstream secondary messenger, cAMP, would mediate hematopoietic emergence *in vitro*. Cyclic AMP is a secondary messenger for a large range of other G- protein coupled receptors, including those relevant for murine HSC homeostasis and development, such as Adenosine (Jing et al., 2015), PGE2 receptor (Diaz et al., 2015) [also tested in clinical trials to enhance engraftment of CB HSCs in patients (Cutler et al., 2013)] and CXCR4. In this paper we therefore used small molecules to artificially induce intracellular cAMP production in hPSC-derived cultures, at the time of HSC emergence, and assessed its effect on hematopoietic output.

Addition of forskolin (activator of Adenylyl Cyclase) together with IBMX (inhibits degradation of cAMP) resulted in a marked increase in the frequency of HSC-like cells, as well as in CFU-E and CFU-G numbers indicating that cAMP signaling is also involved in progenitor lineage specification. This was coupled to increased expression of CXCR4 on HSC-like cells, to a general decrease in intra-cellular reactive oxygen species (ROS) and to increased expression of genes involved in the antioxidant response. These effects have a particular relevance, as they may directly impact the ability of HSCs to engraft, owing to a better homing ability (with increased CXCR4 levels) and survival (lower ROS levels).

To address the signaling downstream from cAMP, both the EPAC and the PKA axes were individually inhibited, and only inhibition of the EPAC axis had a negative effect in blood emergence. As EPAC inhibition also led to a decrease in endothelial spread from the embryoid bodies, and in frequency of endothelial immunophenotypes, this further strengthens the notion of an endothelial precursor to blood in our hPSC differentiation protocol.

Paper IV

Reactive oxygen species impair the function of CD90+ hematopoietic progenitors generated from human pluripotent stem cells

In steady-state, adult HSCs reside in the bone marrow, and have been shown to be a particularly sensitive cell type when it comes to oxidative stress (Ito et al., 2004; Tothova et al., 2007). As HSCs containing higher levels of ROS are compromised in their repopulation ability, this project was designed to evaluate the impact of intracellular ROS levels in the hematopoietic progenitors produced in our standard *in vitro* culture conditions.

We show that hPSC-derived hematopoietic cells display elevated levels of intracellular ROS compared to primary CB hematopoietic cells. Side-by-side evaluation of hPSC-derived ROS^{low} and ROS^{hi} hematopoietic cells indicated a correlation of an elevated ROS phenotype with increased levels of DNA damage, decreased CFU potential and impaired growth potential. Together, these data strongly indicate that the hematopoietic cells generated in our standard culture conditions are functionally hampered by elevated intracellular ROS levels. Addressing this issue is therefore crucial for the prospect of generating engrafting HSCs from hPSCs.

We therefore designed an approach for lowering intracellular ROS levels targeting multiple sources of ROS. This strategy included culturing the cells in hypoxia [4% O_2, within the range of oxygen tension reported within the bone marrow niche (Mohyeldin et al., 2010)], as well as adding a cocktail of antioxidants and of small molecules specifically inhibiting enzymatic ROS production as well as ROS-inducing stress pathways. This strategy had positive effects at multiple levels. In terms of developmental progression, it specifically increased the percentage of early immunophenotypic HSC-like progenitors without affecting non-hematopoietic cells nor downstream mature hematopoietic cells. Overall HSC-like cell numbers were also increased. When it comes to intracellular ROS levels, this strategy led to a 10-fold increase in cells with a ROS^{lo} phenotype and an 18-fold increase in numbers of ROS^{lo} HSC-like cells. Importantly this significantly improved the quality of the produced cells, enabling a 22-fold increase in numbers of ROS^{lo} HSC-like cells displaying robust growth potential.

Transplantation of cells generated in ROS-lowering conditions into immunocompromised mice did not elicit human engraftment, suggesting that further ROS reduction and/or additional developmental cues are still needed for the successful production of hPSC-derived bona fide HSCs. Nevertheless, this report highlights the

significance of monitoring and handling oxidative stress during *in vitro* generation of hematopoietic cells as an essential requirement for the generation of functional HSCs.

General Discussion and Future Perspectives

During the last years, considerable attention has been given to the step of endothelial-to-hematopoietic transition, and to the identification of the hemogenic endothelial cell, as the most relevant sources of information towards the generation of hPSC-derived HSCs. However, deeper consideration on the papers presented in this thesis, together with the latest publications in the field, indicate that a broader approach needs to be taken in order to achieve this goal.

1. Glancing upstream – considerations on Mesoderm specification

When analyzing the gene expression in EHT-related populations in **Paper I**, we observed that most of the $CD34^{+}CD43^{-}$ endothelial cells already have detectable expression levels of a subset of the hematopoietic program, including key genes such as *HOXB4*, *GATA2*, *TAL1*, *PBX1*, *BMI1*, although at lower levels than the hematopoietic cells (see Figure 1D of **Paper I**). It is therefore plausible that even though not all these endothelial cells have hemogenic potential, in a developmental setting they may all be generated with a transcriptional wiring more or less permissive to the onset of a hematopoietic program if given the right external cues. This could indicate that this wiring for hemogenic potential is already established prior to EHT, during endothelial specification or maybe even during mesodermal patterning. The latter hypothesis has been strengthened by recent independent publications demonstrating that at the end of the differentiation protocol, the ontogeny of hPSC-derived *in vitro* produced blood (namely in terms of globin expression) can be significantly altered by the growth factor signaling provided at the start of differentiation, between day 2 and 4 during mesoderm specification (Kennedy et al., 2012; Ng et al., 2016). Ng et al. (2016) have namely reported significant changes in gene expression and chromatin availability in

endothelial and hematopoietic cells resulting from mesoderm where Wnt signaling was activated and Activin inhibited during this narrow window of early *in vitro* development. If signaling directing mesoderm specification impacts downstream hemogenic endothelium and hematopoietic progenitors, it could therefore also hold some of the keys for downstream HSC engraftment.

It is also noteworthy that during development, *de novo* hematopoiesis has been reported to occur from derivatives of presumably several different mesoderm subtypes – extra-embryonic mesoderm in the case of YS and placenta, splanchnic lateral plate mesoderm in the AGM region, paraxial mesoderm in the head, even cardiac mesoderm in the case of the heart (Nakano et al., 2013). The precise identity of the precursor cell as well as the mechanisms of hematopoietic emergence seem to differ in these different hematopoietic emergence sites (Lee et al., 2016; Li et al., 2016; Yzaguirre and Speck, 2016). It also remains to be clarified whether these temporally and anatomically separate hematopoietic events all generate identical hematopoietic progeny, or if they give rise to cells with different hematopoietic potential, contributing for the heterogeneity in terms of lineage bias observed among the progenitor and HSC pool after birth (Benz et al., 2012; Crisan and Dzierzak, 2016; Yu et al., 2016). If in each of these settings hematopoietic cells emerge from endothelium through an EHT event, it would be interesting to find out if there is a common EHT molecular mechanism, in which case the resulting hematopoietic output would vary depending on the mesodermal cell of origin, instead of depending on different EHT processes. Interestingly, when in **Paper I** we sorted out sub-populations of $CD34^{+}CD43^{int}$ cells (the EHT cluster in the process of upregulating hematopoietic genes and of downregulating endothelial signature), we observed differences in hematopoietic sub-lineage output when they were sub-fractionated according to *WAS* gene expression levels. This prompted us to propose that hematopoietic sub-lineage commitment/restriction happens during the process of EHT, prior to complete downregulation of the endothelial transcriptional signature. Although this could be due to different EHT molecular mechanisms that pre-pattern the emergent hematopoietic cells towards specific hematopoietic sub-lineages, we cannot exclude the possibility that this could be due to the presence of derivatives from multiple mesoderm sub-types undergoing EHT.

Now that we could observe heterogeneity among EHT-undergoing cells, elucidation of upstream mesoderm heterogeneity in the well is therefore missing and would provide novel insights towards hematopoietic emergence and *in vitro* HSC generation. For this, single-cell transcriptional analysis at early time-points of the

differentiation protocol during mesoderm specification will provide invaluable information.

2. A single one or several EHT molecular mechanisms during ontogeny?

The quest for the generation of engrafting HSCs has led to an extensive characterization of the mechanisms of HSC emergence in the AGM region. However, similar mechanisms have been described in YS EMP emergence: *Runx1* activation was shown to be essential in both cases, as well as Wnt signaling (Frame et al., 2016; Tran et al., 2010). Interestingly, just as in the E10.5 AGM region EHT is promoted by signals from the ventral endoderm-derived gut tissues, during primitive YS hematopoiesis mesoderm blood-islands were also shown to develop in close proximity, and dependent of signaling from the adjacent primitive endoderm-derived tissues (reviewed in Palis and Yoder, 2001). A deeper molecular understanding of *de novo* blood formation in the YS is required to identify common molecular mechanisms as well as differences between early hematopoietic emergence and *de novo* HSC generation.

Assessment of functional output from pluripotent stem cell-derived hematopoiesis has suggested, in both mouse and human, that these *in vitro* differentiation systems mimic YS early hematopoiesis (Irion et al., 2010; Vanhee et al., 2015; Zambidis et al., 2005). This is further suggested by the low lymphoid outputs, high embryonic and fetal globin expression compared to adult globin, and by the difficulty of generating erythroid cells capable of enucleation (Baron, 2013; Chang et al., 2006).

In **Papers II** and **III** we aimed at influencing hPSC-derived hematopoietic output by targeting EHT events between days 10 and 12 of the differentiation protocol – a time point where we can observe the spread of an endothelial layer where putative blood cell emergence can be detected. In these studies, we applied signals that have been identified in the murine as being relevant for EHT in definitive AGM hematopoiesis [respectively Norepinephrine and stimulators of intracellular cAMP up-regulation (Diaz et al., 2015; Fitch et al., 2012; Jing et al., 2015)], and observed an increase in the frequency of HSC-like cells as well as improvements in CFU numbers and subtype distribution. It remains to be assessed whether application of these AGM-related signals also influences the hematopoietic ontogeny of the generated cells (for instance by evaluating changes in globing gene expression and T-cell differentiation potential). However, these positive

effects of AGM signals at the time EHT in our in vitro hPSC-differentiation system can have two interesting implications: (1) we are modelling hematopoietic emergence as it occurs in the AGM or (2) we are modelling YS hematopoietic emergence, where EHT mechanisms share these mechanistic features with EHT occurring the AGM region. Regarding cAMP signaling, which can promote hematopoietic development in the AGM region and *in vitro* (as we demonstrated in **Paper III**), its downstream transcriptional regulatory circuit was also shown to have a determinant role in early YS hematopoiesis (Oike et al., 1999). Adrenergic signaling, on the other hand, has never been implicated in YS hematopoiesis.

Side-by-side comparison of the molecular mechanisms of EHT in the YS and in the AGM has been hampered by the lack of specific markers for pure populations involved in EHT in these different locations and time points. In **Paper I** we show that single-cell analysis can help overcome this limitation towards dissecting EHT in development. Furthermore, recent progress in single-cell transcriptomics, coupled with novel bioinformatics approaches for the analysis of developmental processes at the single-cell level, will allow to shed a light on the similarities and differences in the mechanism underlying the successive EHT events occurring in development. So far, single-cell transcriptional analysis has only been applied to the study of murine hematopoietic development, specifically to the study of early mesoderm specification and primitive hematopoiesis (Moignard et al., 2015; Scialdone et al., 2016) and to the emergence of HSCs in the AGM (Swiers et al., 2013; Zhou et al., 2016). Although these reports provide a valuable resource for the study of specific molecular mechanisms of EHT in each of these settings, side by side comparisons of the different anatomical locations and different time-points should be performed to identify common features to these separate hematopoietic events, as well as their individual specificities. For this, novel bioinformatics tools should be developed for pattern identification in gene expression dynamics along single-cell EHT continuums. Currently, the use of primary embryonic material, where distinct time-points and anatomical locations can be dissected, would provide a cleaner platform for this specific purpose than *in vitro* hPSCs-derived material. Indeed, our results in **Paper I** suggest that within the hPSC-derived $CD34^+$ fraction there may be a heterogeneous population likely including several different mesoderm derivatives undergoing EHT simultaneously.

In addition to the comparison between EHT events at separate locations, it will be very interesting to compare molecular mechanisms of hematopoietic emergence in the YS during the E7.25 primitive wave, at the time of EMP emergence at E8.5, and the same anatomical location at the time of HSC emergence after E11. This will provide

insights on the molecular pathways and endothelial precursor signatures specific to each hematopoietic wave, and specifically to the definitive HSC producing EHT event, without the confounding variable inherent to the study of different anatomical locations (i.e. AGM vs YS).

3. What is missing for hPSC-derived hematopoietic cells to engraft?

In **Paper IV**, we observed a major feature of our *in vitro* produced hPSC-derived hematopoietic cells, susceptible to affect their engraftment potential: a high intracellular ROS content. We show that *in vitro* produced hematopoietic cells contain similar ROS levels as *in vitro* cultured CB, where high ROS levels are known to hinder engraftment (Mantel et al., 2015; Yahata et al., 2011). Moreover, preliminary data suggests that *in vitro* hematopoietic cells have altered metabolic state compared to CB-derived progenitors as assessed by mitochondrial activity measured by Rhodamine 123 staining (Figure 6A). In **Paper IV** we took antioxidant, O_2 pressure and stress-targeted approaches to lower intracellular ROS levels; it is however likely that modulating the intrinsic metabolism of produced hematopoietic cells may also be required for further lowering ROS levels and elicit engraftment.

We showed in **Paper I** the power of single cell transcriptional analysis for dissecting the EHT process in hPSC-derived cultures. This approach will also be of value in the comparison of *in vitro* hPSC-derived hematopoietic cells and endothelial precursors with their primary counterparts, to identify common transcriptional signatures, and to find the pathways and genes eliciting engraftment of HSCs which are missing in hPSC-derived material. Indeed, although transcriptional comparison has been previously performed (Choi et al., 2012; Schnerch et al., 2013), the use of bulk populations in these reports may limit the reliability of the obtained results.

We have preliminary data comparing our EHT single-cell dataset with cord blood-derived single HSCs (sorted as $CD45^+CD34^+CD38^-CD45RA^-CD90/CD49f^+$ according to Notta et al., 2011) analyzed with the same gene panel as for the analysis of hPSC-derived cells (see Figure 6B and C). Overall, CB-derived HSCs grouped together with the hematopoietic groups (Figure 6B), as they completely lack the expression of a large set of endothelial and mesodermal genes, such as *CDH5*, *KDR*, *NT5E*, *VWF*, *EFNB2*

and *PDGFRA* while expressing key hematopoietic genes in common with *in vitro*-derived blood including *SPI1*, *WAS*, *CBFA2T3*, *RUNX1*, *GATA2* and *GFI1B*.

In order to infer what are the transcripts missing in hPSC-derived cells for obtaining their engraftment, one can look for genes expressed by CB-derived HSCs that are little or not expressed in hPSC-derived newly emerged HSC-like cells (see Figure 6C). These genes fall into two categories: those that are specific to CB-derived HSCs and virtually absent from *in vitro*-derived material (CB-specific), and the ones that are expressed in CB-derived HSCs and in hPSC-derived non-hematopoietic cells, appearing downregulated in hPSC-derived blood (*in vitro* downregulated).

In the CB-specific category we find the *HOXA* cluster and *HLF*, previously identified by other groups as missing in hPSC-derived material (Ng et al., 2016). *HLF* was included in TF cocktails shown to induce HSC functional phenotypes when over-expressed in mature hematopoietic cells (Riddell et al., 2014). Concurrent with the preparation of this thesis, over-expression of *HOXA* cluster members (*HOXA5, 9* and *10*) together with additional TFs was shown to promote engraftment of hPSC-derived hematopoietic cells (Sugimura et al., 2016). This TF cocktail also includes ERG, found here in the category "*in vitro* downregulated". Interestingly, still in the category "CB-specific" we also find the G- protein coupled receptor *GPR56* previously shown to be important for HSC emergence in the mouse AGM and in zebrafish (Solaimani Kartalaei et al., 2015).

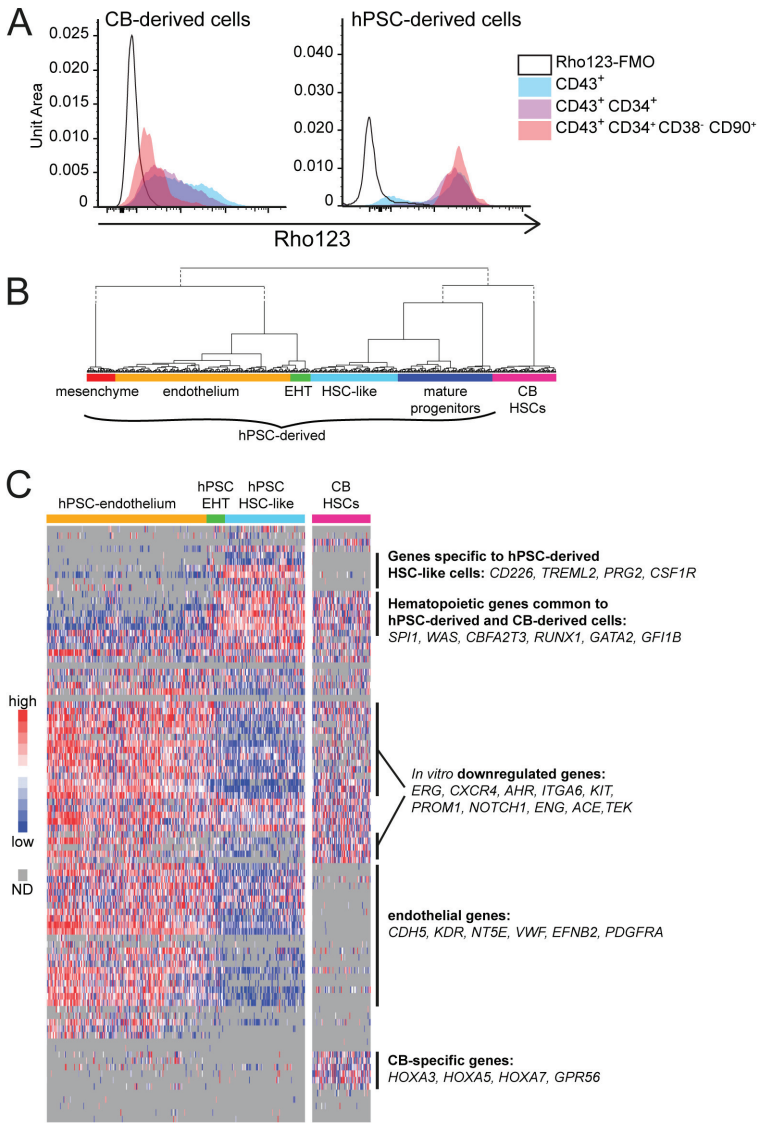

Figure 6: comparison of hPSC-derived HSC-like cells with CB-derived HSCs

A. Levels of Rhodamine123 (Rho123) staining in CB-derived and hPCS-derived hematopoietic cells. While in CB-derived cells Rho123 staining decreases towards more immature HSCs, in the case of hPSC-derived hematopoietic cells Rho123 is uniformly high. This suggests fundamental differences in terms of metabolic state between CB-derived and hPSC-derived hematopoietic cells.

B. Dendogram displaying results of unsupervised hierarchical clustering hPSC-derived single-cells and CB-derived HSCs. Note that the latter cluster with hPSC-derived hematopoietic cells.

C. Heatmap with all qRT-PCR analyzed genes, focusing on clusters corresponding to hPSC-derived endothelial, EHT and HSC-like cells as well as CB-derived HSCs. On the right are listed gene categories of interest, with some

representative genes displaying the represented expression profile. "Genes specific to hPSC-derived HSC-like cells" correspond to the genes expressed in hPSC-derived HSC-like cells and not in cord blood HSCs. "Hematopoietic genes common to hPSC-derived and CB-derived cells" are expressed in both CB HSCs and in hPSC-derived HSC-like cells. "in vitro downregulated genes" are expressed in CB-derived HSCs, and in hPSC-derived endothelial cells prior to hematopoietic commitment, being less/not expressed in hPSC-derived HSC-like cells. "Endothelial genes" are the markers used to establish the endothelial signature; note that they are still expressed at low levels in hPSC-derived HSC-like cells, not in CB-derived HSCs. "CB-specific genes" were found being little or not expressed in hPSC-derived cells, while being expressed in CB-derived HSCs. Data is normalized to GAPDH.

Among the genes in the category of "*in vitro* downregulated" we find genes coding for surface molecules *CXCR4*, *AHR*, *ITGA6*, *KIT*, *PROM1*, *NOTCH1*, *ENG*, *ACE*, and *TEK*. A lower expression of *CXCR4* in *in vitro* derived hematopoietic cells could explain their generally low levels of engraftment in transplantation settings. KIT/SCF signaling is routinely used in HSC *in vitro* maintenance cocktails, and has been implicated in HSC development as well as in HSC migration during development (Christensen et al., 2004; Rybtsov et al., 2014). SCF is part of our hPSC-to-blood differentiation media, and as prolonged exposure to this ligand has been shown to trigger downregulation of its receptor, this may be the reason why the produced hematopoietic cells have lower expression of *KIT*, they may be in the process of desensitization to the pathway. Interestingly, a subset of the genes coding for surface molecules downregulated in HSC-like cells could correspond to an endothelial signature (namely *NOTCH1*, *ENG*, *ACE*, and *TEK*). This would suggest that the *in vitro*-produced HSC-like cells are in a more advanced developmental stage of maturation than primary HSCs. This is corroborated by higher expression in hPSC-derived HSC-like cells of genes suggestive of myeloid maturation *CD226*, *TREML2*, *PRG2*, *CSF1R*, compared to CB-HSC. This suggests that the *in vitro* development of these cells is accelerated, or that the cytokines used in our protocol promote the development of a primed progenitor, skipping the HSC stage. Interestingly, *FOXO1* is also one of the genes found in the "*in vitro* downregulated" category. This gene, together with other *FOXO* family members, was shown to play a central role in HSC response to ROS (Tothova et al., 2007); its *in vitro* down-regulation might contribute for the high ROS levels we observed in hPSC-derived hematopoietic cells (see **Paper IV**). It could therefore also play a role in the poor engraftment potential of *in vitro* derived hematopoietic progenitors.

4. Back to basics: *in vivo* conditions in a dish for functionality of emergent HSCs

In the quest of *in vitro* generation of HSCs, an emergent alternative/complementary approach to stepwise exposure to morphogens for mimicking HSC development, is the concept of transcription factor mediated reprogramming. Similarly to what has been accomplished with iPSCs generation, where virtually any cell can acquire pluripotency and self renewal upon over-expression of a restricted set of transcription factors, the HSC characteristic of multipotency and serial engraftment should be possible to impart following the same strategy. However, a major issue in this reasoning is that while the *in vitro* conditions for long-term maintenance and expansion of pluripotent stem cells have been identified and extensively characterized, the same has not been achieved for HSCs. Hence, in the only instances where transcription factor-mediated programming has successfully generated "induced" HSCs, during TF over-expression the cells had to be exposed to an undefined physiological environment. Riddell et al. induced an HSC phenotype in B-cell progenitors by transiently over-expressing the TFs Run1t1, Hlf, Lmo2, Prdm5, Pbx1, and Zfp37 and injecting the cells into a recipient mouse (Riddell et al., 2014). Similarly, Sugimura et al. reported hPSC-derived hemogenic endothelium could be induced into an HSC phenotype by transient over-expression of ERG, HOXA5, HOXA9, HOXA10, LCOR, RUNX1 and SPI1, as long as the cells were injected into a recipient's bone marrow (Sugimura et al., 2016). While some of these transcription factors are well known regulators of blood development, many tend to be missing in hPSC-derived material (see previous section). This indicates that comparison of hPSC-derived cells with *bona fide* HSCs can provided valuable clues to which cell-intrinsic characteristics (e.g. in terms of gene expression status) are required to obtain functional HSCs. However, these studies also demonstrate that while over-expression of a set of TFs can elicit engraftment, external signals provided by the environment are also playing a determinant role in specifying an HSC phenotype.

The importance of a physiological environment had already been put forward in 2013, when the proof of concept evidence that HSCs can be generated from hPSCs was provided by two articles using hPSC-derived teratomas [see Background section 3b (Amabile et al., 2013; Suzuki et al., 2013)]. These papers report that among other lineages, these teratomas generated HSCs capable of homing to the recipient's bone marrow and provide multilineage lineage reconstitution. Although the stochasticity of

this approach led to high variability in terms of HSC output, it provided a new breath to the field: the correct combination and balance of developmental cues can be achieved for the emergence of HSCs from hPSCs. Of course, the production of HSCs in teratomas is not a viable option for patient transplantation; it however set the stage for the identification of the specific signals provided by the *in vivo* environment to be recapitulated in defined *in vitro* systems.

One of the current bottlenecks in the field is therefore the identification of a defined *in vitro* environment suitable for the last steps of HSC specification and maintenance. In many aspects, this quest is shared with the field of cord blood HSC *ex vivo* expansion (Fares et al., 2014; Hofmeister et al., 2007). Several reports have been testing a growing number of pathways and environmental characteristics possibly involved in HSC generation. For instance, in **Papers II**, **III** and **IV** we tested respectively Norepinephrine signaling, cAMP, and the importance of mitigating levels of oxidative stress. The observed lack of engraftment of resulting material in each approach may however not be due to these pathways not eliciting engraftment, but rather to their implementation in the wrong setting. For instance, more promising results may have been produced by combining cAMP signaling and hypoxia. Presumably, the same applies to most articles published over the last years: several pathways and culture conditions have been tested independently, showing promising results, however the generation of engrafting HSCs clearly requires a complex balance and interplay of all these factors. A systematic approach of combining all findings of the last years in a stepwise, defined, differentiation system may hold the key for HSC generation.

Just as the cell-intrinsic factors (gene expression), discussed above, could be in part determined by comparing *in vitro* produced hematopoietic cell with primary material, one could envision that *in vitro* environment could also be compared with the *in vivo* niches of HSC emergence (in the AGM)/maintenance (in the bone marrow) in terms of extracellular cues and signaling molecules. The composition of these environments to could be determined and compared for instance with approaches such as mass spectrometry imaging (Petras et al., 2017).

Concluding Remarks

Towards the successful generation of functional hematopoietic stem cells from human pluripotent stem cell sources, the work presented in this thesis acts on two fronts:

- the use of developmental and HSC biology knowledge to stimulate signaling pathways likely to elicit HSC specification (in **Papers II** and **III**), and
- a molecular investigation of what actually happens during the differentiation process *in vitro* (in **Papers I** and **IV**).

In the age of "reprogramming" studies, with an increasing resort to transcription factor-mediated induction of a hematopoietic/HSC fate, it is compelling that functional HSC induction still requires a niche providing undefined external cues. The approaches described in this thesis, as well as the results presented, will contribute to moving the field towards a better understanding of these external requirements.

Svensk sammanfattning

Blodet består av olika celltyper, från röda blodkroppar som transporterar syre, till de vita blodkropparna som bildar vårt immunförsvar. Tillsammans bildar blodcellerna det hematopoietiska systemet, som går att jämföra med en gigantisk armé av celler bestående av flera specialiserade truppslag vilka ständigt kämpar för att hålla organismen vid liv. Det hematopoietiska systemet är ett dynamiskt system där förlusterna oupphörligt måste fyllas på under noggrann reglering. Alla mogna blodceller genereras i benmärgen av blodföregångarceller vilka själva härstammar från de celler som finns vid toppen av blodhierarkin vilka kallas för de blodbildande stamcellerna. Förutom att kunna ge upphov till alla olika typer av blodceller har de blodbildande stamcellerna en unik förmåga att förnya sig själva.

Blodstamcellernas förmåga att generera hela blodsystemet möjliggör klinisk benmärgstransplantation, en behandling där blodstamceller från en frisk donators benmärg överförs till en patient där de ersätter det bristfälliga blodsystemet som till exempel vid fall av blodcancer. Det utförs över 50 000 benmärgstransplantationer i världen varje år. Transplantation är dock inte tillgängligt för alla patienter på grund av brist på matchande donatorer.

Målet med min forskning är att generera blodstamceller från pluripotenta stamceller i laboratoriet. De pluripotenta stamcellerna kan expanderas obegränsat och de kan ge upphov till alla typer av celler i kroppen, inklusive blodstamceller. Det finns två typer av pluripotenta stamceller: de embryonala stamcellerna som härstammar från de tidiga stadierna av embryonal utveckling, och de inducerade pluripotenta stamcellerna vilka kan genereras från patienternas egna celler såsom hudceller. Pluripotenta stamceller har därför potential att erbjuda en obegränsad källa av blodstamceller för transplantation.

I vår grupp har vi försökt efterlikna det händelseförlopp som framkallar uppkomsten av de första blodstamcellerna under fosterutvecklingen. Denna uppkomst av de första blodstamcellerna är spännande eftersom de utvecklas under ett kort tidsfönster från speciella endotelceller vilka är strukturceller i vaskulaturen. I det första manuskriptet har vi identifierat de molekylära stegen som ligger bakom omvandlingen

av endotelceller till blod genom att analysera vilka gener som uttrycks i varje enskild cell. De här resultaten är viktiga eftersom jämförelsen av dessa händelser med vad som händer naturligt i embryot kan ge ett recept på hur man kan generera blodstamceller i laboratoriet. I det andra och tredje manuskriptet har vi utvärderat vilken roll det perifera nervsystemet har vid uppkomsten av blodstamcellerna under utvecklingen. Vi observerade att stimulering av den adrenerga signalvägen hos de pluripotenta stamcellerna, med noradrenalin och andra stimulatorer av samma signalväg, ökade antalet av genererade blodföregångarceller. I det sista manuskriptet visar vi att den ofysiologiska syrenivån som de pluripotenta stamcellerna utsätts för utanför kroppen i laboratoriet har en negativ påverkan på funktionen hos de framodlade blodstamcell-lika cellerna.

Sammanfattningsvis tar våra fynd oss närmare möjligheten att generera patientspecifika och skräddarsydda blodstamceller.

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Paper I

Cell Reports
Report

Single-Cell Analysis Identifies Distinct Stages of Human Endothelial-to-Hematopoietic Transition

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SUMMARY

During development, hematopoietic cells originate from endothelium in a process known as endothelial-to-hematopoietic transition (EHT). To study human EHT, we coupled flow cytometry and single-cell transcriptional analyses of human pluripotent stem cell-derived CD34$^+$ cells. The resulting transcriptional hierarchy showed a continuum of endothelial and hematopoietic signatures. At the interface of these two signatures, a unique group of cells displayed both an endothelial signature and high levels of key hematopoietic stem cell-associated genes. This interphase group was validated via sort and subculture as an immediate precursor to hematopoietic cells. Differential expression analyses further divided this population into subgroups, which, upon subculture, showed distinct hematopoietic lineage differentiation potentials. We therefore propose that immediate precursors to hematopoietic cells already have their hematopoietic lineage restrictions defined prior to complete downregulation of the endothelial signature. These findings increase our understanding of the processes of de novo hematopoietic cell generation in the human developmental context.

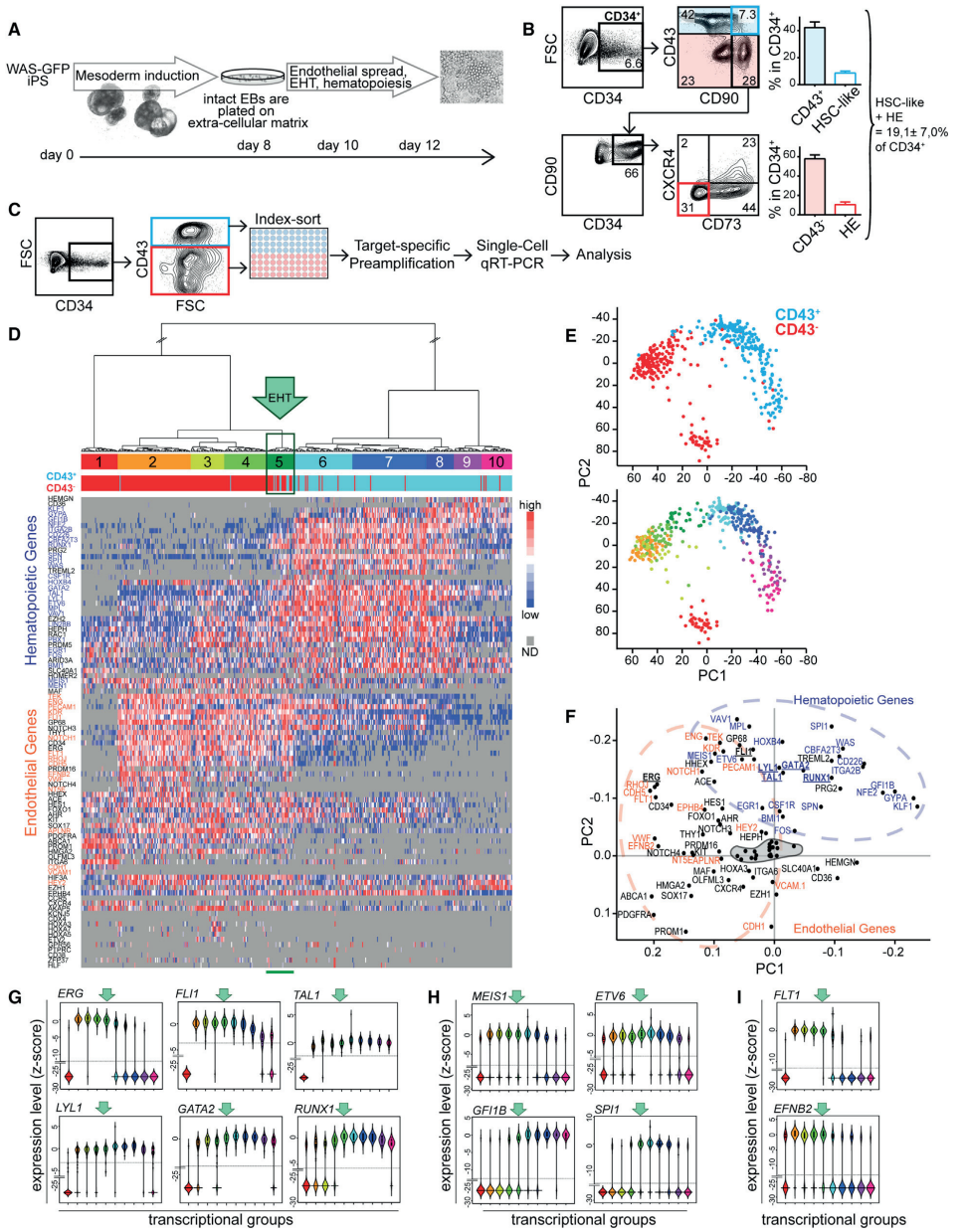
INTRODUCTION

During embryonic development, hematopoietic cells emerge from a precursor in the endothelial layer lining the ventral side of the dorsal aorta in the aorta-gonad-mesonephros (AGM) region. This conversion of endothelium into hematopoietic cells, termed endothelial-to-hematopoietic transition (EHT), has been extensively verified in various animal models (Bertrand et al., 2010; Boisset et al., 2010; Chen et al., 2009; Jaffredo et al., 1998; Kissa and Herbomel, 2010; Zovein et al., 2008) and has been observed in live-cell imaging studies of human pluripotent stem cell (hPSC) differentiation in vitro (Choi et al., 2012; Ditadi et al., 2015; Rafii et al., 2013). Although EHT was reported in the human embryo (Oberlin et al., 2002), no detailed molecular analysis of human cells undergoing EHT has been performed. Limitations to the study of EHT in human embryos make in vitro differentiation of hPSCs an attractive model for dissecting this developmental process. Two independent studies using hPSC differentiation reported a population enriched for the endothelial precursor of definitive hematopoiesis, termed hemogenic endothelium (HE), and highlight its transient nature and low frequency (Choi et al., 2012; Kennedy et al., 2012).

However, the molecular mechanisms regulating de novo emergence of blood cannot be fully understood until the transitional states in EHT are identified and characterized. Moreover, murine AGM imaging and transplantation studies show that while numerous hematopoietic clusters emerge on the walls of the dorsal aorta, very few of these cells are hematopoietic stem cells (HSCs) capable of adult multi-lineage reconstitution (Boisset et al., 2015; Kumaravelu et al., 2002; Taoudi et al., 2008; Yokomizo and Dzierzak, 2010), indicating heterogeneity among in vivo emerging blood.

Single-cell transcriptional analysis (reviewed in Hoppe et al., 2014) is an ideal tool to investigate lineage hierarchies in the heterogeneous populations undergoing EHT. Recent single-cell studies in the mouse have identified the onset of a hematopoietic regulatory program in the early stages of embryonic development (Moignard et al., 2015) and characterized a putative HE population from murine AGM (Swiers et al., 2013). In this latter study, distinct stages in the EHT process can be visualized for different populations at successive developmental time-points. Evaluation of the roles of specific transcription factors (TFs) in EHT at the single-cell level identified novel players in this process (Thambyrajah et al., 2016; Wilkinson et al., 2014). Recently, single-cell RNA sequencing was used to study the heterogeneity of prospectively isolated murine populations, including endothelial cells, pre-HSCs, and HSCs (Zhou et al., 2016).

To characterize EHT in the human developmental context, we performed single-cell transcriptional analysis of hPSC-derived CD34$^+$ cells. We identified a rare subset of cells sharing high numbers of transcripts of both endothelial and hematopoietic signatures, and determined this EHT subpopulation to be developmentally between HE and newly emerged hematopoietic cells. Moreover, we demonstrate that hematopoietic lineage differentiation potentials are already restricted in this narrow EHT window,



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concurrent with the upregulation of the hematopoietic and downregulation of the endothelial programs. Using single-cell analysis, we provide further insight into endothelial and hematopoietic lineage commitment in the human developmental context.

RESULTS

hPSC-Derived CD34$^+$ Fraction Is a Suitable Population for Single-Cell Transcriptional Analysis of EHT

To dissect human EHT, we used in vitro differentiation of induced pluripotent stem cells (iPSCs) to the hematopoietic lineage (Rönn et al., 2015) (Figures 1A and S1A–S1C). Time-course fluorescence-activated cell sorting (FACS) analysis for the expression of HE markers and CD43$^+$ developing hematopoietic cells (Vodyanik et al., 2006) suggested that day 10 was an ideal time point to capture EHT cells (Figure S1D). At this time point, the iPSC-derived CD34$^+$ fraction contained the CD43$^-$CD34^{hi}CD90^{hi}CD73$^-$CXCR4$^-$ HE phenotype (10% of CD34$^+$) as well as our previously described HSC-like phenotype, CD43$^+$CD34$^+$CD90$^+$ (also 10% of CD34$^+$) (Majeti et al., 2007; Rönn et al., 2017) (Figure 1B). The remaining 80% of cells in the CD34$^+$ fraction include a broad repertoire of other known human endothelial and hematopoietic cells (Ivanovs et al., 2014; Kaufman et al., 2001; Kennedy et al., 2012; Tavian et al., 1996) and presumably as-yet-unidentified EHT-related cells.

The hPSC-Derived CD34$^+$ Cell Fraction Contains a Continuum Spanning Endothelial and Hematopoietic Transcriptional States, Including a Cell Population with a Dual Endothelial and HSC Signature

Day 10 iPSC-derived CD34$^+$ cells containing both endothelial and hematopoietic fractions (CD43-negative and positive cells, respectively) were index sorted for subsequent single-cell transcriptional analyses (Figure 1C). The iPSC line used had previously been transduced with a lentiviral vector expressing GFP under the control of the pan hematopoietic *WAS* gene promoter sequences. This WAS-GFP reporter was previously shown to track early hematopoietic commitment during hPSC differentiation (Muñoz et al., 2012) (Figures S1E–S1G). As index sorting allows us to determine the FACS phenotype of each transcriptionally analyzed single cell, the cells were simultaneously analyzed for WAS-GFP and for CD90, CD73, and CXCR4, the additional markers relevant in HE and HSC-like phenotypes. As expected, a fraction of the 437 analyzed CD34$^+$ cells consisted of either HE or HSC-like cells (Figures S1H and S1I). Expression levels of 91 genes related to endothelium, hematopoietic stem and progenitor cells (HSPCs), as well as EHT were assessed on each cell (Table S1).

Spanning endothelial and hematopoietic programs, the cells were divided into ten groups by unsupervised hierarchical clustering (Figure 1D). Principal-component analysis (PCA) distributed the cells from nine of the ten groups in a continuum (Figure 1E). Retrospective coloring based on CD43 sorting phenotype revealed that hematopoietic and non-hematopoietic cells grouped separately, with groups 1–5 being almost exclusively CD43$^-$ and groups 6–10 being CD43$^+$ (Figures 1D and 1E). No plate-specific bias of independently run qRT-PCR analyses was seen (Figure S1J), and index-sort data confirmed consistency between protein expression levels of surface markers and their transcriptional state. Notably, a similar correlation was observed between the WAS-GFP reporter fluorescence and *WAS* transcript levels (Figure S1K).

We proceeded to analyze the identified transcriptional signatures. Groups 2–5 had a clear endothelial program, where group 2 expressed all endothelial genes, and as the hierarchically ordered groups proceeded, there was a progressive downregulation of endothelial gene subsets (Figures 1D and S2Ai). Groups 6–8 had a hematopoietic signature, including many genes relevant for HSC function (Figures 1D and S2Aii). Most of these HSC-affiliated genes were downregulated in groups 9 and 10, which in turn expressed higher levels of genes associated with erythro-myeloid maturation (Figures 1D and S2Aiii). Interestingly, group 5 represented a population at the interface of endothelial and hematopoietic groups that, in addition to an endothelial signature, also displayed increased levels of key hematopoietic genes (green arrow in Figure 1D; see also Figure S2Aiv). Group 1 clustered separately from the transcriptional continuum (Figures 1D and 1E), lacked expression of most endothelial and

Figure 1. The hPSC-Derived CD34$^+$ Cell Fraction Contains a Continuum of Transcriptional States Spanning Endothelium to Blood

(A) Overview of iPSC-to-blood differentiation protocol.

(B) FACS analysis of iPSC-to-blood cultures at day 10 of the differentiation protocol with gating strategy for the immunophenotypes corresponding to HSC-like (CD34$^+$CD43$^+$CD90$^+$, blue outline) and HE (CD43$^-$CD34^{hi}CD90^{hi}CD73$^-$CXCR4$^-$, red outline). Bar graphs show the percentage of CD43$^+$ and CD43$^-$ fractions (in blue fill and pink fill, respectively, corresponding to the shades in the FACS plot), as well as the HSC-like and HE immunophenotypes (in empty columns, blue and red outlines, respectively) within the CD34$^+$ fraction of day 10 iPSC-to-blood cultures (n = 3 independent experiments; data represent mean ± SEM).

(C) Workflow for single-cell surface marker and transcriptional analysis of CD34$^+$CD43$^{+/-}$ fractions of day 10 iPSC-to-blood cultures. Using index sort, the cells were also analyzed for WAS-GFP, CD90, CD73, and CXCR4.

(D) Unsupervised hierarchical clustering, where each group is colored. Second row: retrospective coloring according to CD43-based sorting. On the left, the list of genes is color-coded; hematopoietic genes are in blue, endothelial genes are in orange, and genes that were not expected to help the distinction between endothelium and blood (i.e., implicated in both or none of these two cell types) are in black.

(E) PCA of single cells. Top: cells retrospectively colored by CD43 sorting phenotype. Bottom: cells colored by groups as identified by unsupervised hierarchical clustering in (D).

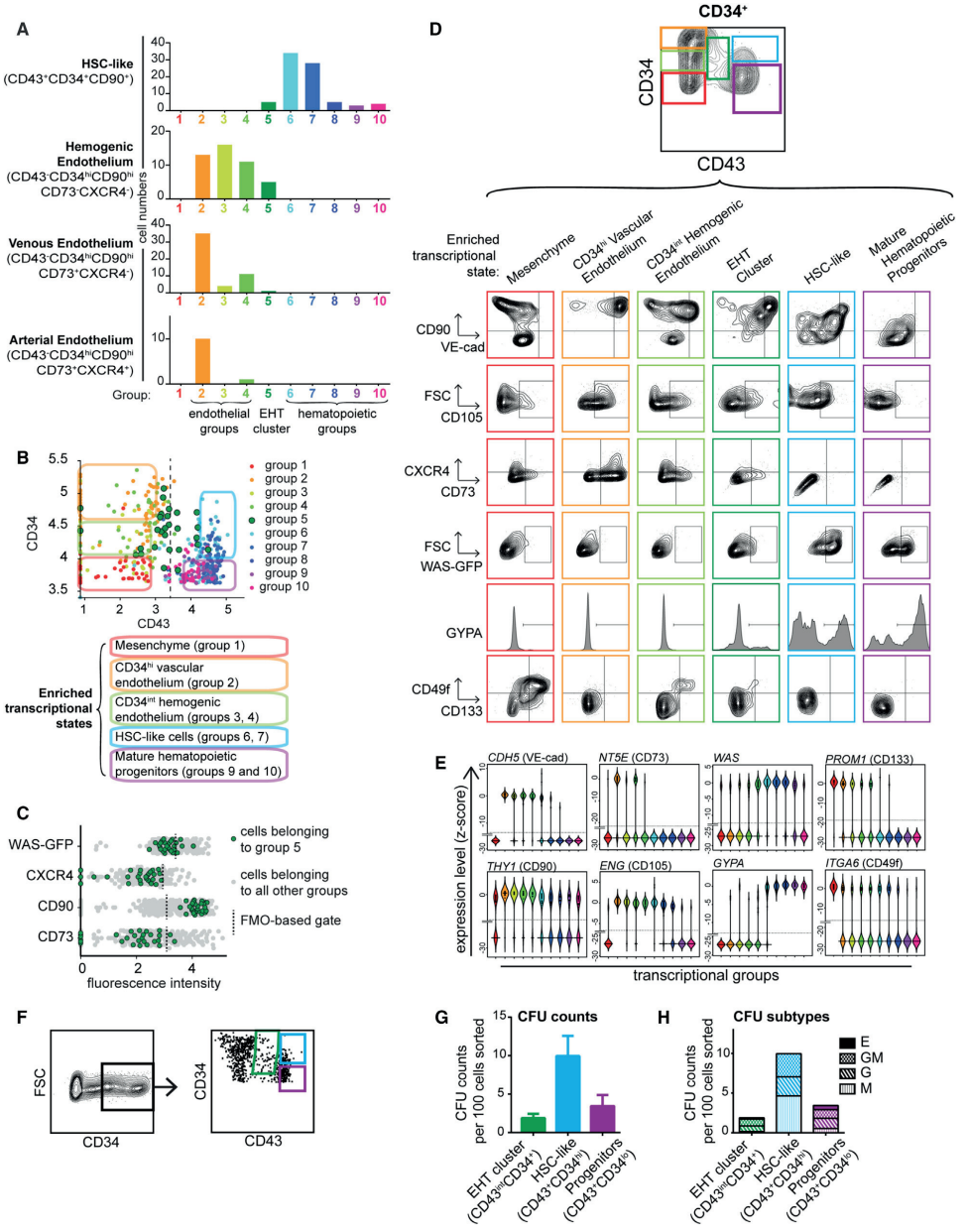
(F) Gene loadings of the PCA analysis. Members of the heptad are underlined. Genes in the center of the plot (with loading values close to 0) are *ZFP37*, *RAC1*, *PTPRC*, *PRDM5*, *PBX1*, *MEN1*, *LIN28B*, *KCNJ5*, *HOXA7*, *HOXA5*, *HOMER2*, *HLF*, *HIF3A*, *GPR56*, *EZH2*, *ETV2*, *CDX4*, *CD38*, *CCR5*, *ARID3A*, and *AKAP5*.

(G) Bean plots for the heptad genes included in this analysis.

(H) Bean plots for additional HSC-affiliated genes.

(I) Bean plots for endothelial genes.

Green arrows above bean plots highlight EHT cluster (group 5). See also Figures S1 and S2 and Table S1.



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hematopoietic genes, and expressed genes suggestive of a mesenchymal cell (Figures 1D and S2Av) (Bakondi et al., 2009; Lee et al., 2009; Mabuchi et al., 2013; Moignard et al., 2015).

To gain further insight into the gene expression dynamics, we examined the gene loadings of the PCA and observed a separation between endothelial and hematopoietic genes. Heptad complex members (Beck et al., 2013; Wilson et al., 2010) were distributed in the upper portion of the gene loadings plot (Figure 1F, genes in underlined font). *TAL1*, *LYL1*, *GATA2*, and *RUNX1* appeared to cluster in the gene loadings plot, suggesting a cooperation in human EHT, and were notably expressed in group 5 (Figure 1G). *ERG* and *FLI1* were also expressed in group 5 and were clearly defining the endothelial groups, while *FLI1* was also expressed in the first hematopoietic groups (Figure 1G). Other key hematopoietic TFs (e.g., *MEIS1*, *ETV6*, *GFI1B*, and *SPI1*) displayed distinct expression patterns in group 5 and surrounding groups (Figure 1H). Finally, two endothelial genes (*FLT1* and *EFNB2*) were both highly expressed in groups 2–5 while being less expressed or not detected in the hematopoietic groups 6–10 (Figure 1I).

Taken together, we hypothesize that the group 5 cells, at the interface of the endothelial and hematopoietic programs, are in a narrow window of the EHT process, just prior to becoming bona fide hematopoietic cells, and we henceforth designate this transcriptional cluster as the EHT cluster.

The EHT Cluster and Surrounding Transcriptional States Identified by Single-Cell qRT-PCR Correspond to Distinct Endothelial and Hematopoietic Populations as Assessed by FACS

To elucidate how previously identified FACS-based populations related to our identified transcriptional groups, we used the index-sort data for backtracking the surface phenotype of each analyzed cell. We observed that cells with the HSC-like immunophenotype were predominantly found downstream of the EHT cluster, with groups 6 and 7 (Figure 2A) showing highest expression levels of HSC-affiliated genes (Figures 1D, 1G, and 1H). We also observed that HE cells were found scattered throughout groups 2 to 5 (highest in group3), indicating heterogeneity of this immunophenotype. Cells corresponding to arterial and venous endothelium as described in Ditadi et al. (2015) were found almost exclusively in group 2 (Figure 2A).

We then used the index-sort data to define sortable populations of EHT cluster cells and of other identified transcriptional states and observed that the different transcriptional groups already clustered in distinct populations based on CD34 and CD43 surface expression (Figure 2B). Specifically, the $CD43^{-}CD34^{hi}$ population enriched for the vascular endothelium (group 2), $CD43^{-}CD34^{int}$ enriched for HE cells (groups 3 and 4), $CD43^{+}CD34^{hi}$ enriched for HSC-like transcriptional signature (groups 6 and 7), and $CD43^{+}CD34^{low}$ enriched for the erythro-myeloid-committed signature (groups 9 and 10) (Figure 2B). Interestingly, we observed that the EHT cluster localized mostly at the interface between the $CD43^{-}$ and $CD43^{+}$ populations (Figure 2B, large green dots). These EHT cluster cells were almost exclusively $CD90^{hi}$, $CXCR4^{-}$, and $CD73^{-}$ and contained both WAS-GFP^{-} and WAS-GFP^{+} cells (Figure 2C). This enrichment strategy and the endothelial and hematopoietic cell identities of the transcriptional groups were confirmed by FACS analyses in additional independent experiments (Figure 2D). Notably, the transcript and protein levels as assessed by FACS for new surface markers were highly correlated and similarly up- or downregulated in the corresponding groups, including the WAS-GFP reporter and endogenous *WAS* transcript, whose expression was initiated in some cells within the EHT cluster and increased in the hematopoietic groups (Figures 2D and 2E). Interestingly, glycophorin A (GYPA) expression was also initiated in the EHT cluster. Moreover, sorting the identified transcriptional groups (EHT cluster, HSC-like, and erythro-myeloid committed cells) and plating into a colony-forming unit (CFU) assay revealed differences in both colony numbers and subtypes, demonstrating functional differences between the populations (Figures 2F–2H).

The EHT Transcriptional Cluster Comprises Subgroups with Distinct Levels of Hematopoietic Commitment Both Transcriptionally and in Differentiation Potential

To further dissect EHT, we performed a new single-cell qRT-PCR analysis on EHT cluster-enriched cells ($CD43^{int}CD34^{+}CD90^{hi}$) (Figure 3A). Combining this expression data with that of the initially sorted material increased the complexity of the EHT cluster, which could then be divided into three subgroups by hierarchical clustering (Figure 3B). These subgroups had progressively increased expression of a subset of hematopoietic genes, including endogenous *WAS*, *GFI1B*, and *ETV6* (Figure 3C). These differences correlated with a progressive increase in CD43

Figure 2. The Different Transcriptional Groups Identified by Single-Cell Analysis Correspond to Distinct Populations as Assessed by FACS

(A) Distribution, throughout the ten transcriptional groups identified in Figure 1D, of the single cells displaying the previously characterized surface phenotypes (using index-sort data) stated on the left.

(B) Scatterplot of the single cells according to surface expression of CD34 and CD43 using index-sort data. The single cells are colored according to the transcriptional group they belong to. Putative FACS gates were drawn to enrich for the transcriptional groups stated in the legend below. Fluorescence intensity in $\log_{10}$.

(C) FACS phenotype characterization of transcriptional group 5. Expression levels for cells belonging to group 5 EHT cluster cells are in dark green, and the remaining cells are in gray. Fluorescence intensity in $\log_{10}$.

(D) Validation of putative gates drawn in (B). Below, the color of the frame of the plots displays their correspondence to the gate of same color in the above CD34/CD43 FACS plot. Plots are representative of two to four independent experiments.

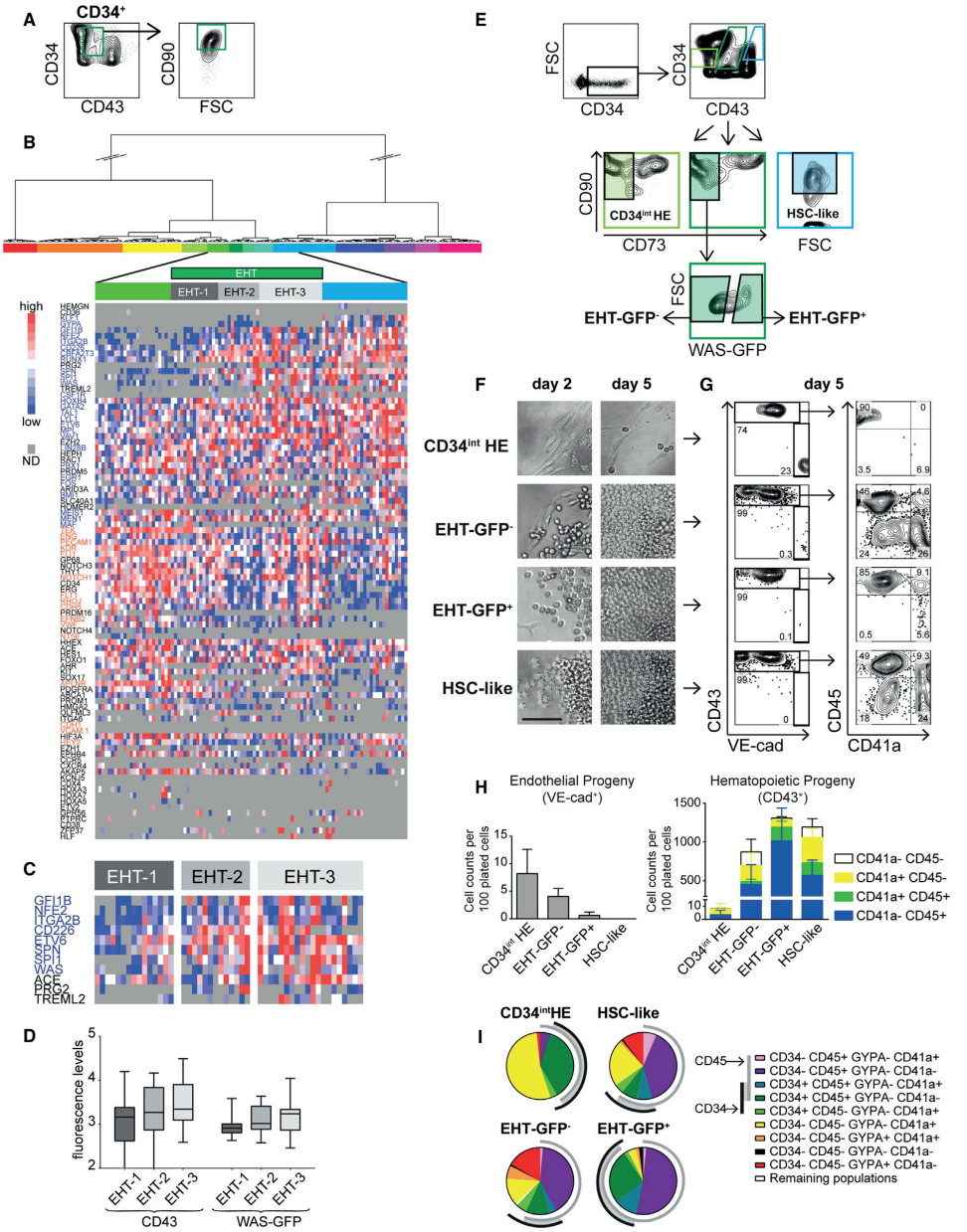
(E) Bean plots from the transcriptional analysis of Figure 1, the transcripts of the surface proteins analyzed in (D).

(F) Gating strategy for sorting the $CD43^{+}CD34^{+}$ identified subpopulations corresponding to EHT cluster (outlined in green), HSC-like (blue), and erythro-myeloid-committed cells (purple).

(G) CFU counts per 100 plated cells sorted using the gating strategy outlined in (F).

(H) CFU counts per 100 plated cells sorted using the gating strategy outlined in (F), with average representation of colony subtypes.

Data represent mean ± SEM; n = 2 independent experiments.



(legend on next page)

surface expression and in the WAS-GFP reporter expression (Figure 3D), suggesting that the originally identified EHT cluster contained cells with different levels of hematopoietic commitment. We therefore sought to elucidate if sorted WAS-GFP$^-$ and WAS-GFP$^+$ EHT cluster cells would exhibit different hematopoietic potentials.

We sorted EHT cluster cells (CD34$^+$CD43^{int}CD90^{hi}CD73$^-$) separating WAS-GFP$^-$ (EHT-GFP$^-$) from WAS-GFP$^+$ (EHT-GFP$^+$) fractions for subculture assays (Figures 3E and S3A–S3C). These EHT cluster sorted populations displayed cells of both spindle-shaped endothelial and round hematopoietic morphologies 2 days after plating (Figure 3F). Tracking WAS-GFP expression in the cultured EHT fractions, we observed instances of WAS-GFP$^+$ cells with endothelial morphology, including from EHT-GFP$^-$ sorted cells (Figure S3D, arrowheads). Surface marker analysis revealed high frequency of hematopoietic cells from both these sorted fractions by day 5 (Figure 3G). In comparison, the CD34^{int} HE sorted cells had exclusively endothelial morphology shortly after plating (day 2) and developed round hematopoietic cells by day 5 of subculture. As expected, the HSC-like population subculture resulted almost exclusively in blood cells and no endothelial progeny (Figures 3F–3H). Interestingly, there was increased blood/endothelium ratio from the analyzed populations the more transcriptionally committed they were toward the hematopoietic fate (Figures 3G and 3H, left).

These results confirm enrichment for cells undergoing EHT in the EHT-cluster-sorted populations and suggest a developmental progression within the EHT cluster.

The EHT Cluster Subpopulations Possess Distinct and Overlapping Hematopoietic Development Markers and Lineage Potentials

To evaluate the hematopoietic differentiation potentials of the above EHT-related populations, we analyzed the hematopoietic (CD43$^+$) fraction resulting from the 5-day-subcultured cells for the expression of CD45 (as an indicator of developmental progression within hematopoietic ontogeny), CD41a and GYPA (as indicators of erythro-myeloid lineage commitment), and CD34 as a marker of progenitors. The HSC-like population gave rise to blood cells with all combinations of CD45/CD41a immunophenotypes, suggesting a broad repertoire of developmentally distinct and lineage committing hematopoietic cells (Figures 3G and 3H, right). However, the progeny of EHT-GFP$^+$, the closest EHT cluster population according to gene expression, was almost exclusively CD45$^+$, suggesting a developmentally more mature hematopoietic progression (Figures 3G and 3H, right). The more distant EHT-GFP$^-$ cells had a progeny very similar to that of HSC-like cells, suggesting EHT-GFP$^-$ cells to be full contributors to all developmental stages and committed lineages from the HSC-like fraction. Analyzing GYPA further confirmed the similarities between the HSC-like and EHT-GFP$^-$ cells; both are capable of generating an early-committing erythroid lineage cell, but the EHT-GFP$^+$ sorted fraction did not have this potential (Figure 3I, red dataset in pie charts). This suggests that the EHT-GFP$^-$ cells possess a broader hematopoietic differentiation potential, generating more developmentally immature CD45$^-$ as well as GYPA$^+$ cells, as compared to the EHT-GFP$^+$ cells. These results also indicate that hematopoietic lineage differentiation potential is already defined prior to complete downregulation of the endothelial program during EHT.

We then set out to combine this differentiation potential data with gene expression correlation analysis in order to infer lineage relationships between the identified transcriptional groups (Figure 4A). We observed high correlation coefficients within the endothelial groups 2–5, hematopoietic groups 6–8, and mature hematopoietic groups 9 and 10. We then combined this with the index-sort data and differentiation potential assays to propose a model with the different transcriptional states identified in the hPSC-derived CD34$^+$ population as well as lineage relationships using gene expression correlation as well as validation through subculture (Figure 4B).

DISCUSSION

Using qRT-PCR analysis of index-sorted hPSC-derived CD34$^+$ single-cells enabled molecular characterization of transcriptional states throughout human EHT as well as the identification and sorting of populations of cells undergoing this process that upon culture possess differing hematopoietic lineage potentials. Our results show heterogeneity of the cells undergoing EHT, as they downregulate their endothelial programs and upregulate their hematopoietic programs. The EHT cluster could be subdivided based on increasing expression of eight genes, including *GFI1B*, shown in the murine system to be a key player

Figure 3. Differentiation Potential of the Identified EHT Transcriptional States

(A) Gating strategy enriching for the EHT cluster.

(B) Top: hierarchical clustering integrating EHT-cluster-enriched cells together with overall analysis of CD34$^+$ cells. Bottom: heatmap of gene expression for the three subgroups identified within the EHT cluster.

(C) Expression levels of genes upregulated along the three EHT cluster subgroups identified in (B).

(D) CD43 surface expression and WAS-GFP levels detected by FACS (using the index-sort data) in the three EHT cluster subgroups identified in (B).

(E) Sorting strategy used for subculturing populations enriched for CD34^{int} HE, EHT cluster further divided in WAS-GFP$^-$ and WAS-GFP$^+$, and HSC-like transcriptional groups.

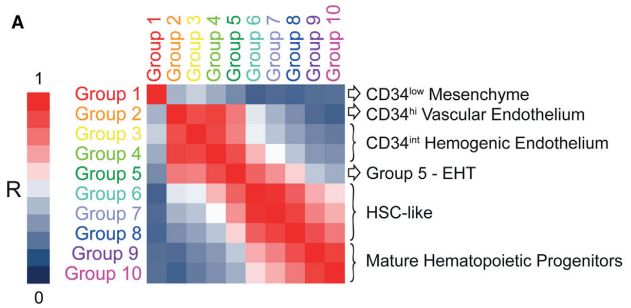
(F) Phase-contrast pictures of sorted cells during subculture. Day 2 corresponds to the day following aggregate plating; day 5 is the day of harvest for FACS analysis. Scale bar, 100 μm.

(G) FACS analysis of day 5 sub-cultures, corresponding to the micrographs in the same rows in (F). Plots are representative of three independent experiments.

(H) Left: endothelial progeny per 100 plated cells. Right: stacked bar graph of the CD41a/CD45 hematopoietic progeny resulting from each subpopulation. Data represent mean ± SEM; n = 3 independent experiments.

(I) Pie charts representing the average composition, in three independent experiments, of the CD43$^+$ blood fraction at the end of subculture for each sorted population. Each color corresponds to each population resulting from all CD45, CD41a, CD34, and GYPA surface marker combinations.

See also Figure S3.



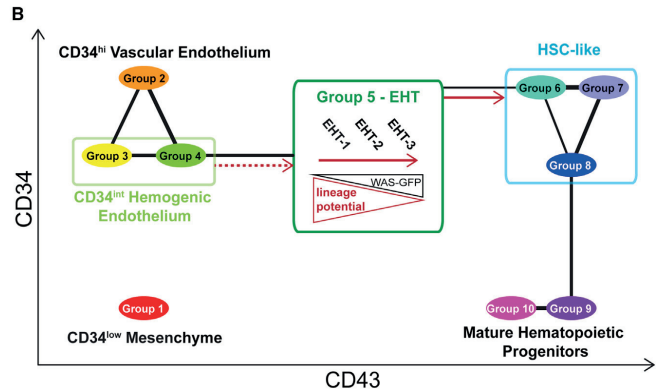


Figure 4. Inferring Population Hierarchy Using Single-Cell Analysis

(A) Pearson's correlation analysis calculated with the average expression values for each gene within each group. FACS-sorted populations, listed on the right, correspond to visibly correlating clusters. (B) Combining differentiation potential assay and Pearson's correlation data to infer lineage relationships between the identified groups. Groups are placed according to their CD34/CD43 phenotype. Black connections correspond to Pearson's correlation between groups with R > 0.8 (thickness proportional to R value). Red arrows summarize lineage relationships confirmed (solid arrows) or inferred (dashed arrows) from the differentiation potential assay.

for the loss of endothelial identity during EHT (Lancrin et al., 2012) and for hematopoietic maturation within AGM cells undergoing EHT (Thambyrajah et al., 2016). The *WAS*-negative (EHT-GFP$^-$) cells within this EHT population displayed the broadest hematopoietic lineage potential similar to that of the HSC-like population, while the *WAS*-expressing (EHT-GFP$^+$) fraction gave rise to a more restricted hematopoietic progeny, almost exclusively CD45$^+$, with no GYPA$^+$ cells. This led us to propose that the hematopoietic differentiation potential in the developmental context is already determined within unique cell populations at the EHT stage. This also implies that separate outcrops of hematopoietic cells emerge from EHT subsets with different lineage propensities depending on the expression levels of specific hematopoietic genes. In support of our findings, a recent study of murine AGM pre-HSCs also demonstrates heterogeneity during emergence at the single-cell level (Zhou et al., 2016). The heterogeneity within EHT could allow for yet another level of control of hematopoietic lineage contributions during development.

The CD34^{int}HE population is enriched in transcriptional group 3, the most abundant transcriptional group in the cells displaying the immunophenotype corresponding to that of previously characterized HE (Ditadi et al., 2015). Interestingly, among the endothelial transcriptional groups, group 3 had specifically high expression of genes, including a subset of hematopoietic TFs and epigenetic modifiers (see Figure S2Avi). These genes may be part of the regulatory network conferring hemogenic capacity to these cells. The hematopoietic progeny of this CD34^{int}HE subset contained similar populations as that of EHT cluster and HSC-like fractions (albeit with different frequencies), with nearly every CD45$^+$ cell co-expressing CD34 (Figure 3I). This suggests that CD34^{int}HE population may give rise to the EHT cluster and HSC-like populations (from which only a small subset of resulting CD45$^+$ cells co-expressed CD34).

Expression dynamics of the analyzed heptad members suggested a pattern with cooperation of *TAL1*, *LYL1*, *GATA2*, and *RUNX1* and separate activity of *FLI1* and *ERG*. This corroborates a recent study showing stronger correlation between *TAL1*, *LYL1*, *GATA2*, and *RUNX1* in terms of DNA-binding patterns, with *FLI1* and *ERG* displaying a separate binding partnership (Wilson et al., 2016). The first four TFs have been shown to synergize for HSC emergence in the murine setting (Chan et al., 2007; Chen et al., 2009; Nottingham et al., 2007), and it is therefore plausible that they also cooperate during human EHT.

Our approach of profiling a heterogeneous subset of cells at the single-cell level proved here to be highly useful to dissect sequential transcriptional states during human EHT. It can be applied to the study of primary material from developing human tissues to decipher the molecular mechanisms of human EHT in vivo and ultimately generate transplantable HSCs in vitro. Our findings set the stage for further exploration of heterogeneity during human hematopoietic cell emergence.

EXPERIMENTAL PROCEDURES

iPSC Expansion and Differentiation

The human iPSC-CB1RB9 cell line was expanded and differentiated toward the hematopoietic lineage as described previously (Rönn et al., 2015) (see Supplemental Experimental Procedures). Briefly, mesoderm-biased embryoid bodies were generated and plated on extracellular matrix to develop an

endothelial-like cell monolayer, from which presumptive hematopoietic cells emerge. Mesototal medium (Primorigen Biosciences) was applied throughout the procedure.

Single-Cell qRT-PCR

At day 10 of the differentiation protocol, the cells were harvested and stained as described in Supplemental Experimental Procedures. Single $CD34^+$ cells were sorted directly into lysis buffer using the index-sorting function of the Diva Software. Target-specific pre-amplification mastermix was then added to each sample for reverse transcription (RT) and pre-amplification. Pre-amplified samples were diluted and analyzed in Fluidigm 96.96 arrays on a Biomark device (Fluidigm), with the TaqMan assays listed in Table S1. For further details and reagents, see Supplemental Experimental Procedures.

Single-Cell qRT-PCR Data Analysis

The data were first analyzed with Fluidigm Real-Time PCR Analysis Software for evaluation of the amplification curves, and Ct thresholds were set automatically using the "auto detector" function. Reactions with Ct > 30 were considered negative. In assays where amplification was detected in no-RT controls, all reactions displaying Ct > [min($Ct_{noRTcontrols}$) − 2] were considered negative. The data were then analyzed using the SCexV online tool (http://stemsysbio.bmc.lu.se/SCexV/; Lang et al., 2015), together with the index-sort data, for performing PCA and hierarchical clustering based on the gene expression data. Cells displaying a Ct value > 25 in the housekeeping gene GAPDH were excluded from the analysis, as well as the positive control samples. Subsequent analysis was then performed for the remaining cells (representing over 90% of the total sorted cells), using *Z* score transformed expression data of all assays excluding the housekeeping gene GAPDH. Unsupervised hierarchical clustering results were used for defining the groups of interest.

Subculture Assay

At day 10 of iPSC-to-blood differentiation, the indicated populations were sorted and cultured in an assay adapted from Ditadi and Sturgeon (2016). Specifically, the sorted cells aggregated overnight in ultra-low adherence U-shaped-bottom 96-well plates, and aggregates were then transferred onto Matrigel-coated flat-bottom 96-well plates. At 5 days post-sort, the cells were collected and analyzed by FACS. For details, see Supplemental Experimental Procedures.

ACCESSION NUMBERS

The accession number for the single-cell qRT-PCR data for the hPSC-derived $CD34^+$ cells reported in this paper is GEO: GSE87422.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.03.023.

AUTHOR CONTRIBUTIONS

C.G. and N.-B.W. conceived and designed the project. The single-cell transcriptional analysis procedure was optimized by C.B. and G.K. in T.E.'s laboratory. R.E.R. and S. Saxena participated in the design of the experiments. C.G. performed experiments and analyses. C.G., S. Soneji, and S.L. performed in silico analyses. C.G. and N.-B.W. wrote the manuscript, which was reviewed and edited by all authors.

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Supplemental Information

Single-Cell Analysis Identifies Distinct Stages of Human Endothelial-to-Hematopoietic Transition

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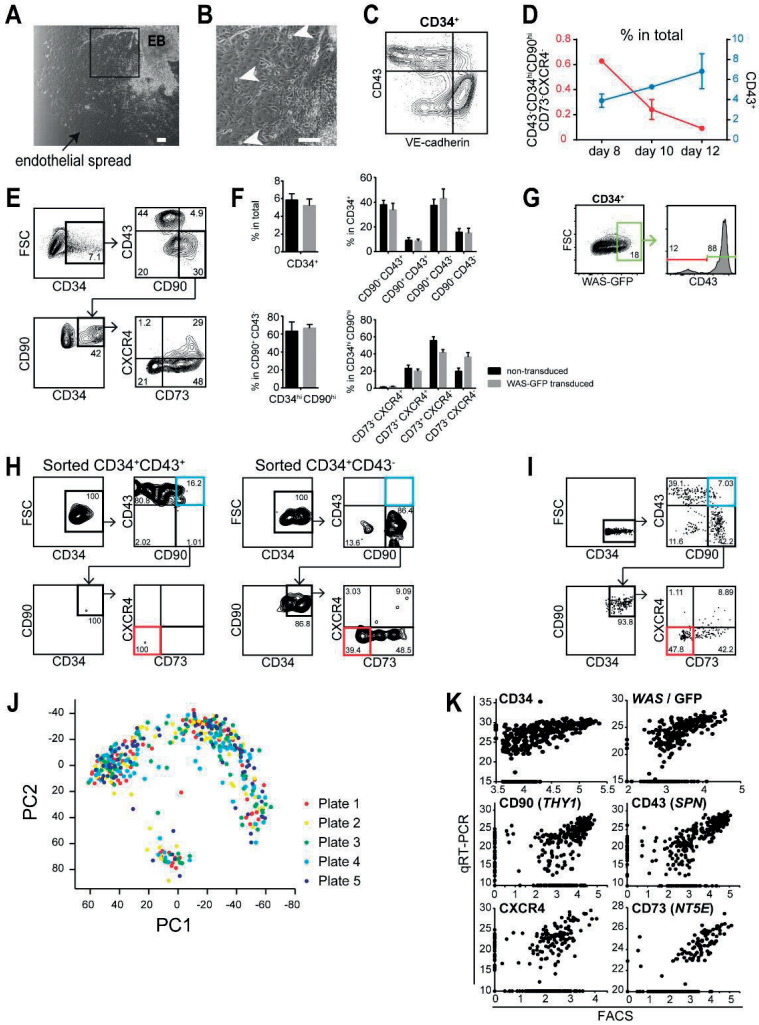

Figure S1. iPS differentiation cultures contain cells undergoing EHT, related to Figure 1

(A) Bright field micrograph of a representative endothelial spread, at day 10 of the differentiation protocol, from a plated EB.

(B) Close-up into the area outlined in A where emerging round cells can be observed (arrowheads). Scale bars: 100µm

(C) FACS profile showing CD43 and VE-cadherin expression in $CD34^+$ cells at day 10 of the differentiation protocol, including double-positive cells. Representative plot of n = 8 independent experiments.

(D) Time-course analysis of the Hemogenic Endothelium according to Ditadi et al. (2015) and general blood $CD43^+$ immunophenotypes in iPS-to-blood cultures; n = 2 independent experiments. Data is represented as mean ± SEM. Time-course analysis of this hematopoietic emergence phase revealed increasing frequencies of cells positive for the marker of early human hematopoietic cell commitment CD43, and decreasing frequencies of the previously

described HE. We have observed that hematopoietic cell emergence occurs in a broad temporal window in human iPS cell differentiation cultures, and since both endothelial monolayers and hematopoietic emergence can be observed at day 10, we predicted that this time-point would be optimal to study EHT, allowing us to capture cells of the whole EHT spectrum, from the HE cell to the more mature hematopoietic progenitors.
(E) Representative flow cytometry plots of non-transduced iPS line at day 10 of differentiation showing no difference in surface markers compared with the WAS-GFP transduced line (depicted in Figure 1B)
(F) Percentage of every intermediate gate depicted in (E), each bar graph positioned in accordance to the corresponding FACS plot. No significant difference was observed between transduced and non-transduced lines. Data is represented as mean ± SEM of n = 3 independent experiments.
(G) Representative flow cytometry plots of WAS-GFP expression in the $CD34^+$ fraction of day 10 iPS-to-blood cultures, displaying as expected a clear enrichment for CD43; representative of n = 3 independent experiments. The WAS-GFP reporter system expressing GFP under the control of proximal and distal promoters of the hematopoietic-specific Wiscott-Aldrich syndrome gene (*WAS*) has been developed to track hematopoietic emergence in hPSCs (Munoz et al., 2012). According to Munoz et al., in this system GFP correlated with hematopoietic potential and since its expression was also seen in $CD31^+CD45^-$ cells it constituted a promising tool for tracing EHT in hPSC differentiation cultures.
(H) Purity check for the two sorted populations, $CD34^+CD43^+$ and $CD34^+CD43^-$.
(I) Surface expression profile of the analysed cells, using the Index-sort data.
(J) Principal Component Analysis of all single cells, colored by individually run qRT-PCR plate.
(K) Consistency between expression levels of surface markers assessed by FACS markers and corresponding qRT-PCR-assessed transcripts.

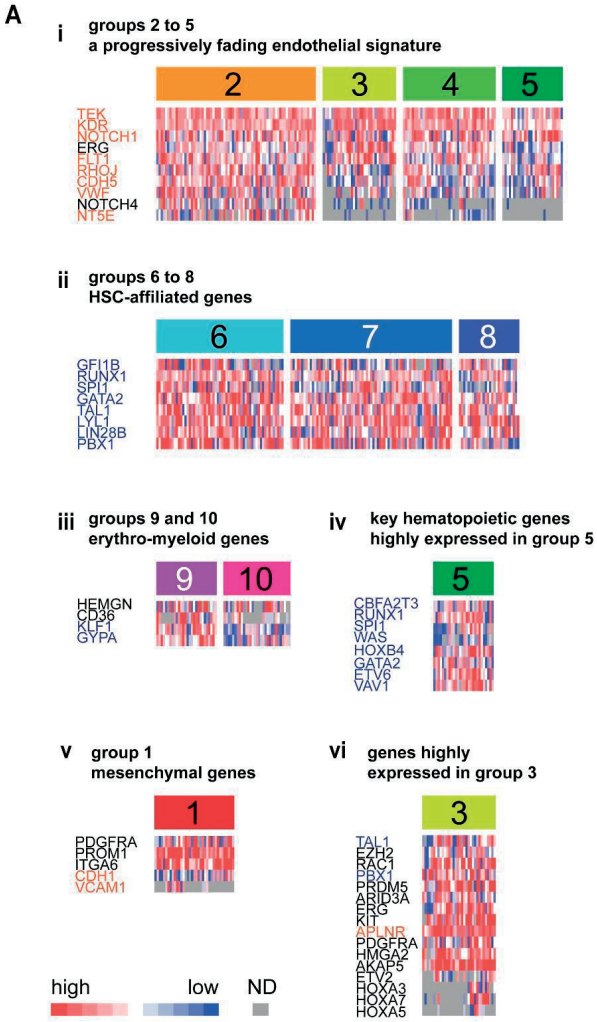

Figure S2. Several signatures can be identified in the single-cell gene expression analysis, related to Figure 1

(A i-vi) Magnification of specific gene lists relevant for the different transcriptional groups identified by single-cell qRT-PCR.

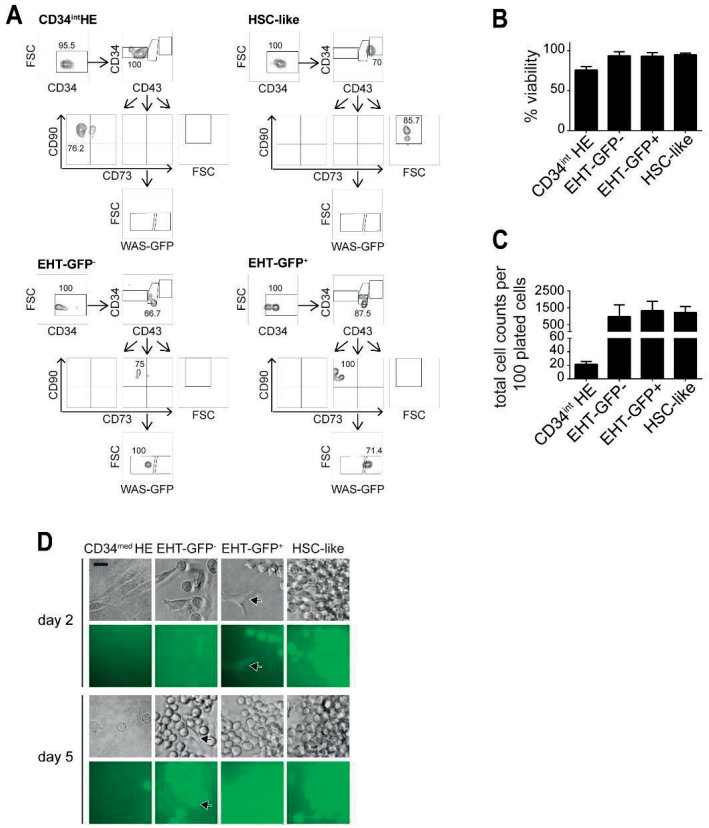

Figure S3. Differentiation potential assessment of identified transcriptional groups, related to Figure 3

(A) Purity check of sorted cells assessed in the subculture assay, representative of the 3 independent experiments.

(B) Percentage of viable cells resulting from 5 day sub-culture of each population. Data is represented as mean ± SEM of n = 3 independent experiments

(C) Total viable cell numbers per 100 plated cells after subculture of each cell population. Data is represented as mean ± SEM of n = 3 independent experiments

(D) Microscope pictures representing the morphology and GFP fluorescence of sorted cells during subculture of each sub-population. Arrowheads: endothelial spindle-shaped cells displaying GFP fluorescence. Day 2 corresponds to the day following aggregate plating; day 5 is the day of harvest for FACS analysis (see Experimental Procedures). Scale bar: 20µm.

Supplemental Table 1. List of genes analysed for single-cell qRT-PCR with corresponding reference and Taqman Assay, related to Figure 1

Gene	Reference	Taqman Assay #
ABCA1	GSE37000 (McKinney-Freeman et al., 2012)	Hs01059137_m1
ACE	(Zambidis et al., 2008)	Hs01104604_m1
AHR	(Oshima et al., 2011)	Hs00169233_m1
AKAP5	GSE37000(McKinney-Freeman et al., 2012)	Hs00270581_s1
APLNR	(Choi et al., 2012)	Hs00270873_s1
ARID3A	(Webb et al., 2011)	Hs00193296_m1
BMI1	(Park et al., 2003)	Hs00995536_m1
CBFA2T3	(Fischer et al., 2012)	Hs00602520_m1
CD226	(Choi et al., 2012)	Hs01040804_m1
CD34	(Majeti et al., 2007)	Hs00990732_m1
CD36	GSE37000(McKinney-Freeman et al., 2012)	Hs01567185_m1
CD38	(Majeti et al., 2007)	Hs01120071_m1
CDH1	GSE35395(Ruiz-Herguido et al., 2012)	Hs01023894_m1
CDH5	(Wang et al., 2004)	Hs00901463_m1
CDX4	(Lengerke et al., 2007)	Hs00193194_m1
CSF1R	GSE37000(McKinney-Freeman et al., 2012)	Hs00911250_m1
CXCR4	(Lapidot and Kollet, 2002)	Hs00607978_s1
EFNB2	(Wang et al., 1998)	Hs00187950_m1
EGR1	(Kobayashi et al., 2010)	Hs00152928_m1
ENG	(Perlingeiro, 2007)	Hs00923996_m1
EPHB4	(Wang et al., 1998)	Hs01119113_m1
ERG	(Kruse et al., 2009)	Hs01554629_m1
ETV2	(Kataoka et al., 2011)	Hs01012850_g1
ETV6	(Pereira et al., 2013)	Hs00231101_m1
EZH1	(Hidalgo et al., 2012)	Hs00940463_m1
EZH2	(Mochizuki-Kashio et al., 2011)	Hs00544833_m1
FLI1	(Kruse et al., 2009)	Hs00956711_m1
FLT1	(Rafii et al., 2003)	Hs01052961_m1
FOS	(Pereira et al., 2013)	Hs04194186_s1
FOXO1	(Tothova et al., 2007)	Hs01054576_m1
GAPDH	-	Hs02758991_g1
GATA2	(Kobayashi et al., 2010)	Hs00231119_m1
GFI1B	(Pereira et al., 2013)	Hs01062469_m1
GP68	GSE37000(McKinney-Freeman et al., 2012)	Hs00229941_m1
GPR56	(Solaimani Kartalaei et al., 2015)	Hs00938473_m1
GYPA	(Li et al., 2014)	Hs00266777_m1
HEMGN	(Oshima et al., 2011)	Hs01559754_m1
HEPH	GSE37000(McKinney-Freeman et al., 2012)	Hs00953259_m1
HES1	(Shojaei et al., 2005)	Hs00172878_m1
HEY2	(Rowlinson and Gering, 2010)	Hs01012057_m1
HHEX	(Wilson et al., 2009)	Hs00242160_m1
HIF3A	(Prashad et al., 2015)	Hs00541709_m1
HLF	(Shojaei et al., 2005)	Hs00171406_m1
HMGA2	(Kumar et al., 2013)	Hs00971724_m1
HOMER2	GSE37000(McKinney-Freeman et al., 2012)	Hs00191454_m1
HOXA3	(Iacovino et al., 2011)	Hs00601076_m1
HOXA5	GSE37000(McKinney-Freeman et al., 2012)	Hs00430330_m1
HOXA7	(Dou et al., 2012)	Hs00600844_m1
HOXB4	(Kyba et al., 2002)	Hs00256884_m1
ITGA2B	(Choi et al., 2012)	Hs01116228_m1
ITGA6	(Notta et al., 2011)	Hs01041011_m1
KCNJ5	GSE37000(McKinney-Freeman et al., 2012)	Hs00942581_m1
KDR	(Labastie et al., 1998)	Hs00911708_m1
KIT	(Labastie et al., 1998)	Hs00174029_m1
KLF1	(Bianchi et al., 2010)	Hs00610592_m1
LIN28B	(Yuan et al., 2012)	Hs01013729_m1
LYL1	(Wilson et al., 2009)	Hs01089802_g1
MAF	GSE37000(McKinney-Freeman et al., 2012)	Hs04185012_s1
MEIS1	(Kobayashi et al., 2010)	Hs01017441_m1
MEN1	(Novotny et al., 2009)	Hs00365720_m1
MPL	(Qian et al., 2007)	Hs01119304_m1
NFE2	(Wilson et al., 2009)	Hs00232351_m1
NOTCH1	(Kumano et al., 2003)	Hs01062014_m1

NOTCH3	(Bigas et al., 2013)	Hs01128541_m1
NOTCH4	(Bigas et al., 2013)	Hs00965889_m1
NT5E	(Choi et al., 2012)	Hs00159686_m1
OLFML3	GSE37000(McKinney-Freeman et al., 2012)	Hs00220180_m1
PBX1	(Oshima et al., 2011)	Hs00231228_m1
PDGFRA	(Davis et al., 2008)	Hs00998018_m1
PECAM1	(Wang et al., 2004)	Hs00169777_m1
PRDM16	(Aguilo et al., 2011)	Hs00922678_m1
PRDM5	(Riddell et al., 2014)	Hs00924602_m1
PRG2	GSE37000(McKinney-Freeman et al., 2012)	Hs00794928_m1
PROM1	GSE37000(McKinney-Freeman et al., 2012)	Hs01009250_m1
PTPRC	(Tavian et al., 1996)	Hs04189704_m1
RAC1	(Ghiaur et al., 2008)	Hs01588892_g1
RHOJ	GSE37000(McKinney-Freeman et al., 2012)	Hs00368156_m1
RUNX1	(Chen et al., 2009)	Hs01021971_m1
SLC40A1	GSE37000(McKinney-Freeman et al., 2012)	Hs00205888_m1
SOX17	(Kim et al., 2007)	Hs00751752_s1
SPI1	(Huang et al., 2008)	Hs02786711_m1
SPN	(Vodyanik et al., 2006)	Hs01872322_s1
TAL1	(Shivdasani et al., 1995)	Hs01097987_m1
TEK	(Arai et al., 2004)	Hs00945146_m1
THY1	(Majeti et al., 2007)	Hs00264235_s1
TREML2	(DeVilbiss et al., 2013)	Hs01077557_m1
WAS	(Parolini et al., 1997)	Hs00997437_m1
VAV1	(Katzav, 2007)	Hs01041613_m1
VCAM-1	(Marshall and Thrasher, 2001)	Hs01003372_m1
VWF	GSE37000(McKinney-Freeman et al., 2012)	Hs01109446_m1
ZFP37	GSE37000(McKinney-Freeman et al., 2012)	Hs00185659_m1

Supplemental Experimental Procedures

iPS cell expansion

The iPS line was maintained in co-culture with irradiated murine embryonic fibroblasts in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 supplemented with 20% KnockOut-serum replacement, Glutamax, Non-Essential Amino Acids, 50 μM beta-mercaptoethanol and 10 ng/mL Fgf2 (all from Life Technologies) in a humidified incubator at 37ºC, 5% CO2. Cells were passaged every six days using 1mg/ml Collagenase IV (Life Technologies). During two days preceding initiation of the blood differentiation protocol, the cultures were treated with 3 μM CHIR99021 (R&D Systems Europe). For inducing differentiation, the iPSC colonies were incubated in 500 μg/ml Dispase (Life Technologies) for 40 minutes at 37ºC and resulting embryoid bodies (EBs) were transferred into ultra-low attachment culture dishes (Corning) in Mesototal medium (Primorigen Biosciences, Wisconsin). At day eight the EBs were plated on Matrigel-coated dishes (8 μg/cm2, Corning) in order to attach and spread a monolayer. Fresh Mesototal medium was added every second day throughout the protocol. Cell cultures were observed on an Olympus IX51 microscope, and pictures acquired with an Infinity 2 camera and Infinity Analyze software (Lumenera).

WAS-GFP (AWE) transduction

The AWE plasmid (Munoz et al., 2012), eliciting GFP expression under the control of the *WAS* gene promoter sequences, was obtained from Dr. Francisco Martín Molina (Pfizer Center of Granada University, Spain); lentivirus production was performed by the Vector Unit at the Lund Stem Cell Center. Singularized iPS-CB1RB9 cell line was transduced at 200 multiplicity of infection in total volume of 100 μl for one hour with agitation every 15 minutes and plated on mouse embryonic fibroblasts for expansion. Individual colonies were then picked and expanded separately in order to obtain clonal transduced lines, used for subsequent experiments. All lines used in this study were shown to be karyotypically normal.

Flow Cytometry

On the day of analysis the iPS-derived cells were singularized and analyzed on LSRII (BD Biosciences) – for sorting Aria III (BD Biosciences) was used – and flow cytometry data was acquired with Diva Software. The following antibodies were used to stain the cells: CD43-APC-H7 (clone 1G10, BD-Biosciences), CXCR4-BV421 (clone 12G5), CD90-BV605 (clone 5E10), CD34-PECy7 (clone 581), CD45-APC (clone HI30), CD41-APC-Cy7 (clone HIP8), all from BioLegend, CD34-APC (clone 4H11, eBioscience), CD73-PE (clone AD2, BD), CD144-PerCP-Cy5.5 (clone 55-7H1, BD Biosciences), and Glycophorin A –eFluor450 (clone 6A7M, eBioscience); exclusion of dead cells was performed by assessing 7-aminoactinomycin D (Sigma Aldrich Sweden) uptake. Flow cytometry data was analysed on FlowJo Software (Flowjo, LLC).

Single-cell qRT-PCR

Single $CD34^+$ cells were sorted directly into 96-well PCR plates (Sarstedt), using the Index-Sorting function of the Diva Software, into 4 μl of lysis buffer [65 μM of dNTP mix (Takara), 0.4% of NP40 Cell Lysis Buffer, 2.4 mM dithiothreitol, 0.5 U/μl RNAseOUT (Life Technologies) in nuclease-free water]. Target-specific pre-amplification was then performed using CellsDirect One-Step qRT-PCR Kit (Life Technologies). Pre-amplification mastermix (6.25 μl of 2X Reaction Buffer, 1 μl of SuperScript III RT/ Platinum Taq mix, and 1.5 μl of Taqman assays equal volume pool) was then added to each sample and reverse transcription was performed followed by pre-amplification in a Biorad T100 thermal cycler using the following program: 1h at 50ºC, 2 min at 95ºC and 25 cycles of 15 sec at 95ºC and 4 min at 60ºC. No-RT controls were treated in parallel containing only Taq Polymerase (Life Technologies) at 0.5 U/μl. Pre-amplified samples were diluted 1:5 and run in Fluidigm 96.96 arrays on a Biomark device (Fluidigm), with Taqman universal MasterMix (Life Technologies) and the Taqman assays listed in Supplemental Table 1, using the appropriate loading reagents according to manufacturer's instructions. Positive controls (10 cells) and no template controls were also included in each 96-well plate. The transcripts analysed were selected from the literature on endothelium, hematopoietic stem and progenitor cells, EHT and from our own

analysis of two GEO datasets [murine AGM and YS-specific HSC-affiliated genes from GSE37000 (McKinney-Freeman et al., 2012) and genes upregulated during murine EHT from GSE35395 (Ruiz-Herguido et al., 2012)].

CFU assay

At day 10 of differentiation, cells sorted according to the gates in Figure 2F were plated in Methocult H4230 (STEMCELL Technologies) supplemented with 2.5 mg/ml human Stem Cell Factor, 2.5 mg/ml human interleukin-3, 5 mg/ml human granulocyte-macrophage colony-stimulating factor, and 500 U/ml erythropoietin, all recombinant human cytokines from PeproTech. After 12 days of culture in a humidified incubator at 37ºC, 5% CO2, the colonies were scored morphologically.

Subculture assay

For testing endothelial and hematopoietic potential, at day 10 of the differentiation protocol the cells were enriched for $CD34^+$ using the CD34 MicroBead Kit (Miltenyi Biotec), and the indicated populations were sorted and cultured in an assay adapted from Ditadi and Sturgeon (2016): the cells were sorted into serum-free assay media consisting of Stempro34 (Life Technologies) supplemented with L-glutamine (2 mM, Thermo Scientific), 50 µg/ml Ascorbic acid (Sigma), 150 µg/ml Holotransferrin (Sigma), 50 µM beta-mercaptoethanol, 5 ng/ml VEGF (Humanzyme), 5 ng/ml bFGF (Life Technologies), 100 ng/ml SCF, 10 ng/ml IL6, 30 ng/ml TPO, 10 ng/ml FLT3 ligand, 30 ng/ml IL3, 2 U/ml EPO (all from PeproTech), 10 ng/ml BMP4, 5 ng/ml IL11, 25 ng/ml IGF-1, 20 ng/ml Sonic Hedgehog (all from R&D Systems), 10 µg/ml Angiotensin II (Sigma), 100 µM Losartan Potassium (Tocris). Each sample of 200 to 500 cells was then kept overnight in ultra-low adherence U-shaped bottom wells (Sigma-Aldrich Sweden) in assay media containing 0,25% Polyvinyl Alcohol (prepared according to Ng et al. (2008)), to allow the formation of aggregates that were then transferred onto Matrigel (8 µg/cm2, Corning)-coated flat bottom 96-well plates. Pictures were taken every day using an Olympus IX70 microscope equipped with a CellSens DP72 camera and CellSens Standard 1.6 software (Olympus). At five days post-sort the floating fraction of cells was collected and the remaining attached cells were singularized using Tryple Express (Thermo Fisher Scientific) incubation; visual inspection in the microscope ensured that no cells were left behind. For FACS analysis the samples were re-suspended in high volume of FACS buffer (500-1000µl) and the whole volume of sample recorded so that virtually every single cell present in each sample was analysed and quantified.

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Paper II

Human Hematopoietic Cell Emergence during Development is Modulated by Norepinephrine Signaling Independent of β2-Adrenergic Receptor

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Abstract

The Peripheral Nervous System (PNS) has been suggested, in murine models, to be a novel determinant for HSC emergence during embryogenesis, as well as a regulator of HSC movement in and out of the adult bone marrow. Norepinephrine (NE) and its signaling through the β2-adrenergic receptor (ADRB2) have been proposed to mediate both these processes. In this study we show for the first time in the human setting the neurogenic potential of the dorsal aorta region in two human fetuses suggesting the presence of PNS cells in the AGM region at the time of HSC emergence in the embryo. We also show that NE, a factor associated with PNS signaling, added to human iPS differentiation cultures robustly increased the frequency of hematopoietic cells with an HSC-like phenotype, suggesting its involvement in hematopoietic cell emergence. We then proceeded to assess which adrenergic receptor is mediating this effect, using both gene expression analysis and specific inhibitors and agonists for the different receptors. We concluded that the observed NE effect is independent of ADRB2; and propose a mechanism where both ADRB3 and ADRA2c are involved in endothelial-to-hematopoietic transition, while activation of ADRA2a prevents downstream differentiation of emerging immature progenitors. Together, these results show that adrenergic signaling is an important pathway for human HSC specification in development, and specifically *in vitro* from hPS cells.

Introduction

In murine development, the generation of definitive, transplantable Hematopoietic Stem Cells (HSCs) with lympho-myeloid adult repopulating ability occurs at day 10.5 post coitum (E10.5). These cells, capable of adult hematopoiesis, first appear in the aorta-gonad-mesonephros (AGM) region of the embryo proper (Muller et al., 1994), suggesting that particular signals from the AGM region enable *de novo* generation, survival and maturation of transplantable HSCs. In contrast, the hematopoietic progenitors emerging from the yolk sac (YS) at earlier time point during development are not capable of lympho-myeloid reconstitution of adult recipients (Moore and Metcalf, 1970). In the human embryo, a similar pattern of development has been suggested, with the YS giving rise initially to primitive lineage-restricted hematopoietic cells (Huyhn et al., 1995; Tavian et al., 2001), and later the cells emerging in the major vessels of the Splanchnopleura/AGM region being endowed with definitive hematopoietic potential (Ivanovs et al., 2011). In the AGM, specifically, the first HSCs have been shown to emerge from the endothelium lining the ventral side of the dorsal aorta (DA) (Boisset et al., 2010; Ivanovs et al., 2014; Taoudi and Medvinsky, 2007). This process of emergence of the HSCs from an endothelial precursor has been coined endothelial-to-hematopoietic transition (Eilken et al., 2009; Kissa and Herbomel, 2010).

Several studies have recently reported important functions of the peripheral nervous system (PNS) for hematopoiesis, in HSC function as well as in HSC specification during development. Direct interaction of the PNS with HSCs has also been suggested given the expression of the β2-adrenergic receptor (ADRB2) on HSCs, reported in human cord-blood $CD34^+$ $CD38^-$ cells (Spiegel et al., 2007), as well as on nascent HSCs in the AGM region of mice (Fitch et al., 2012). Spiegel et al. (2007) report in addition

that this receptor is up-regulated in mobilized HSCs and upon G-CSF and GM-CSF *in vitro* exposure, suggesting an important role of adrenergic signaling for HSC egress from the BM. Indeed, injection of epinephrine in mice increased HSC egress from the BM. The same article also reported that exposure of cord-blood $CD34^{+}CD38^{-}$ cells to catecholamines enhanced their motility *in vitro* as well as their ability to repopulate NOD-SCID mice (Spiegel et al., 2007).

Likewise, regarding the adult BM niche and HSC mobilization, it has been shown that synchronization of HSC egress from the BM with the circadian rhythm is mediated by norepinephrine (NE)-secreting sympathetic fibers in the bone marrow, which regulate *cxcl12* expression in the niche through activation of β3-adrenergic receptor on stromal cells (Mendez-Ferrer et al., 2008). The relevance of the adult bone marrow PNS components for HSC function was further revealed by the importance of adrenergic innervation for BM hematopoietic recovery following genotoxic insults (Lucas et al., 2013), as well as by the evidence of TGF-β signaling regulation by neural crest-derived non-myelinating Schwann cells, essential for bone marrow HSC maintenance (Yamazaki et al., 2011).

During development, an interaction between the developing PNS and HSC emergence in the AGM was suggested by Nagoshi et al. (2008). In this study the authors use a neural crest-specific reporter mouse model, and show the spatial and temporal correlation of Neural Crest migration and maturation into TH-expressing cells in the vicinity of the dorsal aorta, with the emergence of the first HSCs (Nagoshi et al., 2008). These Neural Crest structures contain the precursors for the peripheral nervous system and are gradually established next to the dorsal aorta (DA), in the mouse, from E8.5 to E14.5. They start maturing and expressing tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine production, from E10.5 onwards [reviewed in (Ruhrberg and

Schwarz, 2010)], coinciding developmentally with the emergence of the first hematopoietic cells from the ventral side of the DA (Muller et al., 1994; Taoudi and Medvinsky, 2007).

The finding of Nagoshi et al. (2008) suggest to us that there is a connection between the developing PNS and the emergence of the first HSCs in the DA; that neural crest-derived cells provide signals to the DA, triggering hematopoietic cell emergence and conceivably promoting their maturation into functional HSCs. This hypothesis was recently confirmed by Fitch et. al (2012), in a Gata3$^{-/-}$ mouse study, where secretion of catecholamines by the developing PNS was indeed demonstrated to be essential for the emergence and function of the first HSCs.

Here we report spontaneous outgrowth of neurospheres following the harvest and culture of human fetal cells from the DA in the AGM region of two human fetuses, revealing neural potential in the vicinity of the human AGM region. Moreover, addition of the most abundant neurotransmitter in the PNS, the catecholamine NE, to our *in vitro* human ES/iPS cell differentiation towards the hematopoietic lineage significantly increases the frequency of cells displaying the immunophenotype of the most immature progenitors. The modulation of this signaling, by addition of specific agonists and antagonists, revealed that both beta- and alpha2-adrenergic signaling could mediate this effect. Addition of the specific ADRB2 antagonist did not revert NE effects, showing that it is unlikely to be mediated through this receptor, contrarily to what has been suggested in murine development.

Results

Detection of neural lineage potential in the AGM of two human fetuses

With the goal to identify the different cell types present in the AGM region during human fetal development, we generated primary cell cultures specifically from the DA and urogenital ridge (UR) regions, of human fetuses (Figure 1A). Two fetuses, at 42 and 58 days of gestation, were dissected. Following harvest and gentle mechanical dissociation of the tissues, cells were plated in adherent cell culture conditions and allowed to expand. Explants from the different regions were plated into separate wells. As expected, for both fetuses, most cells expanding from the explants had stromal or endothelial morphologies (Figure 1B-i, ii). Interestingly, explants from the DA region of both fetuses gave rise to structures reminiscent of neurospheres (Figure1B-iii, iv). Staining of these neurosphere-like structures for MAP2, a pan-neuronal marker for mature neurons, and GFAP, marker for glial cells, revealed neurogenic potential of the starting material (Figure 1C).

The cell culture conditions not being neural lineage-inducing, suggests that the DA and UR derived tissues contained committed neural lineage progenitor cells, able to survive, proliferate and differentiate into both glial and neuronal progenies. Given that the only known structure in the vicinity of the dorsal aorta in the AGM region containing neural lineage cells in the developing embryo are the sympathetic ganglia (Ruhrberg and Schwarz, 2010), we hypothesized that the observed structures resulted from the expansion and maturation of those trunk neural crest stem cell-derived precursors of the sympathetic nervous system. The close proximity of neural lineage cells to sites of *de novo* HSC emergence and maturation led us to hypothesize that these cells play a

role in hematopoietic development, similar to what has been suggested in the mouse (Fitch et al., 2012).

Norepinephrine added to hPS-to-blood at the time of emergence increases frequencies of HSC phenotype and CFU potential

Since we had identified neural crest-derived progeny in the proximity of developmental areas harboring *de novo* hematopoietic emergence, we then decided to test adding the most abundantly secreted factor produced by the neural crest-derived peripheral nervous system, NE, to our hPSC differentiation system. We also investigated whether the cells produced in our differentiation protocol express receptors for NE, which would be indicative of their ability to respond to NE (Figure 2A).

For this we performed RT-qPCR for the 9 adrenergic receptors in sorted populations, enriching for the following cell types: hemogenic endothelium (HE, $CD43^{-}CD34^{hi}CD90^{hi}CXCR4^{-}CD73^{-}$ according to (Kennedy et al., 2012)), HSC-like cells ($CD43^{+}CD34^{+}CD38^{-}CD90^{+}$), hematopoietic progenitors ($CD43^{+}CD34^{+}CD90^{-}$), mature hematopoietic cells ($CD43^{+}CD34^{-}$), mesenchymal stromal like cells ($CD43^{-}CD34^{-}CD90^{+}CD73^{+}$) and the remaining cells (Supplemental Figure 1). Apart from *ADRA1a* and *ADRA2b*, all receptors were detected in at least one of the populations of interest, suggesting these cells should be capable of responding to NE (Figure 2B). Notably, HE expressed *ADRA1d*, *ADRA2c*, *ADRB2* and *ADRB3*, that could play a role in the regulation of blood emergence. In addition to *ADRA2a*, the HSC-like population displayed expression of *ADRB2* in accordance to what has been previously reported (Fitch et al., 2012; Spiegel et al., 2007).

We applied the receptor saturating concentration of NE, 300μM (Belin de Chantemele et al., 2009; Xiao et al., 2003), at the start of visible emergence of hematopoietic cells, continuously until harvest at which point we assessed for hematopoietic output (Figure 2A). Hematopoietic output was then assessed by measuring the frequency of three different hematopoietic populations: whole blood ($CD43/45^+$ cells), $CD34^+$ progenitors within $CD43/45^+$blood, and HSC-like $CD43^+CD34^+CD38^-CD90^+CD45RA^-$ cells. We have previously shown that the latter population harbors cells with lymphoid and myeloid differentiation ability (Ronn et al., 2015) and displays surface makers that are known to be present on engrafting HSCs in the AGM (Ivanovs et al., 2014). We therefore call it HSC-like immunophenotype (Figure 2C). We observed that while the frequency of general blood was not significantly affected by addition of NE, the frequency of $CD34^+$ progenitors in blood showed a slight but significant increase (Figure 2D). Interestingly, this frequency increase was most pronounced in the most immature HSC-like progenitor fraction (Figure 2D). When the obtained cells were plated in methylcellulose for assessing CFU frequency, we also observed a significant increase in colony formation by the cells generated in the presence of NE (Figure 2E), although the distribution of colony types was not affected (Figure 2F).

These data indicate that NE signaling promotes hematopoietic development in hPSC differentiation cultures. We then proceeded to identify which receptors are involved in this signaling.

Modulation of adrenergic signaling affects blood generation, independent of ADRB2

The β2-adrenergic receptor (ADRB2) has been reported previously to be expressed on human cord-blood HSCs (Spiegel et al., 2007), as well as on nascent HSCs in the E10.5 AGM of murine embryos, where it was hypothesized to mediate NE signaling needed for HSC emergence (Fitch et al., 2012). Moreover, we observed that it is expressed in our HE and HAC-like cells (Figure 2B).

We therefore sought out to evaluate the role of beta-adrenergic receptors (ADRBs) in the observed effect of NE. For this, we performed the differentiation assay in the presence of NE as well as of the beta-adrenergic agonist Isoproterenol. We observed that this generic activation of ADRBs appears to mimic the action of NE (Figure 3A-C, green column). Addition of the ADRB generic antagonist Propranolol generally decreased blood generation (Figure 3A, red column). Together these results indicate that ADRBs play a role in hematopoietic generation *in vitro*. Interestingly, addition of a ADRB2-specific inhibitor, ICI118,551 did not reverse the effect of NE, suggesting that this receptor is not a crucial mediator of the observed NE effect (Figure 3A-C, orange column). This is contrary to what has been reported in the murine system. Our data suggests that ADRB3 may mediate this effect (given that according to the transcriptional data, *ADRB1* is not expressed in HE cells, see Figure 2B).

Unlike ADRB and ADRA1 knock-out mice, ADRA2 triple knock-out mouse model has been shown to give rise to an embryonic lethal phenotype, between day E9.5 and E11.5 (Philipp et al., 2002). This indicates that ADRA2 receptors could also be involved in hematopoietic cell emergence. We tested this hypothesis by performing our differentiation assay in the presence of NE as well as ADRA2 agonists and antagonists (Figure 3D-F). Although the addition of the ADRA2 agonist Medetomidine did not have a clear effect in blood generation, the addition of the ADRA2 antagonist Yohimbine increased the frequency of general blood and decreased the frequency of

hematopoietic progenitors (both $CD34^+$ and HSC-like immunophenotype) in a dose-dependent fashion (Figure 3D-F, red columns). This suggests that inhibition of the ADRA2 receptors leads to differentiation of immature progenitors. The general ADRA1 inhibitor Doxazosin had no effect in general blood frequency nor in early progenitors, showing instead general toxicity in the cultures (data not shown), which excluded this set of receptors from our analysis.

Discussion

Nagoshi et al. (2008) report the detection of neural crest stem cells at several developmental hematopoietic locations. These cells were then suggested to migrate to the sites of hematopoiesis during development from the AGM, to the fetal liver, and then to the bone marrow, at the same time as HSC migration to these organs during embryonic development (Nagoshi et al., 2008). We show in the present study that during human development too, neural progenitors are present at proximity of the dorsal aorta in the AGM. We hypothesized from these anatomical associations between hematopoietic cells and neural crest, that there would be a requirement for HSCs to be in proximity to the neural crest stem cells and progeny during their development. In the present study we sought to address the question whether addition of NE, the neurotransmitter most commonly secreted by neural crest derivatives, would support blood emergence during human development.

Catecholamines secreted by the developing peripheral nervous system were shown to regulate emergence of HSCs in the murine AGM region (Fitch et al., 2012). In the model in question, a catecholamine-deficient $Gata3^{-/-}$ phenotype showing reduced levels of HSC emergence in the AGM was rescued using adrenergic receptor agonists. However, the mechanism of this signaling was not verified in the study, being only reported the expression of ADRB2 on the nascent HSCs (Fitch et al., 2012). In the present report, we also show that this receptor is expressed on HSC-like cells. In addition, we tested inhibiting this receptor upon addition of NE and observed that it did not prevent the positive effect of NE on the HSC immunophenotypic fraction in our cultures. Therefore, other adrenergic receptors must be mediating the increase in frequency of the HSC-like fraction. According to our data, it could be ADRB3 given that it is also expressed in HE cells and that the ADRB general agonist Isoproterenol

mimicked NE action. A general role of ADRBs is further supported by a recent publication in our laboratory suggesting that increased intracellular cAMP, secondary messenger downstream from ADRBs, mediates hematopoietic cell emergence and maintenance of HSC-like progenitors (Saxena et al., 2016).

Catecholamine signaling is essential for mammalian development, as suggested by the E11.5 embryonic lethal phenotype of transgenic mice lacking tyrosine hydroxylase (TH), the rate-limiting enzyme for the production of all catecholamines (Zhou et al., 1995). All mouse models lacking individual adrenergic receptors are viable and fertile, with the exception of ADRA2B, for which homozygous animals, although viable and fertile, are born at levels below Mendelian ratios and display poor survival (Link et al., 1996). This receptor was however not detected in our differentiation cultures. Interestingly, while triple knock-out mouse models lacking all β-adrenergic receptors or lacking all three α1-adrenergic receptors are viable (Sanbe et al., 2009; Stockigt et al., 2012), knocking out all three α2-adrenergic receptors gives rise to an embryonic lethal phenotype, between day E9.5 and E11.5, without any cardiac abnormalities (Philipp et al., 2002). Philipp et al (2002) report in addition severe defects in yolk sac and placental blood vessels, particularly with defect in the cohesion of endothelial tissue, and lower amounts of circulating blood cells, relating mortality to defects in placental function. This strong phenotype of ADRA2 triple knock-out mice prompted us to test how modulating these receptors could affect hematopoietic emergence. We observed that while generic activation of these ADRA2 receptors had little or no effect in blood generation, their inhibition increased the frequency of mature blood cells at the expense of immature progenitors. We propose that this is due to differentiation of the progenitors into mature cells, and that it is regulated by ADRA2a (expressed by

HSC-like cells in our cultures, see Figure 2B). This does not exclude that ADRA2c, expressed by HE cells could also be involved in endothelial to hematopoietic transition.

Together, our results suggest that catecholamine signaling has an important role in human hematopoietic development and that this effect is mediated through both ADRB and ADRA2 receptors. As ADRA2 receptor signaling has a mostly antagonizing effect on the downstream cAMP and calcium-mediated pathways, it is possible that on the HE cells these receptors' function is to provide a negative feed-back, counter-balance and modulate ADRB signaling. We therefore propose a model where in HE cells, NE signals through ADRB3 receptor, leading to downstream cAMP production, and that a negative feed-back provided by the simultaneous activation of ADRA2c receptor is required for a balanced regulation of the pathway. After endothelial-to-hematopoietic transition, HSC-like cells express ADRA2a, which seems to play a role in preventing their differentiation into mature progeny (Figure 4).

By showing neural potential in the AGM regions of two human fetuses, and by showing a positive effect of norepinephrine for *in vitro* generation of cells with HSC immunophenotype, we show for the first time that adrenergic signaling is a relevant pathway modulating human hematopoietic development and *in vitro* generation of hematopoietic cells. Moreover, our study reveals an important role of the sympathetic nervous system in hematopoietic development in the human setting.

Material and Methods

Human Fetus dissection and explants cultures

Two fetuses were analyzed, of 42 and 58 days of gestation. After removing the extremities and the fetal liver, the embryo was cut transversally using a cryotome blade to produce a flat middle section at the level of the trunk (between hind limbs and forelimbs). Using sterile 10-100ul pipet tips, the section was punctured to produce small explants from the urogenital ridges, as well as from the aorta and surrounding mesenchyme. These were separately collected in eppendorf tubes containing the following culture media: DMEM/F12 with 15% KO Serum, 1% non-essential aminoacids, 1% Glutamax, BME (0.7 ul), Penicilline/Strepomycin and Fungizone. Following harvest and gentle mechanical dissociation, the resulting suspensions were plated in 24-well tissue culture-treated plates coated with 0.1% gelatin. Media was changed every 24 hours (Fungizone discontinued after 48 hours).

ES and iPS cell expansion and hematopoietic differentiation

Human ES (H1, Hues3 and Hues2) and iPS (RB9-CB1) cell lines were expanded and differentiated towards the hematopoietic lineage as described in (Ronn et al., 2015). After EB plating at day 8 of differentiation, the cultures were monitored daily, to track endothelial sheet spreading and emerging of the first hematopoietic cells. At that time of first emergence were added the adrenergic stimulating/inhibiting compounds (Norepinephrine at 300µM from Sigma, ICI118,551 at 20µM, Isoproterenol at 10µM, Propranolol at 20µM, Medetomidine at 20µM and 100µM, and Yohimbine at 20µM

and 40 μM all from Tocris). The compounds were added twice, every second day before harvest.

Colony Forming Unit assay

CFU potential of the cells obtained at the end of the protocol was assessed by plating 20 000 cells in Methocult H4230 (Stem Cell Technologies) supplemented with IMDM (Thermo Scientific) and human SCF (25ng/ml), GM-CSF (50ng/ml), IL3 (25ng/ml) and Erythropoietin (5U/ml). Cells were incubated in humidified incubator at 37°C with 5% CO_2 for 14 days and colonies were then visualized and quantified using bright-field microscopy.

FACS analysis

At the end of the protocol, the cell culture was harvested and FACS-analysed as described in (Ronn et al., 2015). The antibodies used were the following: CD43-FITC (BD Biosciences), CD45-FITC (BD Biosciences), CD34-PECy7 (Biolegend), CD38-APC (Biolegend), CD90-PE (Biolegend), CD45RA-V450 (BD Biosciences). Neural crest stem cell percentage was assessed with p75 and HNK1. 7-aminoactinomycin D (7AAD) was added to the samples prior analysis for exclusion of $7AAD^+$ dead cells.

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Figure Legends

Figure 1: Detection of neural potential of human fetus AGM

A – Scheme of a transversal section of the AGM region of a human fetus. AGM – aorta-gonad-mesonephros; DA – dorsal aorta; UG – Urogenital Ridges

B – Representative micrographs of cellular growth following culture of AGM explants dissected from human fetuses. i: outgrowth with endothelial morphology; ii: outgrowth of stromal morphology; iii: outgrowth with neurosphere morphology from the DA region of the 42 days-old fetus; iv: outgrowth with neurosphere morphology from the DA region of the 58 days-old fetus. i and ii were taken from explants of the UG region of the 58 days-old fetus

C – Fluorescence microscopy pictures of the neurosphere-like structures stained for MAP2, GFAP and DAPI.

Figure 2: Norepinephrine added to hPS-to-blood at the time of emergence increases frequencies of HSC-like phenotype, and CFU potential

A – Time-line of the hPSC-to-blood differentiation experiment, with Norepinephrine added for the last 4 days of the protocol (from time of emergence to endpoint)

B – RTq-PCR for the 9 adrenergic receptors in sorted hPSC-derived populations

C – Gating strategy for the FACS analysis at the endpoint of the hES-to-blood differentiation protocol

D – Box and whisker plot of the fold difference (NE vs controls) of the stated frequencies: blood in viable, $CD34^+$ in blood, HSC-like immunophenotype in blood, HSC-like immunophenotype in viable. N = 11 independent experiments. * $p<0.05$ (one-sample T-test comparing to theoretical value 1) p values are stated at the bottom.

E – Box and whisker plot of the CFU counts per 20 000 plated cells in methylcellulose, N = 7 independent experiments.

E – Bar graph of the average colony type distributions registered in the experiments shown in D. Error bars correspond to S.E.M. (N = 7).

Figure 3: Dissecting NE signaling through modulation of beta and alpha2-adrenergic receptors

A – Bar graph of fold difference (treated vs control) of the % of blood in viable for the conditions modulating beta-adrenergic signaling stated at the bottom of the Figure.

B – Bar graph of fold difference (treated vs control) of the % of CD34+ in blood for the conditions modulating beta-adrenergic signaling stated at the bottom of the Figure.

C – Bar graph of fold difference (treated vs control) of the % of HSC-like immunophenotype in blood for the conditions modulating beta-adrenergic signaling stated at the bottom of the Figure.

D – Bar graph of fold difference (treated vs control) of the % of blood in viable for the conditions modulating alpha2-adrenergic signaling stated at the bottom of the Figure.

E – Bar graph of fold difference (treated vs control) of the % of CD34+ in blood for the conditions modulating alpha2-adrenergic signaling stated at the bottom of the Figure.

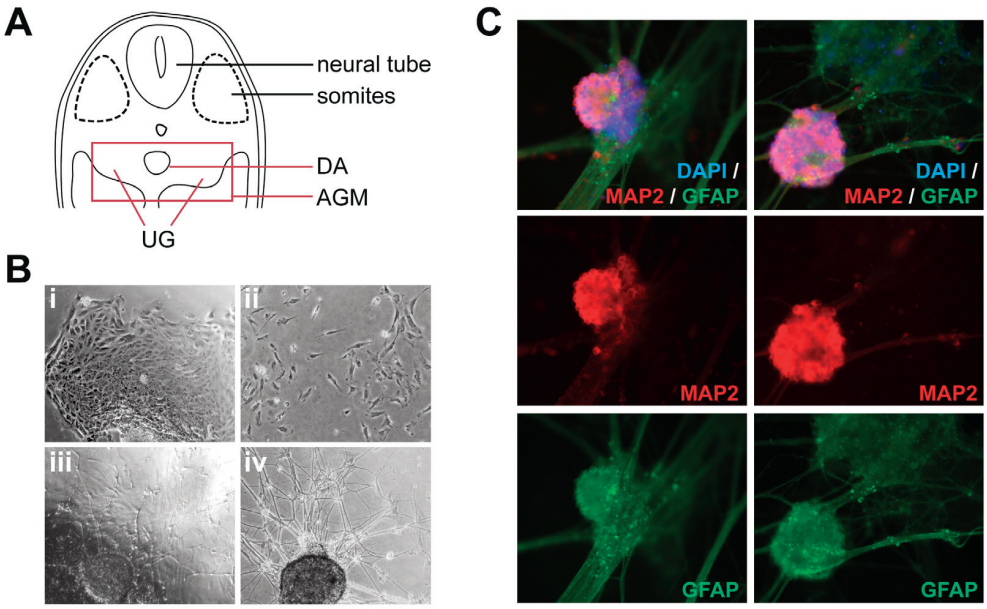
F – Bar graph of fold difference (treated vs control) of the % of HSC-like immunophenotype in blood for the conditions modulating alpha2-adrenergic signaling stated at the bottom of the Figure.

(N = 1 to 5 independent experiments)

Figure 4: Proposed mechanism for adrenergic receptor signaling in hPSC-derived blood emergence

Figure S1 (refers to Figure 2): Gating strategy to sort populations of interest for qRT-PCR

Figure 1


A
neural tube
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DA
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i
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DAPI /
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Figure 2

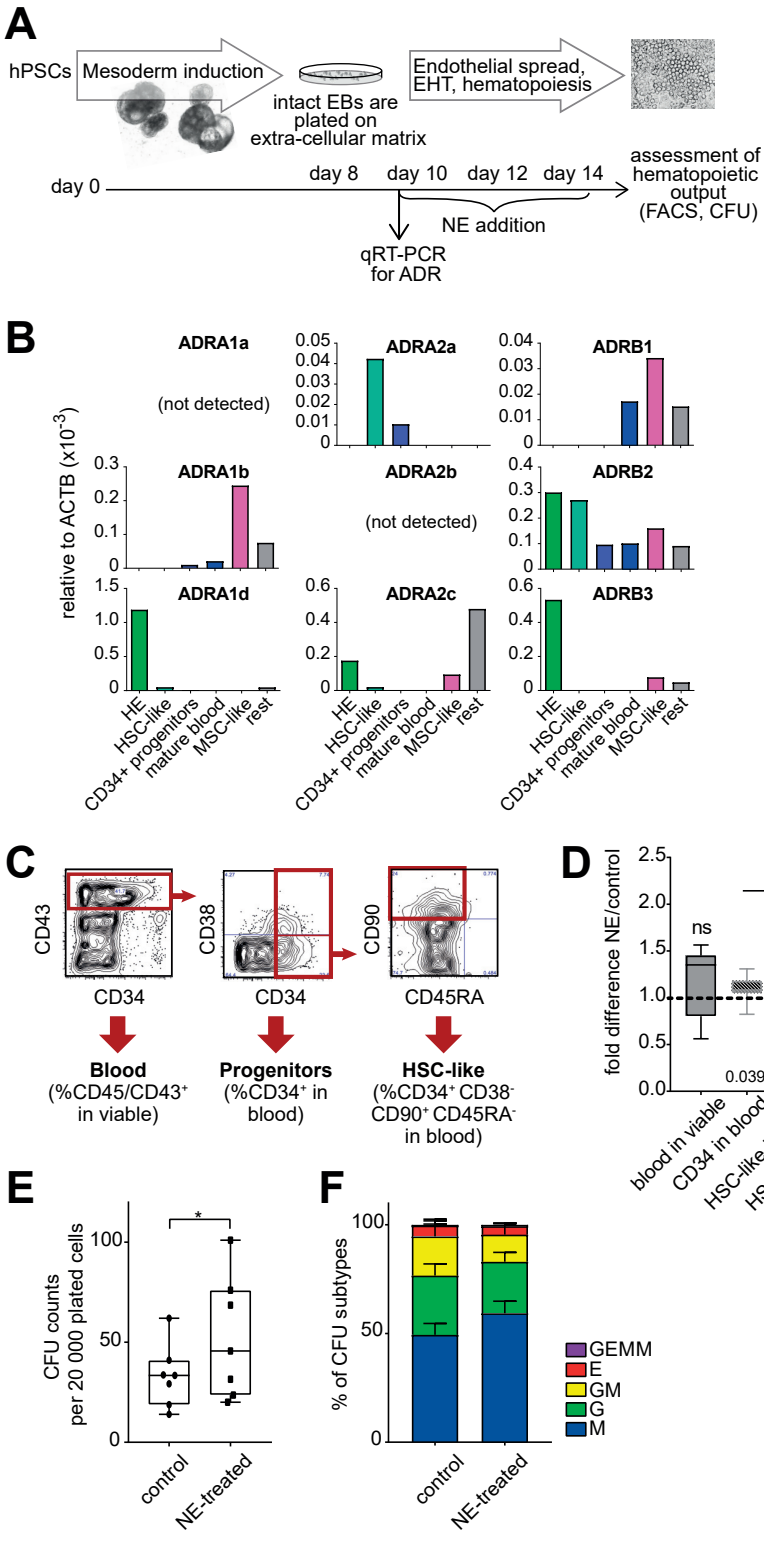

A
hPSCs
Mesoderm induction
intact EBs are plated on extra-cellular matrix
Endothelial spread, EHT, hematopoiesis
day 0
day 8
day 10
day 12
day 14
NE addition
qRT-PCR for ADR
assessment of hematopoietic output (FACS, CFU)
B
relative to ACTB (x10-3)
ADRA1a
(not detected)
ADRA2a
ADRB1
ADRA1b
ADRA2b
(not detected)
ADRB2
ADRA1d
ADRA2c
ADRB3
HE
HSC-like
CD34+ progenitors
mature blood
MSC-like
rest
C
CD43
CD34
CD38
CD34
CD90
CD45RA
Blood
(%CD45/CD43+ in viable)
Progenitors
(%CD34+ in blood)
HSC-like
(%CD34+ CD38- CD90+ CD45RA- in blood)
D
fold difference NE/control
ns
*
0.039
0.022
0.010
blood in viable
CD34 in blood
HSC-like in blood
HSC-like in viable
E
CFU counts per 20 000 plated cells
*
control
NE-treated
F
% of CFU subtypes
GEMM
E
GM
G
M
control
NE-treated

Figure 3

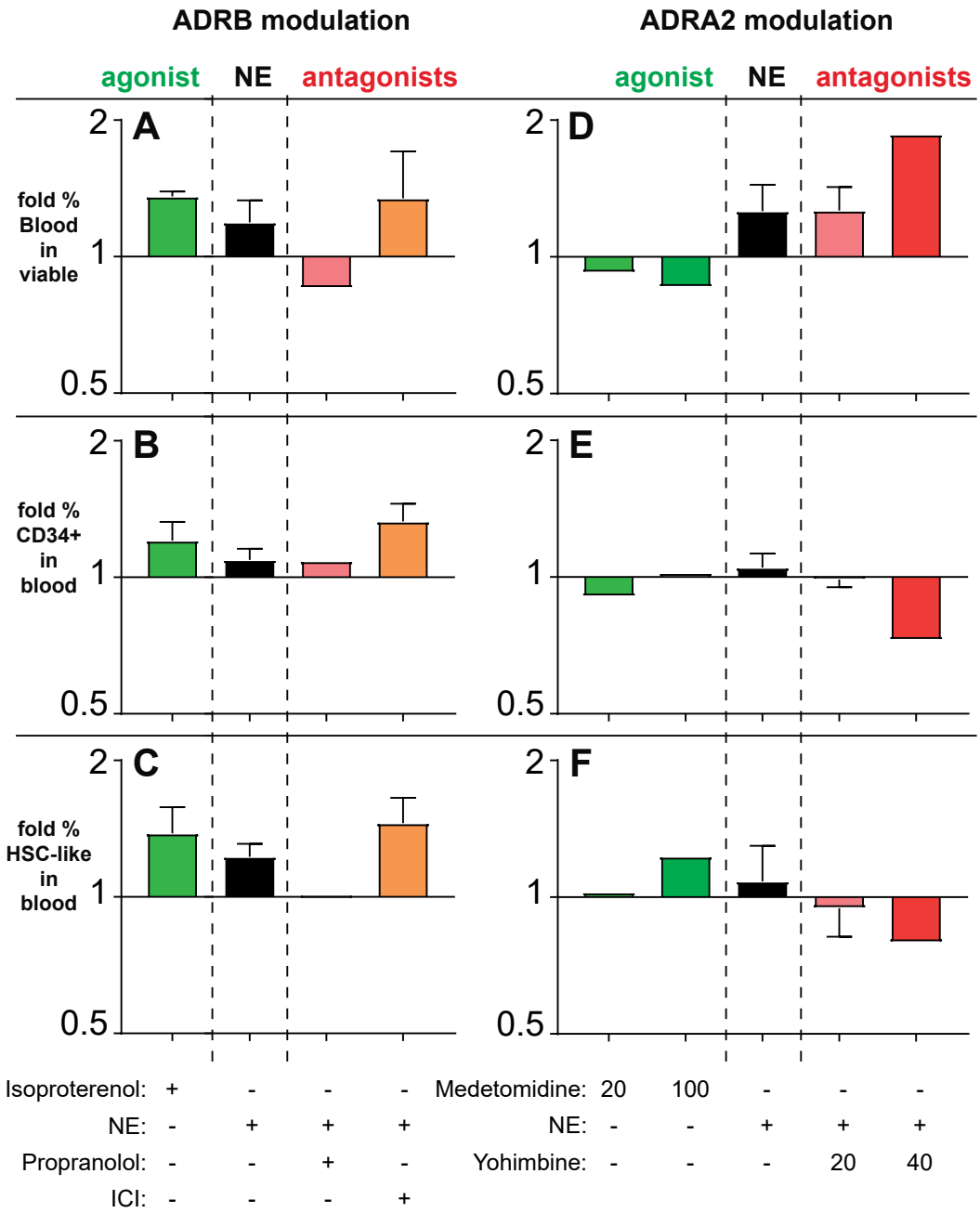

ADRB modulation
ADRA2 modulation
agonist
NE
antagonists
agonist
NE
antagonists
A
B
C
D
E
F
fold % Blood in viable
fold % CD34+ in blood
fold % HSC-like in blood
2
1
0.5
Isoproterenol: + - - -
NE: - + + +
Propranolol: - - + -
ICI: - - - +
Medetomidine: 20 100 - - -
NE: - - + + +
Yohimbine: - - - 20 40

Figure 4

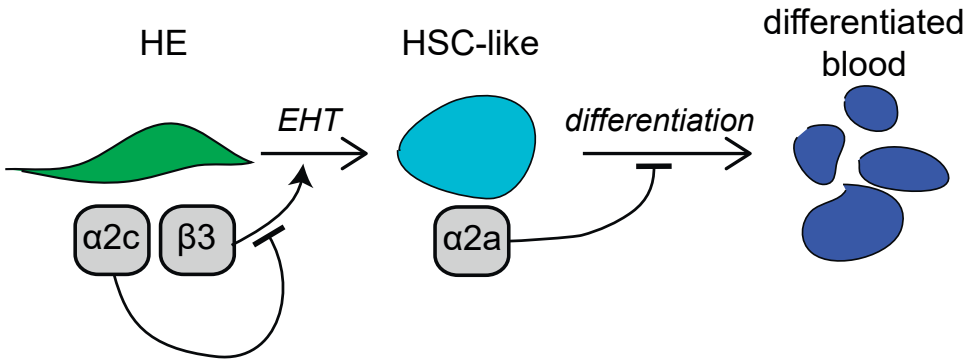
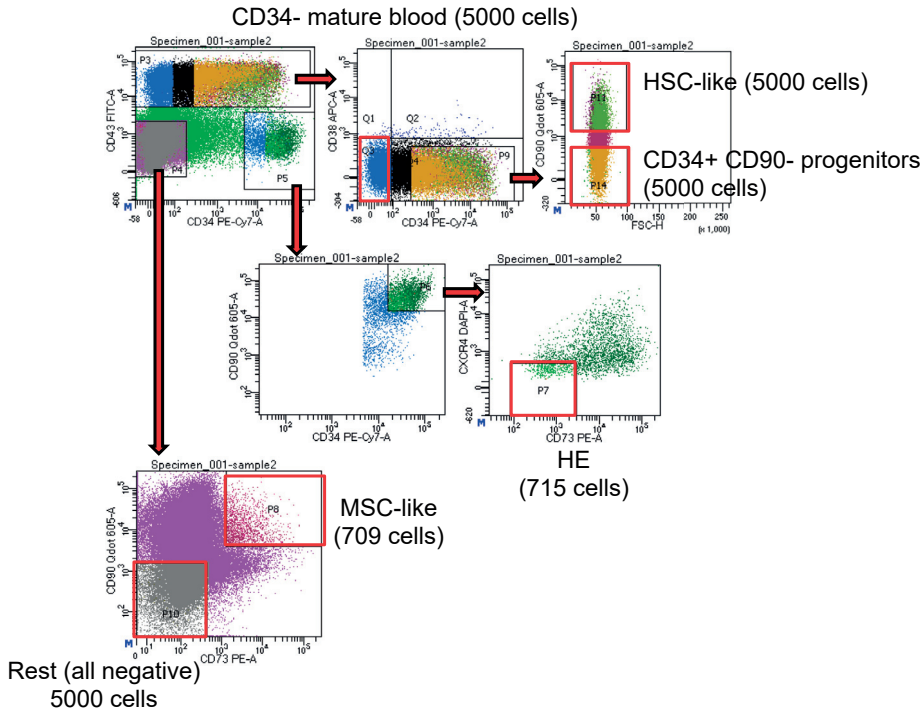

HE
HSC-like
differentiated blood
EHT
differentiation
α2c
β3
α2a

Figure S1


CD34- mature blood (5000 cells)
HSC-like (5000 cells)
CD34+ CD90- progenitors (5000 cells)
HE (715 cells)
MSC-like (709 cells)
Rest (all negative) 5000 cells
Specimen_001-sample2
CD43 FITC-A
CD34 PE-Cy7-A
CD38 APC-A
CD90 Qdot 605-A
FSC-H
CXCR4 DAPI-A
CD73 PE-A
P3
P4
P5
Q1
Q2
P9
P11
P14
P7
P8
P10

Paper III

Stem Cell Reports
Article

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Cyclic AMP Signaling through Epac Axis Modulates Human Hemogenic Endothelium and Enhances Hematopoietic Cell Generation

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SUMMARY

Hematopoietic cells emerge from hemogenic endothelium in the developing embryo. Mechanisms behind human hematopoietic stem and progenitor cell development remain unclear. Using a human pluripotent stem cell differentiation model, we report that cyclic AMP (cAMP) induction dramatically increases HSC-like cell frequencies. We show that hematopoietic cell generation requires cAMP signaling through the Exchange proteins activated by cAMP (cAMP-Epac) axis; Epac signaling inhibition decreased both hemogenic and non-hemogenic endothelium, and abrogated hematopoietic cell generation. Furthermore, in hematopoietic progenitor and stem-like cells, cAMP induction mitigated oxidative stress, created a redox-state balance, and enhanced C-X-C chemokine receptor type 4 (CXCR4) expression, benefiting the maintenance of these primitive cells. Collectively, our study provides insights and mechanistic details on the previously unrecognized role of cAMP signaling in regulating human hematopoietic development. These findings advance the mechanistic understanding of hematopoietic development toward the development of transplantable human hematopoietic cells for therapeutic needs.

INTRODUCTION

Hematopoietic stem cells (HSCs) replenish the hematopoietic system throughout the lifetime of an individual, and can be transplanted into patients to treat malignant and non-malignant blood disorders. The need to develop an alternative source of HSCs to matched adult donors, such as HSCs generated in vitro from pluripotent stem cells, requires increased understanding of the mechanisms of HSC development. During development, the first hematopoietic cells emerge from hemogenic endothelium in the embryonic aorta-gonad-mesonephros (AGM) region through endothelial-to-hematopoietic transition (EHT) (Zovein et al., 2008). The concurrence of neural crest stem cells in the AGM region coincides with the time of HSC emergence, suggesting a link between neural crest/catecholamines and hematopoietic development (Nagoshi et al., 2008). Recently, catecholamine signaling was reported to regulate HSC emergence in the AGM region, as the deletion of GATA binding protein 3 (GATA3), a crucial regulator of catecholamine production, compromised HSC development, which could be rescued with administration of catecholamine derivatives (Fitch et al., 2012). However, the mechanism of catecholamine signaling, through its second messenger, cyclic AMP (3′-5′-cyclic AMP; cAMP) and its downstream signaling pathways have not been critically evaluated in the context of hematopoietic development.

In the adult hematopoietic system, a situation parallel to the hematopoietic developmental context exists. Catecholamines and sympathoadrenergic innervation (Afan et al., 1997; Mendez-Ferrer et al., 2010) of the bone marrow (BM) niche regulates HSC mobilization and migration (Katayama et al., 2006; Lucas et al., 2013; Mendez-Ferrer et al., 2008) of catecholamine receptor-expressing hematopoietic stem and progenitor cells (Heidt et al., 2014; Spiegel et al., 2007). Together, these studies during developmental hematopoiesis and adult hematopoiesis provide evidence for neural regulation of hematopoietic cells and establish catecholamine-mediated signaling as a key component of the hematopoietic program.

Activation of specific G-protein-coupled receptors by catecholamines, as well as neurotransmitters, growth factors, and hormones, activate the cAMP-signaling pathway (Beavo and Brunton, 2002; Sutherland and Rall, 1958), followed by cell-type dependent responses mediated by cAMP effectors protein kinase A (PKA) (Walsh et al., 1968) and Exchange proteins activated by cAMP (Epac) (de Rooij et al., 1998). Epac have been shown to modulate endothelial cell remodeling, enhance endothelial cell adhesion, and regulate the integrity of endothelial cell junctions (Cullere et al., 2005; Fukuhara et al., 2005; Kooistra et al., 2005). However, the role of Epac signaling in hemogenic endothelium is unknown.

cAMP-mediated regulation of adult hematopoiesis is emphasized in studies showing that cAMP increases C-X-C chemokine receptor type 4 (CXCR4) expression and motility of hematopoietic progenitors (Goichberg et al., 2006), HSCs from $G_s\alpha$-deficient mice do not engraft (Adams et al., 2009), and $G_s\alpha$-deficient osteocytes alter the BM niche, leading to defective hematopoiesis (Fulzele et al., 2013). In human hematopoietic cells, prostaglandin E2

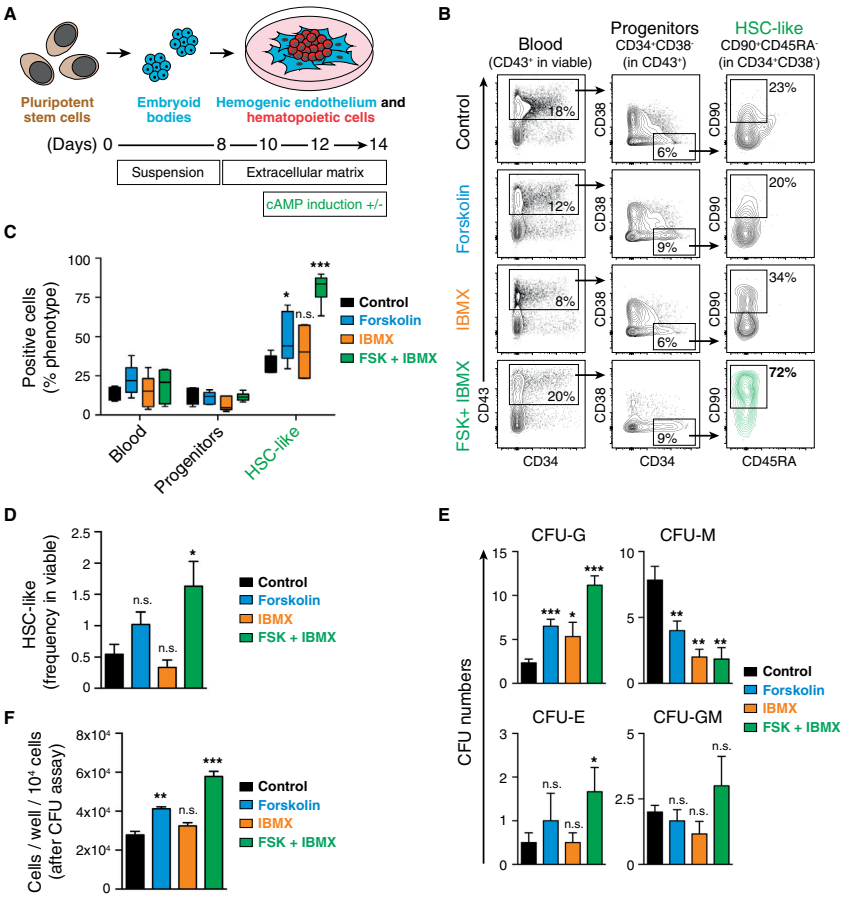

Figure 1. cAMP Induction Increases HSC-like Frequency during hPSC-to-Hematopoietic Differentiation

(A) Conditions and timeline applied to differentiate hPSCs toward mesoderm commitment and hematopoietic differentiation.

(B) Flow cytometric analysis of hematopoietic cells at day 14 of differentiation. Representative flow cytometry plots (biexponential axis) of cells cultured in control medium (MesoTotal), and cells treated with forskolin, IBMX, or forskolin + IBMX are shown.

(C) Percentage of the hematopoietic surface phenotypes indicated in (B). Data represent mean ± SEM of three independent experiments. Statistical analysis was performed using the t test. Significance compared with the control setting: *$p < 0.05$, ***$p < 0.001$; n.s., not significant.

(D) Frequency of the putative HSC-like cells (in viable fraction). Data represent mean ± SEM of three independent experiments. Statistical analysis was performed using the t test. Significance compared with the control setting: *$p < 0.05$; n.s., not significant.

(E) Total CFU numbers after 12-day CFU assay of differentiated hematopoietic cells treated with forskolin, IBMX, and forskolin + IBMX. The CFU distribution of three independent experiments is shown as mean ± SEM CFU-G (granulocyte), CFU-M (macrophage), CFU-E (erythroid), CFU-GM (granulocyte/macrophage). Statistical analysis was performed using the t test. Significance compared with the control setting: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

(legend continued on next page)

(PGE2)-mediated cAMP activation enhances human cord blood engraftment (Cutler et al., 2013; Goessling et al., 2011). Recently, cAMP was shown to regulate hematopoietic emergence and homing in studies where cAMP was upregulated by adenosine in zebrafish and mouse (Jing et al., 2015), PGE2 in zebrafish and mouse (Diaz et al., 2015; Goessling et al., 2009; Hoggatt et al., 2009; North et al., 2007), and shear stress in murine AGM (Kim et al., 2015). However, the role and mechanism of cAMP signaling, as mediated through PKA and Epac, in regulating human developmental hematopoiesis has not been adequately studied, and no study has been performed on the role of cAMP in the human hematopoietic developmental context.

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (Thomson et al., 1998) and induced pluripotent stem cells (iPSCs) (Takahashi et al., 2007), provide an ideal in vitro model to recapitulate human hematopoietic development. We have shown that hPSC-derived HSC-like cells possess lymphoid and myeloid differentiation ability, a key feature of HSCs (Ronn et al., 2015). Recent studies have functionally demonstrated an endothelial precursor to blood (hemogenic endothelium) from hPSC differentiation cultures (Ditadi et al., 2015; Slukvin, 2013), further establishing hPSCs as a suitable model to study human hematopoietic cell development. However, the signals regulating hemogenic endothelium and newly emergent HSCs in the human developmental context remain undefined. In addition, for functional transplantable HSCs it is vital to reduce reactive oxygen species (ROS) and oxidative stress, as reduced ROS is crucial for HSC functionality (Ito et al., 2006; Jang and Sharkis, 2007; Yahata et al., 2011).

As cAMP-mediated regulation of human hematopoietic cell emergence remains elusive, we set out to investigate the role of cAMP signaling in the development of hematopoietic progenitors from hPSCs. Here, we demonstrate that cAMP induction during hPSC-to-hematopoietic differentiation increases the frequency of cells with HSC-like surface phenotype and increases the colony-forming unit (CFU) potential. We demonstrate that cAMP regulation of hemogenic endothelium is dependent on the cAMP-Epac signaling axis. Furthermore, we propose that the cAMP-mediated increase in HSC-like cells is in part coupled to cAMP-mediated mitigation of oxidative burden and increasing hematopoietic cell function.

RESULTS

cAMP Induction Increases the Frequency of HSC-like Cells Derived from hPSCs

To assess the role of cAMP signaling in human hematopoietic development, we differentiated hPSCs using our previously described protocol (Ronn et al., 2015) whereby mesoderm-biased embryoid bodies (EBs) were plated onto the extracellular matrix allowing for adherence and expansion of hemogenic endothelium, to generate hematopoietic cells (Figure 1A). To elevate intracellular cAMP, we applied a combination of forskolin and 3-isobutyl-1-methylxanthine (IBMX) from day 10. Forskolin specifically increases intracellular cAMP levels by activating the catalytic subunit of adenylyl cyclase (Seamon et al., 1981). IBMX is a phosphodiesterase (PDE) inhibitor that specifically prevents PDE-mediated dephosphorylation of cAMP to AMP (Beavo et al., 1970). Thus, combining forskolin with IBMX elevates intracellular cAMP by increasing cAMP production and preventing its dephosphorylation. Fluorescence-activated cell sorting (FACS) analysis of the hPSC-derived hematopoietic cells revealed that cAMP induction with forskolin + IBMX significantly increased the numbers and frequency of our previously described HSC-like cells ($CD43^+CD34^+CD38^-CD90^+CD45RA^-$) (Figures 1B–1D).

To confirm that the upregulation of HSC-like cells was specifically due to increased cAMP levels, we used two different synthetic cAMP analogs, dibutyryl-cAMP and 8-Br-cAMP, to elevate intracellular cAMP during hPSC-to-hematopoietic differentiation. Both cAMP analogs upregulated the HSC-like phenotype (Figure S1A). Also, to rule out any unspecific effects of forskolin we used 1,9-dideoxyforskolin, an inactive analog of forskolin that does not activate adenylyl cyclase. We observed that HSC-like phenotype was induced only with forskolin + IBMX, not with 1,9-dideoxyforskolin + IBMX (Figure S1B), thus verifying the specificity of forskolin-mediated effects through cAMP.

Assessment of the differentiation capacity of hematopoietic cells in a CFU assay showed an increase in granulocyte CFU (CFU-G) and erythroid colonies, while the numbers of macrophage CFU (CFU-M) were decreased (Figure 1E). cAMP induction, although decreasing the numbers of CFU-M colonies, significantly increased the total cell number resulting from the colony assay (Figure 1F) compared with the non-induced control.

(F) Cell numbers obtained per well (per 1×10^4 seeded cells) after CFU assay. Data represent mean ± SEM of three independent experiments. Statistical analysis was performed using the t test. Significance compared with the control setting: $^{**}p < 0.01$, $^{***}p < 0.001$; n.s., not significant.
See also Figure S1.

Together, these data show that elevation of intracellular cAMP upregulates hPSC-derived HSC-like surface phenotype, and also alters the distribution of colony types, favoring mixed, granulocyte, and erythroid colonies at the expense of macrophage colonies.

cAMP Signaling through the Epac Axis Is Required for HSC-like Cell Generation from an Endothelial Cell Intermediate

Intracellular cAMP downstream effectors, PKA and Epac, influence cellular functions by regulating the activation of various transcription factors and signaling molecules (Cheng et al., 2008). We set out to elucidate the mechanism of cAMP-mediated upregulation of phenotypic HSC-like cells during hPSC differentiation toward the hematopoietic lineage. Epac inhibition abrogated the hematopoietic cell generation efficiency as assessed by FACS measuring the frequency of pan-hematopoietic marker $CD43^+$ cells and HSC-like cells (Figures 2A, 2B, and S2A). Inhibition of PKA, on the other hand, had no negative effect on hematopoietic cells (Figures 2A, 2B, and S2A). The cell viability after PKA and Epac inhibition was similar to that of controls, at 90%–95% (Figure S2B). We observed that inhibition of Epac in the absence or presence of forskolin + IBMX reduced the radial spreading of adherent endothelial-like cells (emerging from the plated EBs) while PKA inhibition did not have any effect (Figure 2C), the radial spread being measured as indicated in Figure S2C. The reduced EB radial spread after Epac inhibition was in accordance with a trend in reduced total cell number at day 14 after Epac inhibition (Figure S2D), suggesting that Epac inhibition affected cell proliferation in a manner not associated with cell survival. As bona fide hematopoietic cells are reported to emerge from such endothelial spread (Eilken et al., 2009), we evaluated the effect of Epac inhibition on the frequency of the previously described hemogenic endothelium phenotype cells ($CD43^-CD34^+CXCR4^-CD73^-VEcad^+$) (Ditadi et al., 2015), whereby we applied the cAMP modulators at days 6 and 8 and analyzed the cells at day 10 (schematic regimen described in Figure S2E and Experimental Procedures). Epac inhibition in the absence or presence of forskolin + IBMX decreased the hemogenic endothelial fraction (Figure 2D) without compromising the viability of hemogenic endothelium (Figure S2F). Epac inhibition also reduced the non-hemogenic endothelium ($CD43^-CD34^+VEcad^+$) (Figure 2E) and arterial endothelium ($CD43^-CD34^+CD90^+CD73^+CXCR4^+$) (Figure 2F). In contrast, the various endothelial cell types were insensitive to PKA inhibition (Figures 2D–2F and S2A).

These results indicate that the cAMP-Epac axis plays a pivotal role in the development of hematopoietic progenitors and stem-like cells in hPSC differentiation cultures by regulating hemogenic endothelial cell expansion.

cAMP Induction Reduces Oxidative Stress and Induces CXCR4 Upregulation in hPSC-Derived Hematopoietic Cells

The deleterious effects of elevated ROS and its ensuing oxidative damage on the function of mammalian HSCs are well documented (Ito et al., 2006; Jang and Sharkis, 2007; Suda et al., 2011; Yahata et al., 2011). As cAMP induction decreased the prevalence of macrophages in our system (Figure 1E), and as cAMP elevation coupled with PDE inhibition has been reported to promote immune homeostasis (Katakami et al., 1988; Sinha et al., 1995), we rationalized that cAMP induction with forskolin + IBMX might mitigate the ROS burden in our hPSC-to-hematopoietic differentiation system, thus protecting the HSC-like cells from ROS-mediated effects mentioned above. Analysis of ROS levels showed that cAMP induction decreased the ROS levels in hPSC-derived hematopoietic cells compared with the control setting (Figure 3A). cAMP induction along with PDE inhibition (forskolin + IBMX), and protecting endogenously available cAMP with PDE inhibition alone (IBMX), significantly lowered the ROS levels in various hematopoietic phenotypes, including the HSC-like cells (Figure 3B), thus indicating reduced oxidative stress of these hematopoietic cellular fractions.

The decreased ROS level after cAMP induction prompted us to determine the status of genes that regulate the redox state of cells and thus help to reduce the oxidative stress. Transcriptional analysis of the redox-state-regulating genes in hPSC-derived hematopoietic cells showed that nuclear factor (erythroid-derived 2)-like 2 (*NFE2L2*) was upregulated after cAMP induction with forskolin + IBMX (Figure 3C). *NFE2L2* is a global regulator of the oxidative stress response, as it binds to anti-oxidant response element in the upstream promoter region of several anti-oxidative genes and initiates their transcription (Itoh et al., 1997; Tsai et al., 2013), thus initiating the mitigation of ROS-induced oxidative stress in the cells. In our system, *NFE2L2* upregulation after cAMP induction was in concert with the upregulation of anti-oxidant genes, such as superoxide dismutase (*SOD1*, *SOD2*), glutathione peroxidase (*GPX2*) (18-fold increase), catalase (*CAT*), and glutathione S-reductase (*GSR*) (Figure 3C).

Stress conditions activate p38 mitogen-activated protein kinases (*p38MAPK*) (Raingeaud et al., 1995). ROS/stress-mediated *p38MAPK* activation leads to HSC self-renewal defects and reduced HSC long-term repopulation potential (Ito et al., 2006; Jang and Sharkis, 2007). Thus we analyzed the levels of stress-activated p38 pathway components *P38-MAPK*α, δ, and γ and found that cAMP induction generally reduced the levels of these p38MAPK isoforms (Figure 3D), indicative of reduced stress. As elevated ROS levels have been shown to induce differentiation of hematopoietic progenitors toward mature lineages (Jang and Sharkis,

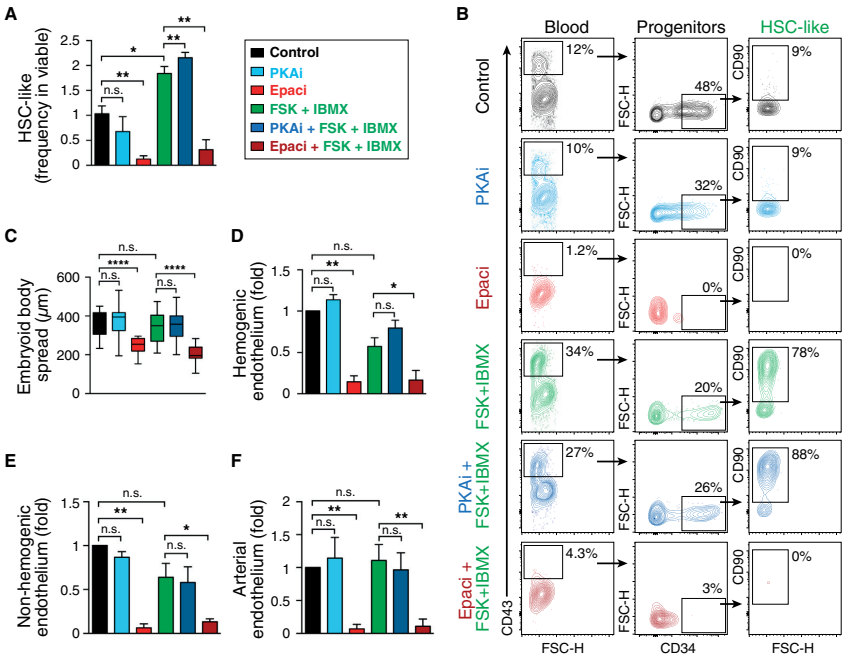

Figure 2. cAMP-Mediated HSC-like Upregulation Occurs through the cAMP-Epac Axis

(A) Quantification of HSC-like frequency in viable (at day 14) after PKA or Epac inhibition. Data represent mean ± SEM of three independent experiments. Statistical analysis was performed using the t test. *p < 0.05, **p < 0.01; n.s., not significant.

(B) Representative flow cytometry plots (biexponential axis) showing the $CD43^+$ blood cells and HSC-like cells (day 14) generated after forskolin + IBMX-mediated cAMP induction and PKA or Epac inhibition (PKAi, Epaci), with or without forskolin + IBMX.

(C) Quantification of radial spread of EBs (distance between center of EB and outer edge of the cellular spread, day 13). Data represent mean ± SEM of 100 EBs from three independent experiments. Statistical analysis was performed using the t test. ****p < 0.0001; n.s., not significant.

(D) Analysis of hemogenic endothelial ($CD43^-CD34^+CXCR4^-CD73^-VEcad^+$) phenotype (day 10), after cAMP induction and PKA or Epac inhibition (PKAi, Epaci) with or without cAMP induction. Data represent mean ± SEM of three independent experiments; mean fold change respective to control is shown. Statistical analysis was performed using the t test. *p < 0.05, **p < 0.01; n.s., not significant.

(E) Analysis of general (non-hemogenic) endothelium ($CD43^-CD34^+VEcad^+$) at day 10, after cAMP induction and PKA or Epac inhibition (PKAi, Epaci) with or without cAMP induction. Data represent mean ± SEM of three independent experiments; mean fold change respective to control is shown. Statistical analysis was performed using the t test. *p < 0.05, **p < 0.01; n.s., not significant.

(F) Analysis of arterial endothelium ($CD43^-CD34^+CD90^+CD73^+CXCR4^+$) at day 10, after cAMP induction and PKA or Epac inhibition (PKAi, Epaci) with or without cAMP induction. Data represent mean ± SEM of three independent experiments; mean fold change respective to control is shown. Statistical analysis was performed using the t test. **p < 0.01; n.s., not significant. For (D)–(F) the analysis was done at day 10, as the hemogenic endothelium is less abundant after this time point.

See also Figure S2.

2007; Suda et al., 2011), we analyzed the abundance of mature hematopoietic cells ($CD43^+CD34^-CD38^+$) in our differentiation assay. cAMP induction reduced the prevalence of mature hematopoietic progenitors (Figure 3E), indicating the role of cAMP in preventing cell maturation and agreeing to the reduced ROS levels after cAMP induction (Figures 3A and 3B). Together, these data indicate that cAMP induction with forskolin + IBMX upregulated anti-oxidant defense mechanisms and downregulated stress-activated genes, thus creating a redox balance in our system that is favorable for hematopoietic progenitor and stem-like cell maintenance.

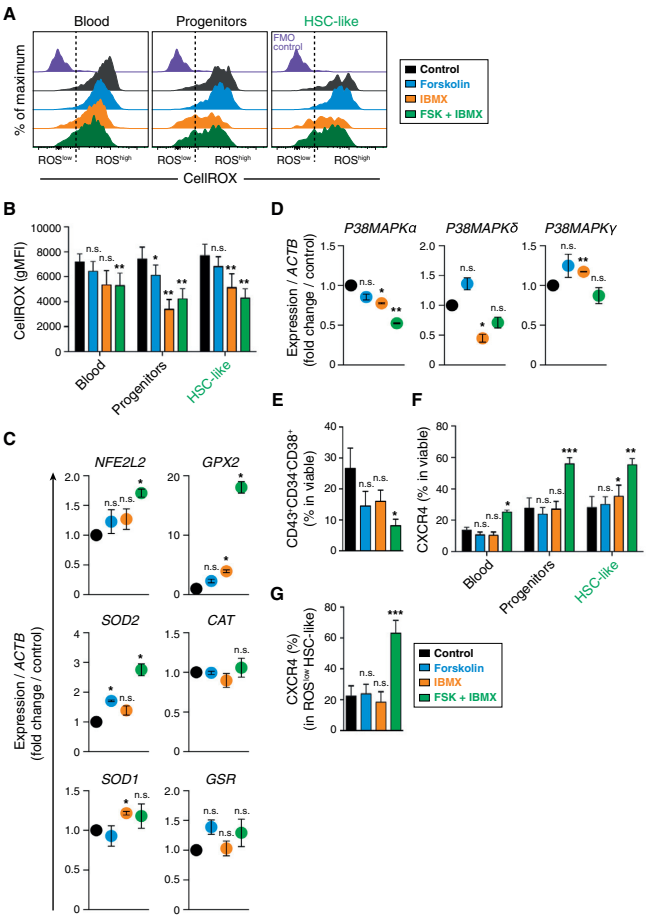

Figure 3. cAMP Induction Reduces Oxidative Stress and Induces CXCR4 in hPSC-Derived Hematopoietic Cells

(A) Flow cytometry analysis for detection of reactive oxygen species (ROS) in differentiated hPSC-to-hematopoietic cells at day 14 of differentiation. Representative flow cytometry plots (biexponential x axis) show ROS levels in the hematopoietic surface phenotypes. FMO control, fluorescence-minus-one (staining control).

(B) Quantification of geometric mean fluorescence intensity (gMFI) of CellROX dye as indicated in (A). Data represent mean ± SEM of three independent experiments. Statistical analysis was performed using the t test. Significance compared with the control setting: *p < 0.05, **p < 0.01; n.s., not significant.

(C and D) qRT-PCR expression analysis of the indicated redox-state-regulating genes (C) and p38MAPK-related genes (D) in PSC-derived hematopoietic cells. Relative expression of each gene to housekeeping gene *ACTB* (*β-ACTIN*) was calculated, and mean fold change respective to control condition (set at 1) is shown. Data represent mean ± SEM of three independent experiments. Statistical analysis was performed using the t test. Significance compared with the control setting: *p < 0.05, **p < 0.01; n.s., not significant.

(E) Analysis of mature hematopoietic progenitors ($CD43^+CD34^-CD38^+$) after cAMP induction (day 14). Data represent mean ± SEM of three independent experiments. Statistical analysis was performed using the t test. Significance compared with the control setting: *p < 0.05; n.s., not significant.

(F) Expression of CXCR4 across indicated hematopoietic surface phenotypes. Data represent mean ± SEM of three independent experiments. Statistical analysis was performed using the t test. Significance compared with the control setting: *p < 0.05, **p < 0.01, ***p < 0.001; n.s., not significant.

(G) Expression of CXCR4 in HSC-like surface phenotype (ROS^{low} fraction). Data represent mean ± SEM of three independent experiments. Statistical analysis was performed using the t test. Significance compared with the control setting: ***p < 0.001, n.s., not significant. See also Figure S3.

The chemokine receptor CXCR4 is pivotal for retaining quiescent HSCs in the BM niche, as well as for HSC homing to BM (Lai et al., 2014; Nie et al., 2008; Peled et al., 1999). Given that cAMP and PGE2 treatment has been shown to increase CXCR4 expression in adult HSCs/human cord blood $CD34^+$ cells (Goessling et al., 2011; Goichberg et al., 2006; Hoggatt et al., 2009), we analyzed CXCR4 levels in our hPSC-derived hematopoietic cells following cAMP induction. Analysis of CXCR4 expression revealed that cAMP induction with forskolin + IBMX enhanced CXCR4 expression across various hematopoietic surface phenotypes, including the HSC-like fraction (Figure 3F). Importantly, CXCR4 expression was enhanced in the ROS^{low} HSC-like surface phenotype upon treatment with forskolin + IBMX (Figure 3G). We investigated whether cAMP-mediated CXCR4 modulation was regulated through PKA or Epac. cAMP-induced upregulation of CXCR4 was insensitive to inhibition of PKA, indicating that CXCR4

regulation does not depend on PKA. On the contrary, Epac inhibition reduced CXCR4 expression, indicating that CXCR4 regulation was mediated by the cAMP-Epac axis (Figure S3). Our data showing that PKA does not regulate CXCR4 expression agree with a previous finding where cAMP-mediated CXCR4 elevation (in human mobilized peripheral blood $CD34^+$ cells) was insensitive to PKA inhibition (Goichberg et al., 2006).

Together, these findings show that cAMP induction reduced the oxidative burden by creating a redox-state balance in hPSC-derived hematopoietic cells and upregulated CXCR4 in the HSC-like cells, both essential for HSC functionality.

DISCUSSION

Using an hPSC differentiation system to model human hematopoietic cell emergence and development, our findings suggest that cAMP and signaling through its Epac axis is an important factor for the generation of hemogenic endothelium, from which the first hematopoietic cells arise. Thus cAMP is a crucial second messenger molecule regulating the in vitro hPSC-derived HSC-like cells. Interestingly, cAMP induction upregulated the frequency of HSC-like cells in the differentiation cultures and also mitigated oxidative stress, created redox-state balance, and enhanced CXCR4 expression in hPSC-derived hematopoietic cells, thus suggesting multiple independent functions of cAMP signaling in endothelial and hematopoietic cells. Because of the importance of low ROS levels in maintaining repopulating human HSCs (Jang and Sharkis, 2007; Yahata et al., 2011), the identification of cAMP-mediated ROS reduction in hPSC differentiation systems provides a mechanism for how ROS can be regulated in in vitro systems to better mimic in vivo HSC development. We speculate that the presence of mature immune cells elevates ROS activity in our differentiation system. The reduction in mature cells and decreased macrophage numbers following cAMP induction agrees with cAMP-mediated redox-state balance, leading to decreased ROS levels. ROS reduction was in concert with the increase of anti-oxidant gene response, decrease in the p38 stress pathway components, and reduced prevalence of mature hematopoietic cells. In terms of cAMP-mediated ROS reduction and reduction of mature progenitors, the reduced abundance of differentiated cells could in part result in decreased ROS, or, alternatively, low ROS levels could be playing a role to reduce differentiation, either way both benefiting the system for increasing the prevalence of HSC-like cells.

Thus, benefits of cAMP, in terms of decreased ROS/p38 and maintenance of redox balance together, created a better environment for the HSC-like cells, presumably helping their survival, preventing premature senescence, and maintaining their functionality, as has been shown for adult HSCs. cAMP induction reduced p38 signaling, which is beneficial for the maintenance of human hematopoietic stem and progenitor cells (Baudet et al., 2012; Zou et al., 2012). The ability of cAMP to regulate inflammation and ROS has been shown previously, either by reducing pro-inflammation cytokines and enhancing the T helper 2 responses in inflamed human blood cells (Harris et al., 2002; Snijdewint et al., 1993; Yoshimura et al., 1997) or via suppressing inflammation/ROS through the cAMP-Epac axis (Remans et al., 2004; Xu et al., 2008). In our assay, cAMP-mediated ROS/p38 reduction, increased anti-oxidant response, and reduced differentiation could in part be attributed to these inflammation-regulating actions of cAMP.

We speculate that ROS reduction has an important, but not exclusive, regulatory role in the cAMP-mediated maintenance of HSC-like cells. Moreover, cAMP-mediated ROS/p38 reduction and increased CXCR4 receptor expression provide a correlational benefit to HSC-like cells, as low ROS levels specifically increased the $CXCR4^{high}$ HSC-like cells. These findings suggest that cAMP induction imparts important functional properties to the derived hematopoietic cells, as low oxidative stress (Ito et al., 2006; Jang and Sharkis, 2007; Yahata et al., 2011) and high CXCR4 expression (Nie et al., 2008; Peled et al., 1999) are properties of HSCs with long-term transplantation potential.

Multiple factors, through binding to GPCRs, orchestrate the regulation of cAMP levels in a cell- and context-dependent manner, an effect that is most likely to be preserved during embryonic hematopoietic emergence and development (Diaz et al., 2015; Goessling et al., 2011; Hoggatt et al., 2009; Jing et al., 2015; Kim et al., 2015; Li et al., 2014; North et al., 2007). Thus, the regulatory networks involved in cAMP-mediated regulation of hematopoietic development are likely to be multifactorial and interconnected. Detailed dissection of such signals is therefore required to elucidate the signaling networks that trigger cAMP-mediated benefits to hematopoietic cells.

Our findings on the pro-hematopoietic effects of cAMP on hPSC-derived hematopoietic cell specification are in agreement with reports describing the pivotal role of cAMP signaling in promoting and enhancing mouse and zebrafish hematopoietic development (Diaz et al., 2015; Jing et al., 2015; Kim et al., 2015), as well as human $CD34^+$ cord blood/mobilized peripheral blood survival and engraftment (Goessling et al., 2011; Li et al., 2014). These studies demonstrated that cAMP activation via fluid shear stress in the murine system (Diaz et al., 2015; Kim et al., 2015), adenosine signaling in the zebrafish hematopoietic system (Jing et al., 2015), and PGE2 in human $CD34^+$ cells (Goessling et al., 2011; Li et al., 2014), instructs hematopoietic specification, mediated through the cAMP-PKA axis. In our in vitro model

of human hematopoietic development, using an hPSC differentiation system, we show that by inhibiting the downstream effectors of cAMP signaling, PKA and Epac, only Epac signaling is required for hematopoietic development, notwithstanding that apart from affecting the underlying endothelium, Epac inhibition appears to affect the newly formed HSC-like cells. As experimental modulation of cAMP signaling to instruct HSC fate from hPSCs has not yet been achieved (Traver, 2015), our finding describing the pro-hematopoietic benefits of cAMP on hPSC-derived human hematopoietic cells is a key step toward refining modalities of human HSC generation using cAMP. Toward understanding the in vivo emergence and development of human HSCs, our findings on human PSC-derived hematopoietic cell generation necessitate further evaluation of cAMP and its signaling components during definitive human hematopoiesis, i.e., either directly in human fetuses or by developing culture systems to propagate human AGM region in vitro, notwithstanding the ethical and technical challenges related to this pursuit.

cAMP-mediated Epac upregulation has been reported to reorganize cortical actin, enhance vascular endothelial cadherin-mediated cell adhesion, and induce integrin-mediated cell adhesion, leading to decreased endothelial cell permeability and enhanced endothelial barrier function (Cullere et al., 2005; Fukuhara et al., 2005; Rangarajan et al., 2003). Developmentally, in the early embryo hematopoietic cells (including cells with the potential to form HSCs) emerge from hemogenic endothelium in the AGM region through EHT (Chen et al., 2009; Zovein et al., 2008). From our Epac inhibition experiments, we demonstrate the critical role of Epac signaling in modulating hPSC-derived hemogenic endothelium and HSC-like cells. Epac being one of the downstream effectors of cAMP that regulates endothelial cell-cell adhesion, permeability, and barrier functions may suggest a link between endothelial cell mechanobiology and hemogenic endothelial cell function. However, due to the inability to currently evaluate pure populations of human endothelial cells with hematopoietic potential, confirmation of Epac's role, whether specifying hematopoietic function at the hemogenic endothelium (HE) cell stage, or acting indirectly on HE via an upstream cell intermediate, is still necessary.

Collectively, our findings suggest that cAMP regulates the generation and function of human HSCs via multiple separate mechanisms in both the endothelial and hematopoietic cell fractions. By demonstrating the role of cAMP-Epac axis in hematopoietic cells and HSC-like cells using an in vitro human development model, our study provides insights into understanding the previously unknown role of the cAMP-signaling component in human hematopoietic development. Taken together, these findings advance our current understanding of human hematopoietic developmental mechanisms toward the development of transplantable hematopoietic cells for therapeutic purposes.

EXPERIMENTAL PROCEDURES

Human Pluripotent Stem Cell Culture

Human iPSC line RB9-CB1 derived from cord blood endothelial cells (Ronn et al., 2015; Woods et al., 2011) was cultured on irradiated mouse embryonic fibroblasts in DMEM/Nutrient Mixture F-12 (DMEM/F12) supplemented with 20% KnockOut-serum replacement (KO-SR), 2 mM L-glutamine, 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol, and 10 ng/ml basic fibroblast growth factor, all from Thermo Fisher Scientific. The cells were incubated in a humidified incubator at 37°C and 5% CO_2.

Embryoid Body Formation and Hematopoietic Differentiation

EBs were prepared after incubating the pluripotent stem cell colonies with 2 mg/ml Dispase (Thermo Fischer Scientific), followed by gentle pipetting. The detached colonies were washed twice with 20% KO-SR containing DMEM/F12, before plating in ultra-low-adherence suspension culture dishes (Corning Life Sciences) to form EBs for 8 days. During suspension culture, the MesoTotal HPC/HSC Differentiation System (Primorigen Biosciences) was used to specify the EBs toward mesoderm commitment. At day 8, EBs were plated on 0.08 μg/mm^2 Matrigel (BD Biosciences), and further differentiation toward hematopoietic cells was carried out until day 14 in MesoTotal medium. Application of fresh medium and cAMP induction with 10 μM forskolin (Stemgent) and 500 μM IBMX (3-isobutyl-1-methylxanthine; Santa Cruz Biotechnology) was carried out at days 10 and 12 of differentiation. For inhibition of PKA and Epac, 50 μM PKA inhibitor (Rp-8-CPT-cAMPS) (Staples et al., 2001; Yu et al., 2014) and 20 μM Epac inhibitor (ESI-09) (Almahariq et al., 2013; Zhu et al., 2015) (both from BioLog Life Science Institute) were used (20 min pre-treatment followed by cAMP induction) on days 10 and 12 of differentiation, and the cells were analyzed on day 14. These inhibitors occupy cAMP-binding domains of their respective receptor (PKA or Epac), thus blocking further cAMP binding and inhibiting the functions of PKA or Epac. For analysis of hemogenic endothelium, PKA and Epac were inhibited on day 6 (EB stage) and day 8 (adherent stage) and the cells were analyzed on day 10 (schematic regimen, Figure S2E). We selected day 10 to analyze hemogenic endothelium, as this population is less abundant after day 12 (data not shown). For measurement of EB radial spread, the distance between center of an EB and outer edge of its cellular spread was analyzed on day 13 of differentiation (Figure S2C) using ImageJ (developed at the NIH).

Colony Formation Assay

After 14-day hPSC-to-hematopoietic differentiation, the differentiated hematopoietic cells were plated (1×10^4 cells/9.5 cm^2) in Methocult H4230 (STEMCELL Technologies) supplemented with 2.5 μg/ml human Stem Cell Factor, 2.5 μg/ml human interleukin-3, 5 μg/ml human granulocyte-macrophage colony-stimulating factor, and 500 U/ml erythropoietin, all recombinant

human cytokines from PeproTech. For cells treated with forskolin or IBMX, or a combination of both, similar treatment was continued in the CFU assay (chemicals added to the CFU medium on the first day of CFU assay), and after 12 days hematopoietic colonies were scored microscopically to evaluate various CFU phenotypes.

Flow Cytometry

For analysis of surface markers, cells harvested using TrypLE Select (Thermo Fisher Scientific) were labeled with primary antibodies for 30 min at 4°C. The following fluorophore-conjugated antibodies were used: CD43-FITC (catalog #555475, clone 1G10), CD45RA-V450 (#560363, clone HI100), CD73-PE (#550257, clone AD2), anti-VE-cadherin-Percp-Cy5.5 (#561566, clone 55-7H1) (all from BD Biosciences), and CD34-PE.Cy7 (#343515, clone 581), CD38-APC (#303509, clone HIT2), CD90-PE (#328110, clone 5 × 10^{10}), and CXCR4-BV421 (#306517, clone 12G5) (all from BioLegend). After incubation with the antibodies for 30 min, cells were washed, resuspended in 2% fetal bovine serum (Thermo Fischer Scientific) containing PBS, and acquired with BD FACSCanto (BD Biosciences). To detect oxidative stress, we used CellROX Deep Red (#C10422, Life Technologies) according to the manufacturer's instructions. For live/dead cell discrimination, 7-amino-actinomycin D (BD Biosciences) was applied to the cells before acquisition. Dot plots were derived from gated events based on size and scatter characteristics and doublet-exclusion, fluorescence-minus-one controls were used to identify gating boundaries. Acquired events were analyzed using FlowJo software.

RNA Isolation and qRT-PCR

Total RNA from cells was extracted using an RNeasy Micro Kit (Qiagen) and 500 ng of total RNA was reverse transcribed to cDNA using SuperScript III reverse transcriptase (Life Technologies) according to the manufacturer's instructions. qPCR was performed with gene-specific primers (Table S1) (Dannenmann et al., 2015) using SYBR GreenER qPCR SuperMix (Life Technologies) in a 7900HT Fast Real-Time PCR system (Life Technologies), and the relative expression to housekeeping gene *β-ACTIN* was analyzed by comparative C_T method (Livak and Schmittgen, 2001).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software). Quantitative data represent mean ± SEM, unless otherwise stated, and n represents the number of biological replicates. For statistical evaluation, Student's t test (two-tailed) was used, statistical significance in the figures being indicated by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.03.006.

AUTHOR CONTRIBUTIONS

Conceptualization, S.S. and N.B.W.; Investigation and data presentation, S.S.; Data analysis, S.S. and N.B.W.; Writing – Original Draft, S.S. and N.B.W.; Writing – Review & Editing, S.S., R.R., C.G., N.B.W.; Methodology, S.S., R.R., C.G., R.M., N.B.W.; Funding Acquisition, N.B.W.; Supervision, S.S. and N.B.W.

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Paper IV

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REGENERATIVE MEDICINE

Reactive Oxygen Species Impair the Function of CD90$^+$ Hematopoietic Progenitors Generated from Human Pluripotent Stem Cells

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Key Words. Reactive oxygen species • Hematopoietic stem cells • Human pluripotent stem cells • Hematopoietic development • Functional impairment

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ABSTRACT

Cell stressors, such as elevated levels of reactive oxygen species (ROS), adversely affect hematopoietic stem cell (HSC) reconstituting ability. However, the effects of ROS have not been evaluated in the context of hematopoietic development from human pluripotent stem cells (hPSCs). Using our previously described in vitro system for efficient derivation of hematopoietic cells from hPSCs, we show that the vast majority of generated hematopoietic cells display supraphysiological levels of ROS compared to fresh cord blood cells. Elevated ROS resulted in DNA damage of the CD34$^+$ hematopoietic fraction and, following functional assays, reduced colony formation and impaired proliferative capacity. Interestingly, all the proliferative potential of the most primitive hematopoietic cells was limited to a small fraction with low ROS levels. We show that elevation of ROS in hPSC-derived hematopoietic cells is contributed by multiple distinct cellular processes. Furthermore, by targeting these molecular processes with 4 unique factors, we could reduce ROS levels significantly, yielding a 22-fold increase in the most primitive CD90$^+$ CD34$^+$ hematopoietic cells with robust growth capacity. We demonstrate that the ROS reducing factors specifically reduced ROS in more primitive hematopoietic fractions, in contrast to endothelial cells that maintained low ROS levels in the cultures. We conclude that high levels of ROS in in vitro differentiation systems of hPSCs is a major determinant in the lack of ability to generate hematopoietic cells with similar proliferation/differentiation potential to in vivo hematopoietic progenitors, and suggest that elevated ROS is a significant barrier to generating hPSC-derived repopulating HSCs. STEM CELLS *2017;35:197–206*

SIGNIFICANCE STATEMENT

Cell stressors, such as elevated levels of reactive oxygen species (ROS), adversely affect hematopoietic stem cell reconstituting ability. However, the effects of ROS have not been evaluated in the context of hematopoietic development from human pluripotent stem cells (hPSCs). Our results show for the first time that high levels of ROS, resulting from mechanisms associated with in vitro hPSC differentiation systems, occurs and functionally impairs the most primitive of the newly generated hematopoietic cells, and that ROS regulation is a requirement for generating functional primitive hematopoietic cells in vitro. We provide strong evidence that efforts to further normalize ROS toward physiological levels will be crucial to generate functionally equivalent human hematopoietic stem and progenitor cells in vitro as compared to their in vivo counterparts.

INTRODUCTION

Hematopoietic stem cells (HSCs) give rise to all blood types throughout the life span of an adult. Although largely quiescent, these cells are sensitive to genotoxic insult and upon reentry into cell cycle are vulnerable to genotoxicity responses aimed at preventing cancer cell development [1]. With the goal of understanding human hematopoietic development and ultimately being able to provide an alternative source of HSCs for therapeutic purposes, progress has been made to generate transplantable hematopoietic cells in vitro by directed differentiation of human pluripotent stem cells (hPSCs) and direct reprogramming to the hematopoietic lineages [2–8]. However, achieving robust engraftment of in vitro generated human HSCs has been extremely difficult. Adult HSCs, both human and murine, have been demonstrated to be highly sensitive to increased levels of reactive oxygen species

(ROS), where ROS-mediated oxidative damage impairs the self-renewal and long-term (LT) engraftment capacity of these cells [9, 10]. Thus the evaluation of the levels of ROS in pluripotent stem cell differentiation cultures is highly warranted. ROS is a collective term for oxygen containing molecules that, due to unpaired valence shell electrons, are highly reactive, causing oxidative damage to components of the cell including DNA, proteins, and lipids [11], and results in cell cycle arrest, premature senescence, or apoptosis. It has been suggested that this accumulation of oxidative damage to biomolecules contributes to phenotypes and diseases associated with aging and cancer, as argued by the free radical theory of aging [12, 13]. In normal cells, the vast majority of ROS originates from the mitochondria as a by-product of cellular metabolism through oxidative phosphorylation [14]. The intracellular levels of ROS are regulated through an intricate system of factors including available nutritional antioxidants (such as vitamin C, vitamin E, and selenium), reducing cofactors and peptides (glutathione, thioredoxin, and nicotinamide adenine dinucleotide phosphate), and antioxidant enzymes (Superoxide dismutase, Catalase, and Glutathione peroxidase) [15]. Additional mechanisms have also been shown to influence cellular ROS, including active ROS detoxification by neighboring cells in the niche [16, 17], the distance of the cell to the microvasculature [9, 10, 18], proliferative activity of the cells [11, 19], and the release of ROS in the niche by innate immune cells. At controlled physiological levels, cells depend on ROS for oxidative turnover of proteins required for signal transduction, with ROS as a central mediator in signaling pathways involved in proliferation, differentiation, and quiescence [12, 20, 21]. Thus, it has been hypothesized that there is a cellular "redox window" where an appropriate ROS level is required for physiological cellular function, while increased ROS can contribute to cellular dysfunction and pathological conditions [14, 18].

Depending on the concentration of oxygen, ROS can result from spontaneous oxidation [22]. Furthermore, effector cells of innate immunity, such as granulocytes, can enzymatically release ROS into the extracellular space [23, 24]. In addition to being a cause of stress, ROS has also been reported to be produced in cells intended for apoptosis or senescence, with active ROS generation identified as downstream of p53 activation [25], endoplasmic reticulum stress [26], and by the p38-mediated stress response [27], indicating that ROS is a central and shared feature between the various classical pathways of stress signaling. It has been demonstrated that primary (noncultured) hematopoietic progenitors from cord blood rapidly transit from a ROS^{lo} to a ROS^{hi} phenotype when cultured in vitro, leading to impairment in LT engraftment capacity of HSCs [9, 10]. Thus, the physiological ROS^{lo} phenotype is a primary requisite for the functionality of adult HSCs. Given that hPSC-derived hematopoietic cells have persistently demonstrated proliferative capacity deficits compared to adult and neonatal counterparts (bone marrow and cord blood progenitors), we hypothesized that hPSC-derived hematopoietic cells are functionally impaired as a direct result of the elevated ROS levels and its associated consequences to cell proliferation, and survival. In this study, using our previously established protocol for generating hPSC-derived hematopoietic progenitors [5], we demonstrate that the vast majority of hematopoietic progenitors generated during in vitro hPSC-to-blood differentiation possess high levels of intracellular ROS as compared to noncultured cord blood progenitors. Functional evaluation of hPSC-derived hematopoietic progenitors revealed that ROS^{hi} cells have higher levels of DNA damage and reduced colony forming capacity as compared to ROS^{lo} cells. Furthermore, we show that ROS^{lo} hPSC-derived $CD90^+$ $CD34^+$ hematopoietic cells have superior proliferative potential cultured in an expansion assay, demonstrating a ROS sensitivity of the most primitive iPS-derived hematopoietic cells with a functional dependence on physiological (low) ROS levels. We assess four functionally separate ROS reducing strategies, including; (a) the use of antioxidants, (b) blocking of innate immunity (c) inhibition of the p38-mediated stress response, and (d) reduced oxygen tension, and demonstrate that the high ROS levels in the in vitro differentiation cultures is contributed to by multiple distinct cellular processes and not only from metabolic activity. Furthermore, the use of the combination of all four ROS reducing factors (All Factors Combined [AFC]), enabled a 60% reduction of ROS, and significantly increased the generation of ROS^{lo} $CD90^+$ $CD34^+$ hematopoietic progenitors with robust growth capacity. These results show for the first time that high levels of ROS, resulting from mechanisms associated with in vitro hPSC differentiation systems, occurs and functionally impairs the most primitive of the newly generated hematopoietic cells, and that ROS regulation is required for generating functional hematopoietic progenitor cells in vitro.

Materials and Methods

Cell Culture

hPSCs were routinely maintained as colonies on murine embryonic fibroblasts (MEFs) (Merck Millipore, Darmstadt, Germany) until start of differentiation. Additionally, the hPSCs were cultured with 3 μM CHIR99021 (R&D Systems, McKinley Place, MN), during a 48-hour period before start of differentiation, to prime them for mesodermal commitment [28]. Cell lines used were human ES cell lines H1, HUES 2, and HUES 3 (obtained from WiCell, Madison, WI, and Harvard University, respectively, under material transfer agreements), and the iPS cell line RB9-CB1 (derived from cord blood endothelial cells transduced with tet-inducible lentiviral vectors expressing *OCT4*, *SOX2*, *LIN28*, *KLF4*, *C-MYC*) [29]. All pluripotent cell lines were karyotypically normal and have earlier been demonstrated to be pluripotent by in vivo teratoma assay and polymerase chain reaction. Mycoplasma testing was performed routinely to assure that all lines were free of contamination. Cell media used for differentiation of pluripotent stem cells toward blood was MesoTotal (Primorigen Biosciences, Madison, WI). At day 0 of differentiation colonies of hPSCs were separated from MEFs by incubation with 0.5 mg/ml Dispase (Invitrogen) in DMEM at 37°C for 30 minutes. Detached colonies were collected, washed, and resuspended in MesoTotal before placed into Corning Costar Ultra-low attachment Plates/Dishes (Sigma-Aldrich, St. Louis, MO) after which the colonies formed Embryoid Bodies (EBs) over night. On day 1 and 2 of differentiation the EBs were collected, washed, and received full media changes. On day 4 and 6 50% of the used MesoTotal was replaced with fresh media. On day 8 the EBs were collected and resuspended in 100% fresh MesoTotal before distributed into wells coated with Matrigel (STEMCELL

Technologies, Vancouver, BC, Canada), after which the EBs attached and layers of cells began to spread. On day 10, 12, and 14 100% of MesoTotal was replaced. On day 15, only 90% of the media was replaced to limit loss of free-floating hematopoietic progenitors. On day 16, the cultures were harvested for analysis. All media was collected before the differentiated cells were washed with PBS, singularized using TrypLE (Thermo Fisher Scientific), passed through a 21G needle, and filtered using 30 μm sterile Cup Filicons (BD Biosciences). The cells were then centrifuged at 350G for 8 minutes before resuspended and counted. Growth media used for sub-culture of CD34-enriched cord blood cells was standardized cord blood expansion media SFEM (STEMCELL Technologies), supplemented with 100 ng/ml each of the following cytokines; hTPO, hSCF, and hFLT3 (all from PeproTech, Rocky Hill, NJ). The following factors were added to the media if indicated: L-Ascorbic acid (Sigma, St. Louis, MO) at 0.378 mg/ml, and Citric acid monohydrate (ACROS Organics/Thermo Fisher, NJ) at 0.158 mg/ml. Also myeloperoxidase (MPO) blocker 4-Aminobenzoic acid (Sigma) at 100 μM, and p38 mitogen-activated protein kinase (MAPK) inhibitor LY2228820 (Selleckchem, Houston, TX) at 500 nM, were dissolved in Dimethyl sulfoxide (DMSO) (Sigma) before addition to media. DMSO was used as vehicle control.

All cells were cultured at 37°C at 5% CO_2. Oxygen level was either at atmospheric concentration (21%) or kept at 4% by use of a hypoxic incubator (BINDER GmbH, Tuttlingen, Germany). All media was allowed to preincubate for 8 hours before addition to cell cultures.

Flow Cytometry

Differentiated cells were washed in PBS before singularization using TrypLE (Thermo Fisher Scientific), passed through a 21G needle, and filtered using 30 μm sterile Cup Filicons (BD Biosciences). Cells were treated with 7AAD to exclude dead cells. Cells were stained using the following anti-human antibodies; fluorescein isothiocyanate-conjugated CD45 (eBioscience, 11-0459-42) and CD43 (BD Biosciences, 555475), phycoerythrin-cyanine (PE-Cy7)-conjugated CD34 (Biolegend, 343516), Phycoerythrin (PE)-conjugated CD90 (Biolegend, 328110). CellROX Deep Red Reagent (Thermo Fisher Scientific, C10422), used to detect oxidative stress, was applied according to the manufacturers instruction. Cells were incubated with CellROX Deep Red Reagent, and additional antibodies, for 20 minutes at 37°C in the dark. Cells were acquired on a Flow Assisted Cell Sorter (FACS) LSR II (BD Biosciences) or sorted using a FACS Aria III (BD Biosciences). Analysis was done using FlowJo, version X.0.7 (FLOWJO LLC, Ashland, OR). All FACS gates are based on fluorescence minus one (FMO) controls unless stated otherwise.

Colony Forming Unit Assay

Hematopoietic progenitors were sorted and plated with 1.5 ml of MethoCult H4435 (STEMCELL Technologies) into individual wells on Falcon Tissue Culture six-well plates (Thermo Fisher Scientific) at a ratio of 500 cells per well. No additional cytokines or compounds were added to the methylcellulose. Cultures were incubated for 14 days in a standard humidified incubator at 37°C with 5% CO_2. Colonies were counted, and scored by size, using bright-field microscopy.

Comet Assay

Hematopoietic progenitors were sorted into ice-cold PBS and cast into 40°C 1% low melt point agarose (LONZA, Rockland, ME) on Microscope Slides (Thermo Fisher Scientific). Cells were lysed in NaOH solution at pH $>$ 13 overnight at 4°C in the dark. The slides were then rinsed twice with NaOH, at pH 12.3, followed by submersion in a EASYCAST B1 gel runner (Thermo Fisher Scientific) filled with the same solution and allowed to run for 25 minutes at 20V, 50 mA (0.6 V/cm). The slides were then washed with distilled H_2O followed by a 5-minutes immersion in 70% EtOH before being allowed to air-dry for 15 minutes. The slides were submerged in TE-Buffer solution with 1:10,000X SYBR Green I (Invitrogen, Eugene, OR) and incubated in the dark for 30 minutes, followed by H_2O rinsing, and 15 minutes of air-drying. The slides were then immediately evaluated using an IX70 microscope (OLYMPUS, Shinjuku, Tokyo, Japan), equipped with a DP72 camera (OLYMPUS), and images were captured using the software cellSens Standard 1.6 (OLYMPUS). Brightness and contrast adjustments were un-biasedly carried out for all images using Photoshop CS6 (Adobe Systems Inc., San Jose, CA) prior to image analysis. Automatic comet analysis and OTM scoring was performed using the previously published ImageJ plug-in OpenComet [30] for ImageJ (version 1.48). This protocol is a modified version of the alkaline Comet Assay protocol published previously [31].

Growth Capacity Assay

$CD43/45^+$ $CD34^+$ $CD90^+$ cells were sorted into the following media; IMDM (+Hepes, -Glutamine) (GE Healthcare Bio-Sciences, Little Chalfont, U.K.), 20% heat-inactivated FBS (Thermo Fisher Scientific), 1X L-Glutamine (Thermo Fisher Scientific), 10 μg/ml Penicillin-Streptomycin Solution (GE Healthcare BioSciences), supplemented with the following cytokines; hSCF, hFLT3, hIL3, hTPO, hGM-CSF (all from PeproTech) at the final concentration of 10 ng/ml. Cells were distributed into wells on Nunc MiniTrays (Thermo Fisher Scientific) at 4 cells per well and 20 μl media. Plates were then placed on elevations in sterile plastic boxes containing PBS to prevent media evaporation. Each well was scored for cell growth at 18 hours, 5 days, 9 days, and 13 days postsort by image capturing using bright-field microscopy followed by manual unbiased area coverage estimation.

Statistical Analysis

Statistical analysis was, unless stated otherwise, performed using unpaired Student's *t* test, and the results were considered to be statistically significant at p value $<$ .05. All graphs depict mean ± SEM. The number of biological replicates is indicated by the n value. The graphs and statistical evaluation were done using GraphPad Prism (GraphPad Software, San Diego, CA).

Results

Elevated Reactive Oxygen Species Levels Correlate with the Decreased Function of hPSC-Derived Hematopoietic Progenitors

Since elevated ROS has been shown to impair the function of both murine and human HSCs, and progenitors, and since

these primary cells, when cultured in standard in vitro culture conditions, rapidly shift from a ROS^{lo} to a ROS^{hi} state [9, 10], we assessed the levels of ROS in our pluripotent stem cell differentiation system, previously described [5], and evaluated the impact of ROS on the in vitro generation and functionality of hematopoietic cells. Following 16 days of differentiation from the pluripotent state, hematopoietic cell fractions were identified by their cell surface phenotype using the established markers, as follows: total hematopoietic cell fraction identified as $CD43/45^+$ (combined use of early- & pan-hematopoietic markers) [32], the hematopoietic progenitor fraction as $CD43/45^+$ $CD34^+$, and our more primitive hematopoietic cell fraction, previously described as HSC-like [5], as $CD43/45^+$ $CD34^+$ $CD90^+$. The cell permeable dye CellROX Deep Red, becoming fluorescent upon presence of intracellular ROS, was used to measure ROS in all cell populations. In conjunction with our analyses using our hPSC-to-blood protocol, freshly isolated CD34-enriched cord blood cells were used as a reference point to indicate physiological levels of ROS, which we defined as ROS^{lo} (Fig. 1A, upper left panel, and Supporting Information Fig. S1A, upper panel). The same cells cultured for 3 days in a hematopoietic progenitor specified media rapidly increased ROS levels, with few cells displaying the ROS^{lo} phenotype, which we defined as ROS^{hi} (Fig. 1A, upper middle panel, and Supporting Information Fig. S1A, middle panel). Analysis of ROS in our hPSC-derived hematopoietic progenitors ($CD34^+$) demonstrated the vast majority having the ROS^{hi} phenotype (Fig. 1A, upper right panel, and Supporting Information Fig. S1A, lower panel). Similar levels of ROS were observed in hematopoietic progenitors generated from differing hPSC lines (Supporting Information Fig. S1B).

To evaluate the impact of ROS on the functionality of iPS cell derived hematopoietic progenitors, cells were separated into ROS^{lo} and ROS^{hi} fractions (Supporting Information Fig. S1C, S1D). Since ROS is known to cause DNA damage [33], we sought to directly evaluate the extent of DNA damage in ROS^{lo} and ROS^{hi} hematopoietic progenitors. For this purpose, we used the alkaline Comet assay that allow for measurement of both single- and double-strand breaks [31]. Analysis of sorted hematopoietic progenitors demonstrated that the ROS^{hi} fraction had significantly higher DNA damage as compared to the ROS^{lo} fraction (Fig. 1B, 1C). In addition, hPSC-derived hematopoietic progenitors sorted for ROS^{lo} or ROS^{hi} were evaluated for their growth and differentiation capacities in the colony forming unit (CFU) assay. CFUs were analyzed in terms of number, size, and type (Fig. 1D, Supporting Information Fig. S1E, S1F). ROS^{lo} hematopoietic progenitors ($CD34^+$) yielded higher numbers of CFUs as compared to ROS^{hi} counterparts (Fig. 1D, left panel), and ROS^{lo} cells generated significantly greater numbers of medium and large sized colonies indicating superior proliferative capacity (Fig. 1D, right panel). The difference in ROS level did not change the frequencies of different colony types, however erythroid colonies, which are known to be more sensitive to ROS [34], were only observed in ROS^{lo} progenitor-derived CFUs (Supporting Information Fig. S1F).

Adult HSCs have been demonstrated to be more sensitive to ROS as compared to the downstream hematopoietic hierarchy [35, 36]. Given the difficulties in generating HSCs from hPSC differentiation cultures capable of repopulation in a xenotransplant assay, we developed an alternative assay to assess the cells functional properties. To evaluate the impact of ROS hPSC-derived $CD43/45^+$ $CD34^+$ $CD90^+$ cells were sorted for either the ROS^{lo} or ROS^{hi} phenotype (Fig. 1E, left panel) and distributed into Terasaki plates to measure their growth at several time-points over a 13-day period (Fig. 1F, upper panel). While ROS^{hi} cells demonstrated severely limited cell growth and displayed a flattened morphology indicative of senescence, the ROS^{lo} cells proliferated and gave rise to large clusters of hematopoietic cells (Fig. 1F, lower panel), indicating that the $<3\%$ ROS^{lo} cells (Fig. 1E, right panel) of the total $CD90^+$ progenitor fraction contained all the proliferative potential of these cells generated in the standard condition. These results strongly indicate that ROS^{lo} levels are requisite for the expansion/proliferation of these more primitive hematopoietic cells. Taken together, high ROS levels in hPSC-derived hematopoietic differentiation systems, with its associated increase in DNA damage, significantly impairs the growth capacity of more primitive hematopoietic cells.

ROS Reducing Strategies Specifically Increase ROS^{lo} Hematopoietic Progenitors

In order to identify means to facilitate a physiological level of ROS in hPSC-derived hematopoietic progenitors, we employed four strategies, each aimed at reducing ROS by a separate mechanism (Fig. 2A): (a) ROS scavenging by a cocktail of antioxidants (ascorbic acid and citric acid), (b) MPO inhibition with 4-aminobenzoic acid (4-ABAH) [23] to prevent innate immune-cell mediated ROS release, (c) p38α MAPK inhibition with the small molecule LY2228820 [27] to limit stress-response mediated ROS generation, and (d) reducing ROS by reducing the oxygen concentration to 4% during culture. Applied individually, each of the ROS reducing strategies reduced ROS between 11%-47% (Fig. 2B) indicating that active innate immunity, active stress signaling, and accelerated ROS formation due to a supraphysiological oxygen level, collectively contribute to the increased ROS observed in our cells. To provide broad protection from both spontaneous and enzymatically generated ROS, AFC (All Factors Combined) were used, which facilitated the strongest reduction of ROS levels (Fig. 2B), equivalent to a 60% reduction compared to the hPSC-derived control condition and approaching the ROS level of noncultured cord blood. Importantly, cellular viability was not negatively affected using these ROS reducing strategies (Fig. 2C). However, analysis of the frequencies of total hematopoietic cells ($CD43/45^+$), hematopoietic progenitor cells ($CD43/45^+$ $CD34^+$), and cells with the previously described HSC-like surface markers ($CD43/45^+$ $CD34^+$ $CD90^+$) (Fig. 2D) demonstrated that the AFC significantly increased the frequency of ROS^{lo} total blood cells by twofold, and ROS^{lo} progenitors by fivefold (Fig. 2E). Interestingly, the AFC condition increased the frequency of ROS^{lo} $CD43/45^+$ $CD34^+$ $CD90^+$ cells by 15-fold (Fig. 2E). Furthermore, from equal amounts of pluripotent starting material, the AFC condition produced 18-fold increase in total output of ROS^{lo} $CD43/45^+$ $CD34^+$ $CD90^+$ cells compared to the standard condition (Fig. 2F). There was also a modest (1.5-fold) but statistically significant increase in the frequency of the total ($ROS^{lo} + ROS^{hi}$) $CD43/45^+$ $CD34^+$ $CD90^+$ cells as compared to the standard condition (Fig. 2G) These results together demonstrate that increased ROS in hematopoietic cells cultured in vitro is contributed by multiple mechanisms, and that a combination of

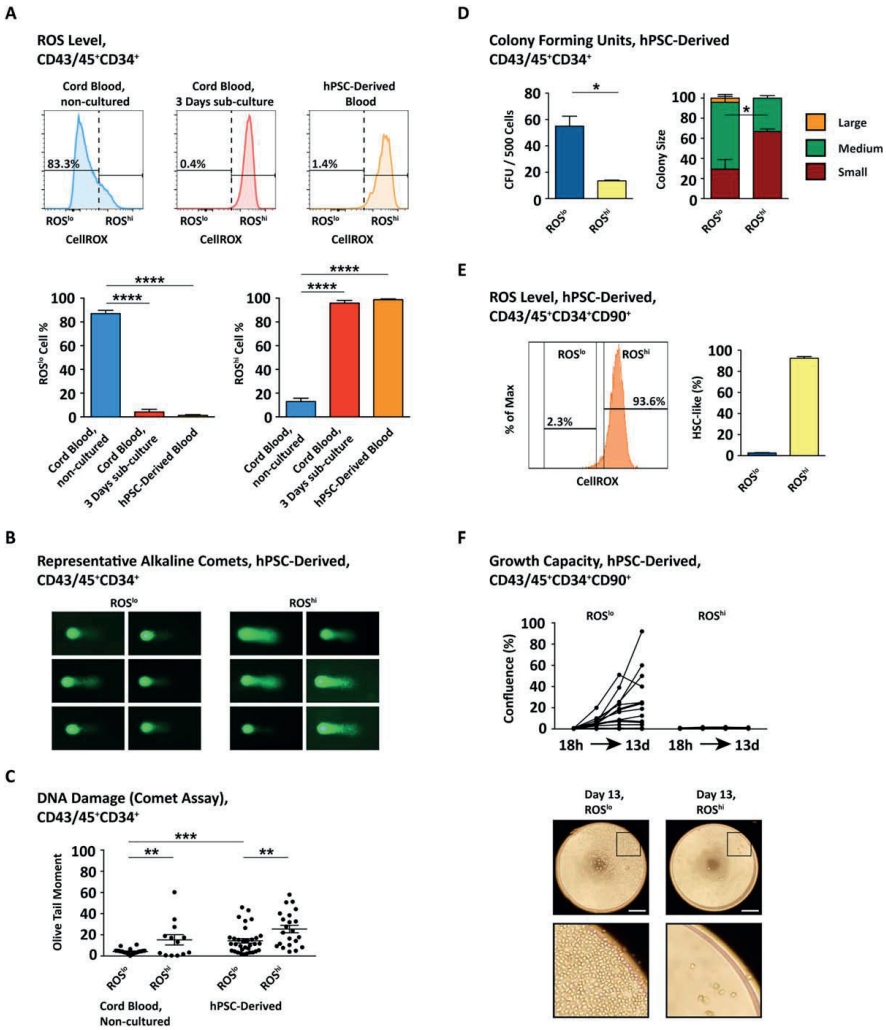

Figure 1. Elevated ROS levels lead to reduced functionality of hPSC-derived hematopoietic progenitors. **(A)**: Representative flow cytometry histograms displaying the ROS levels of the hematopoietic progenitor fraction ($CD43/45^{+}CD34^{+}$) for noncultured cord blood, 3 days subcultured cord blood, and hPSC-derived blood. The gating strategy is detailed in Supporting Information Figure S1A. Lower panel bar graphs show the ROS^{lo} and ROS^{hi} status of hematopoietic progenitors ($n = 3$). **(B)**: Representative alkaline comets of ROS^{lo} and ROS^{hi} hPSC-derived hematopoietic progenitors. **(C)**: Level of DNA damage in ROS^{lo} and ROS^{hi} hematopoietic progenitors, indicated by Olive Tail Moment, for noncultured cord blood, and hPSC-derived hematopoietic progenitors ($n = 3$). **(D)**: Number of CFU obtained from 500 ROS^{lo} or ROS^{hi} hPSC-derived hematopoietic progenitors. Right panel show the size distribution of the CFUs as indicated. **(E)**: Representative FACS sort gates for the ROS^{lo} and ROS^{hi} fractions of the hPSC-derived $CD43/45^{+}CD34^{+}CD90^{+}$ hematopoietic progenitor population. Bar graph showing the distribution of ROS^{lo} or ROS^{hi} cells ($n = 3$). **(F)**: Growth capacity of hPSC-derived $CD43/45^{+}CD34^{+}CD90^{+}$ cells. Upper panel show the growth kinetics of a representative sample group (ROS^{lo} and ROS^{hi} hPSC-derived $CD43/45^{+}CD34^{+}CD90^{+}$ cells) indicated by well confluency at 18 hours, 5 days, 9 days, and 13 days after seeding. Lower panel show representative culture wells, initially seeded with ROS^{lo} or ROS^{hi} cells, after 13 days of subculture (scale bars = 200 μm). Data represents mean ± SEM. Asterisks indicate significant differences (*, $p < .05$; **, $p < .01$; ***, $p < .001$; ****, $p < .0001$; n.s., not significant). See also Supporting Information Figure S1. Abbreviations: hPSC, human pluripotent stem cells; ROS, reactive oxygen species.

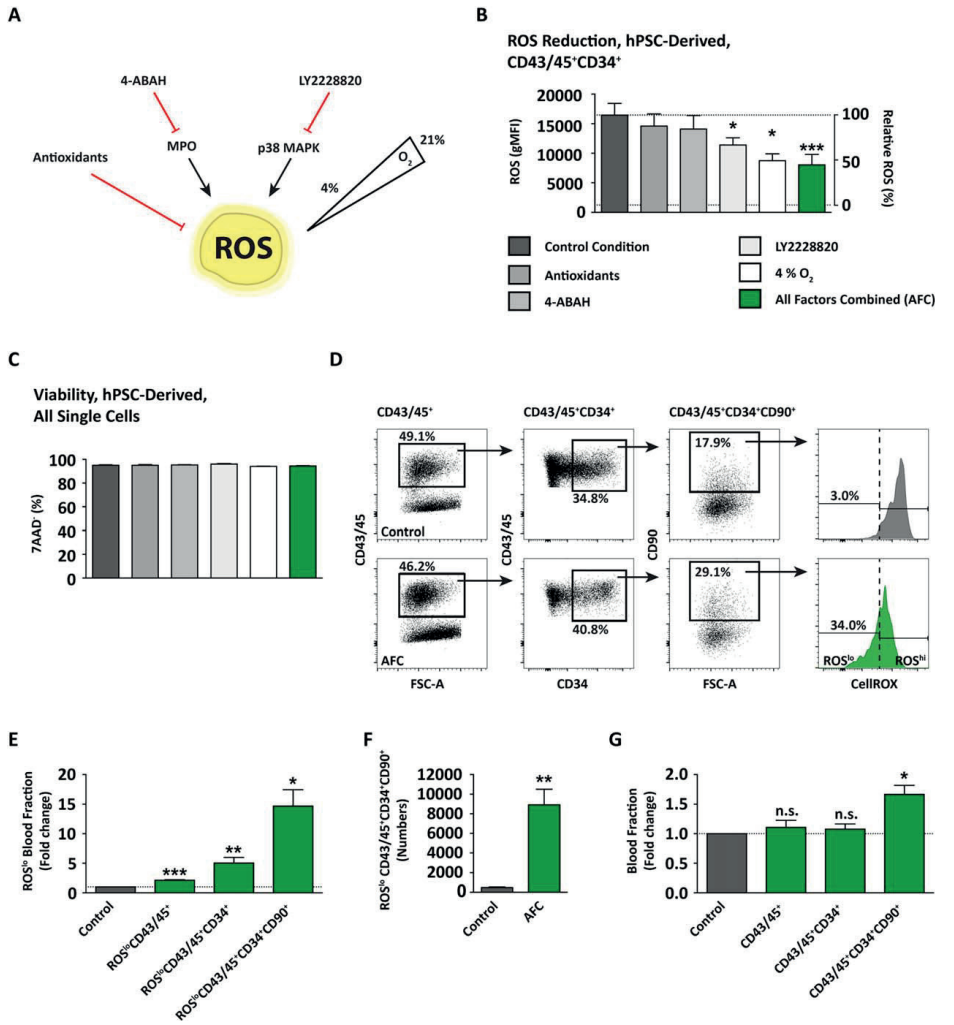

Figure 2. Combination of ROS-reducing strategies leads to increased generation of ROS^{lo} $CD90^{+}$ hematopoietic progenitors. **(A)**: Schematic representation of the four factors used for ROS reduction. **(B)**: ROS gMFI of hPSC-derived hematopoietic progenitors generated with: ROS scavenging by a cocktail of antioxidants (Ascorbic Acid + Citric Acid), MPO blocking by 4-ABAH, p38 MAPK inhibition by LY2228820, reduction of oxygen tension from 21% to 4%, or AFC, and control condition (DMSO) ($n = 3$). Statistics are based on paired parametric *t* test. The right *y*-axis displays extent of ROS reduction as compared to the physiological ROS level of hematopoietic progenitors from noncultured cord blood (set to 0) and the ROS level of hematopoietic progenitors generated in control conditions (set to 100). **(C)**: Viability ($7AAD^{-}$) of hPSC-derived cultures generated with the above described conditions ($n = 3$). **(D)**: Representative flow cytometry dot plots for hPSC-derived total hematopoietic cells ($CD43/45^{+}$), hematopoietic progenitors ($CD43/45^{+}CD34^{+}$), and $CD90^{+}$ hematopoietic progenitors ($CD43/45^{+}CD34^{+}CD90^{+}$), generated in control conditions or with AFC. Gates are based on fluorescence minus one controls. Doublet exclusion and dead cell exclusion (7AAD) were done before applying the gates. The ROS level for the $CD90^{+}$ hematopoietic progenitor fraction is displayed. **(E)**: Fold change in the frequency of ROS^{lo} total hematopoietic cells, ROS^{lo} hematopoietic progenitors, and ROS^{lo} $CD90^{+}$ hematopoietic progenitors. The values are normalized to control (set as 1) ($n = 3$). **(F)**: Bar graph showing the output of ROS^{lo} $CD43/45^{+}CD34^{+}CD90^{+}$ cells generated from equal amounts of initial hPSCs ($n = 3$). **(G)**: Fold change in the frequency of total (ROS^{lo} + ROS^{hi}) hematopoietic cells, the hematopoietic progenitor fraction, and the $CD90^{+}$ hematopoietic progenitor fraction. The values are normalized to control (set as 1) ($n = 3$). Data represent mean ± SEM. Asterisks indicate significant differences (*, $p < .05$; **, $p < .01$; ***, $p < .001$, n.s., not significant). Abbreviations: 4-ABAH, 4-aminobenzoic acid; AFC, All Factors Combined; FSC-A, Forward Scatter-Area; hPSC, human pluripotent stem cells; MPO, myeloperoxidase ROS, reactive oxygen species.

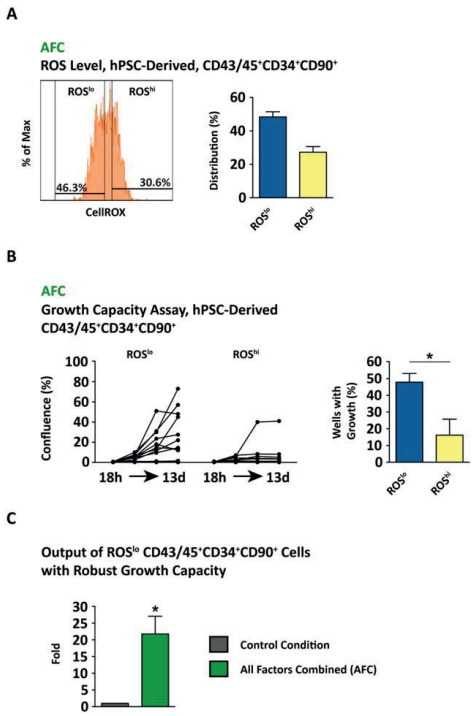

Figure 3. ROS reduction increases the generation of hPSC-derived CD90$^+$ hematopoietic progenitor cells with high growth capacity. **(A)**: Representative FACS gates for sorting the ROS^{lo} and ROS^{hi} fractions of CD43/45$^+$CD34$^+$CD90$^+$ cells generated after AFC. Bar graphs show the distribution of ROS^{lo} and ROS^{hi} cells ($n = 3$). **(B)**: Left panel showing growth kinetics of a representative sample group (ROS^{lo} and ROS^{hi} CD43/45$^+$CD34$^+$CD90$^+$ cells) generated with AFC, as measured by well confluence (%) at 18 hours, 5 days, 9 days, and 13 days postseeding. The right panel bar graph shows the frequency of wells with $>10\%$ well confluence at day 13. **(C)**: Fold change in the generation of ROS^{lo} CD43/45$^+$CD34$^+$CD90$^+$ cells with high proliferative capacity using AFC as normalized to the control condition (DMSO). Data represent mean $\pm$ SEM. Asterisks indicate significant differences (*, $p < .05$, n.s., not significant). Abbreviations: FSC-A, Forward Scatter-Area; hPSC, human pluripotent stem cells; hPSC, human pluripotent stem cells; ROS, reactive oxygen species.

ROS reducing methods allows for significant reduction of ROS, that specifically facilitates increases of ROS^{lo} hematopoietic progenitors, and preferentially, the more primitive CD90$^+$ fraction from hPSCs.

ROS Reduction Increases ROS^{lo} CD90$^+$ Hematopoietic Progenitors with Robust Growth Capacity

To evaluate the function, in terms of growth capacity, of cells generated in the AFC condition, CD43/45$^+$ CD34$^+$ CD90$^+$ cells were separated into ROS^{lo} and ROS^{hi} fractions and were plated into Terasaki wells. Sort-gates were based on the ROS profile of a cord blood sample simultaneously analyzed. Approximately 50% of these hPSC-derived cells displayed a ROS^{lo} phenotype (Fig. 3A). ROS^{lo} CD43/45$^+$ CD34$^+$ CD90$^+$ cells generated with the AFC demonstrated higher growth capacity compared to the limited performance of the ROS^{hi} fraction (Fig. 3B, left panel). The proportion of ROS^{lo} cells capable of proliferation was also increased compared to the ROS^{hi}, thus confirming that the ROS reducing factors not only reduced ROS, but also increased the functional growth capacity of the CD90$^+$ progenitor fraction (Fig. 3B, right panel). Moreover, the total increase in generated ROS^{lo} CD43/45$^+$ CD34$^+$ CD90$^+$ cells with robust growth capacity was 22-fold higher with AFC, as compared to the standard condition (Fig. 3C). These findings demonstrate that high ROS levels limit function in terms of proliferation in the most primitive hPSC-derived hematopoietic progenitors, and that our strategies to reduce ROS significantly increase the output of such hematopoietic progenitors with growth capacity.

A key criterion for functional HSCs is the capacity to serially engraft into immune-compromised recipients [37]. In this study, we also evaluated the engraftment capacity of cells generated either with our standard condition or with the ROS reducing AFC condition. Using a stringent assessment of engraftment in transplanted NSG mice we saw very low levels of human hematopoietic cells that were not discernable over negative controls, indicating that the 60% reduction of ROS reported in this study was insufficient to endow our hPSC-derived hematopoietic cells with engraftment capacity (data not shown). However, a further reduction to physiological levels of fresh cord blood may be required. Nonetheless, the level of reduction achieved demonstrated a critically important feature of HSC function (cellular expansion) to be improved for our hPSC-derived cells with the AFC condition and thus propose that in vitro generation of functionally relevant HSCs will require ROS level regulation.

Endothelial Cells Have Low Levels of ROS, and ROS Reducing Strategies Specifically Reduce ROS in More Primitive Hematopoietic Cell Fractions

The origin of hematopoietic cells both in vivo during embryonic development and in vitro during differentiation from pluripotent stem cells has been shown to occur from endothelial cells with hemogenic potential [2, 4, 38–40]. To evaluate the levels of ROS in other cell types present in the blood directed differentiation cultures, we evaluated ROS levels in the CD43/45$^-$CD34^{hi}CD90^{hi} endothelial cell fraction (Fig. 4A, 4B). Surprisingly, the intracellular ROS levels in endothelial cells derived from hPSCs using the standard culture condition were uniformly low, and were not further reduced with the addition of the AFC (Fig. 4C). This is in contrast to the CD43/45$^+$ CD34$^+$ CD90$^+$ fraction of hematopoietic cells in the same wells, where AFC facilitated a significant reduction of intracellular ROS as compared to the standard condition. Moreover, while the AFC did not significantly change the frequency of the endothelial cells (Fig. 4D), a trend suggested a change in frequency similar to the 1.5-fold increase of hematopoietic CD43/45$^+$ CD34$^+$ CD90$^+$ cells (Fig. 2G). Thus, we cannot exclude that AFC may potentially have some influence on the differentiation toward endothelium. However, this effect was not statistically significant. These observations suggest that there are large differences in the abilities of differing cell types in the differentiation system to regulate their ROS levels; with the broad endothelial cell fraction effectively

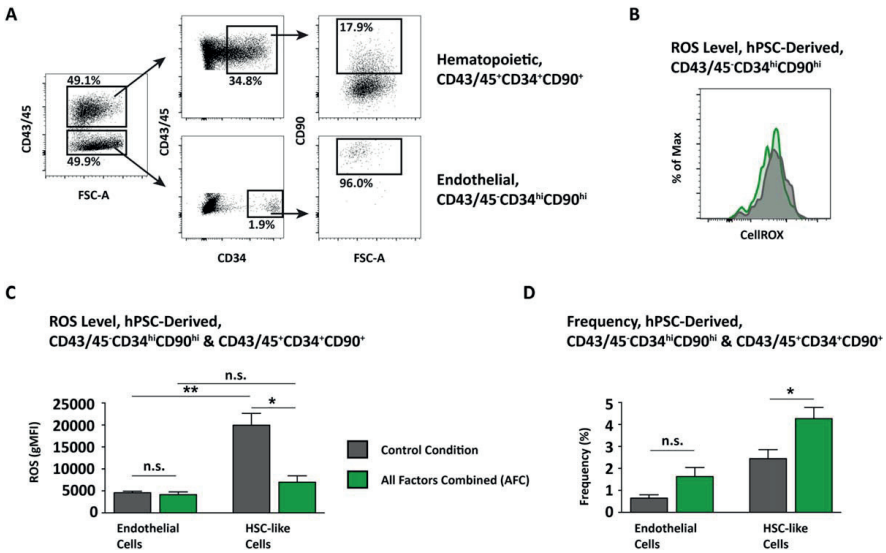

Figure 4. ROS reduction preserve the functionality of CD90$^+$ hematopoietic progenitor cells, without affecting endothelial cells. **(A)**: Representative flow cytometry dot plots showing hPSC-derived CD90$^+$ hematopoietic progenitor cells (CD43/45$^+$CD34$^+$CD90$^+$) and endothelial cells (CD43/45$^-$CD34^{hi}CD90^{hi}). Gates are based on FMO controls, doublet exclusion, and dead cell exclusion. **(B)**: Representative histogram plot displaying the ROS levels of the endothelial population for the DMSO control (gray) and the AFC (green). **(C)**: ROS gMFI of the endothelial and the CD90$^+$ hematopoietic progenitor fractions generated with control condition (DMSO) and AFC ($n = 3$). **(D)**: Frequency of the endothelial and the CD90$^+$ hematopoietic progenitor fractions, generated with control condition and AFC ($n = 4$). Data represent mean ± SEM. Asterisks indicate significant differences (*, $p < .05$; **, $p < .01$, n.s., not significant). Abbreviations: HSC, hematopoietic stem cell; hPSC, human pluripotent stem cells; ROS, reactive oxygen species.

managing its intracellular ROS levels, while hematopoietic progenitors are unable to retain a low ROS profile in culture, and displaying deficits in growth potential as a result. In comparison with CD34$^+$ progenitors, reduction of ROS using AFC had the strongest positive effect on the CD90$^+$ hematopoietic progenitor fraction, yielding the greatest increases in ROS^{lo} cells (Fig. 2E), and resulting in a 22-fold increase in cells with robust growth capacity (Fig. 3C), together suggesting that the most primitive hematopoietic cells in the culture system are the most sensitive to ROS and have the most to gain from ROS reduction strategies.

Discussion

In this study, we explored the impact of ROS on hematopoietic cells generated in vitro from hPSCs. We demonstrate that, in our standard hPSC differentiation system, the vast majority of newly generated hematopoietic cells have supraphysiological levels of intracellular ROS resulting in their functional impairment. The detrimental effects of the high ROS levels included a low frequency of the more primitive hematopoietic cell fractions, reduced colony forming capacity, increased DNA damage, and a complete abrogation of the proliferative capacity of the most primitive (CD43/45$^+$ CD34$^+$ CD90$^+$) hematopoietic progenitor fraction. The application of our four factor ROS reducing strategy was able to reduce the levels of intracellular ROS and restore functional capabilities to the cells, most notably with a 22-fold increase in the number of ROS^{lo} CD43/45$^+$ CD34$^+$ CD90$^+$ cells with robust growth capacity. Since the levels of ROS and their impact on hematopoietic progenitors generated from hPSCs has not previously been evaluated, our finding that newly committed hematopoietic cells are particularly sensitive suggests that hPSC differentiation systems require active regulation of ROS to prevent functional impairment of generated hematopoietic cells. Our ability to reduce ROS using four separate ROS reducing strategies individually demonstrates that ROS build-up in vitro results not only from cellular metabolism, but also from multiple processes including innate immunity, the cellular stress response, and by the elevated atmospheric oxygen tension of standard hPSC differentiation systems. Adult HSCs have previously been described to be more sensitive to oxidative insult as compared to their downstream progeny [35, 36], and the sensitivity to ROS of this more primitive hematopoietic cell fraction, particularly following culturing, is mirrored in our newly generated hematopoietic progenitors. A comparison of CD43/45$^+$ CD34$^+$ CD90$^+$ hematopoietic cells with CD43/45$^-$CD34^{hi}CD90^{hi} endothelial cells from the same wells indicate the ability of endothelial cells to self-regulate and maintain low intracellular ROS levels. This is in contrast to the specific inability of CD43/45$^+$ CD34$^+$ CD90$^+$ hematopoietic progenitors to maintain a low ROS level. Because the endothelial cell fraction did not respond to the ROS reducing factors (no further reduction of ROS, and no significant change in endothelial cell frequencies) it appears unlikely that ROS

levels would have negative effects on endothelial precursors to blood, however when precisely the sensitivity to ROS appears during the transition from endothelial cell to hematopoietic cell remains to be assessed as specific markers of cells that undergo endothelial to hematopoietic transition become available.

Adult HSCs are well-known to lose their LT engraftment capacity when cultured in vitro, and it has been reported that cells with a low level of ROS, a characteristic feature of noncultured and functional HSCs, will rapidly transit toward a ROS^{hi} phenotype upon sub-culture [9, 10]. The same studies confirmed that only ROS^{lo} adult HSCs contained LT engraftment capacity and that these cells also displayed reduced amounts of DNA damage, thus demonstrating an inverse relationship between HSC self-renewal capacity and ROS level. It has recently been demonstrated that even brief exposure to atmospheric levels of oxygen confers a rapid negative effect on the functionality of cord blood HSCs [41], providing evidence that adult human HSCs, in addition to being sensitive to gradual increase of ROS, are also acutely sensitive to in vitro conditions through a shock-like mechanism [42]. Due to the sensitivity of adult HSCs to ROS, it is interesting to speculate what further improvements in function the hematopoietic cells could achieve if ROS levels could be reduced to the levels seen in fresh cord blood. As reported here, AFC only partially normalized the levels of ROS toward the physiological level of noncultured cord blood. Indeed, while we observe a significant difference in DNA damage for ROS^{lo} hPSC-derived hematopoietic progenitors as compared to their ROS^{hi} counterparts, there is still a significant difference in the extent of DNA damage of these cells as compared to ROS^{lo} cells from noncultured cord blood, indicating that further improvements are required if ROS is to be further normalized toward physiological levels. While it is possible to generate hematopoietic cells that are similar to functional HSCs in terms of cell surface markers, as well as in their definitive myeloid/lymphoid differentiation capacity, the hPSC-derived cells lack functional engraftment capacity [2, 4, 5, 43]. It is often assumed that there is a maturation or development signal missing in in vitro differentiation systems that prevents the generation of robust repopulating HSCs. However, it has been demonstrated that functional engrafting HSCs indeed can be generated from hPSCs through stochastic differentiation by means of in vivo teratoma formation [44, 45]. It was suggested that signaling molecules circulating in the blood provided factors that enabled the generation of HSCs in the teratoma, but it is also plausible that the in vivo setting has the ability to help regulate ROS levels of new emergent HSCs allowing for their repopulating function. While we could not successfully generate functional engrafting HSCs, our ROS reducing strategies did not manage to reduce the levels of ROS to that of fresh cord blood. Nonetheless, our results demonstrate that currently used culture conditions do not adequately protect particularly sensitive hematopoietic lineage cell types such as the HSC from oxidative damage, and hence result in reduced function of these cells.

Conclusion

Our findings demonstrate that the reduction of ROS in in vitro culture systems, during de novo generation of hematopoietic cells, restores functional properties to these cells, with the greatest benefit to the most primitive hematopoietic cells. Efforts to further normalize ROS toward physiological levels will be crucial to generate functionally equivalent human hematopoietic stem and progenitor cells in vitro as compared to their in vivo counterparts.

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Author Contributions

R.E.R.: Conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; C.G. and S.S.: Collection and/or assembly of data, data analysis and interpretation; N.-B.W.: Conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflict of Interest

N.-B.W. is board member of Longboat Explorer Inc. R.E.R., C.G., and S.S. indicate no potential conflicts of interest.

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