The Role of EphrinB2 in Hematopoietic Stem/Progenitor Cell Differentiation From an Arterial Hemogenic Endothelium

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Abstract

During development, hematopoiesis develops in temporally distinct waves in the yolk sac (YS) and embryo proper, culminating in the emergence of definitive hematopoietic stem cells (HSCs) from the hemogenic endothelium (HE) of the dorsal aorta. The close association of this aortic endothelium with definitive hematopoiesis suggests a functional relationship between arteriogenesis and blood development, but this association is not fully understood. To gain insight into this relationship, we have chosen to study the role of the "arterial" marker, EphrinB2 (EfnB2) in hematopoietic specification. EfnB2 is a transmembrane protein critical for the development of the arterial vascular system. We find that EfnB2 is expressed in the VE-Cadh^{+}CD41^{-} HE in Day 2 BL-CFC (blast-colony forming cell) culture and Day 6 EBs (embryoid bodies), and that EfnB2 expression in ES cell differentiation enriches for endothelial cells with greater hemogenic capacity. Knock-down experiments in ES cells showed that EfnB2 is not required for endothelial cell commitment and survival. It is also not required for early hematopoietic commitment and differentiation from EBs or BL-CFCs. However, we find that EfnB2 is required for the maturation of ES cells into CD41^{+}/CD45^{+} hematopoietic cells in OP9 co-culture and for definitive hematopoietic colony formation in MethoCult3434 medium. This requirement for EfnB2 expression was confirmed by peptide-mediated blocking of EfnB2 binding to its cognate receptors and by forced expression of a phospho-tyrosine signaling-deficient EfnB2. These results provide evidence for an essential role of endothelial EfnB2 in hematopoiesis.

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Abbreviations

Ac-LDL	Acetylated Low Density Lipoprotein
ACV	Anterior Cardinal Vein
AGM	Aorta-Gonad-Mesonephros
BFU	Blast Forming Unit
bHLH	Basic Helix Loop Helix
BL-CFC	Blast Colony Forming Cell
BM	Bone Marrow
BMP	Bone Morphogenic Protein
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribonucleic Acid
CFU-S	Colony Forming Unit-Spleen
Cx	Connexin
DA	Dorsal Aorta
Dll4	Delta Like Ligand 4
DMEM	Dulbecco's Modified Eagle Medium
E	Erythroid
EB	Embryoid Body
EC	Endothelial Cell
EfnB	EphrinB2
eGFP	Enhanced Green Fluorescent Protein
EHT	Endothelial to Hematopoietic Transition
Ery	Erythroid
ES	Embryonic Stem (Cell)
FCS	Fetal Calf Serum
FACS	Fluorescence Activated Cell Sorting
FL	Fetal Liver
Fl	Floxed
GEMM	Granulocyte Erythroid Macrophage Megakaryocyte
GM	Granulocyte Macrophage
GPCR	G Protein Coupled Protein Receptor
HC	Hematopoietic Cell
HE	Hemogenic Endothelium
Het	Heterozygous

HPC	Hematopoietic Progenitor Cell
HSC	Hematopoietic Stem Cell
HUVEC	Human Umbilical Vein Endothelial Cell
IMDM	Iscove's Modified Dulbecco's Media
iPS	Induced Pluripotent Stem (Cell)
ISV	Intersomitic Vessel
IV	Intravenous
KL	Kit Ligand
KD	Knock-down
KO	Knock-out
LIF	Leukemia Inhibitory Factor
LT-HSC	Long-term Hematopoietic Stem Cell
MACS	Magnetic Activated Cell Sorting
MeC	Methylcellulose
mL	Milliliter
MTG	Monothiol Glycerol
NICD	Notch Intracellular Domain
NK	Natural Killer (Cell)
Nrp	Neuropilin
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline + Tween20
PCV	Posterior Cardinal Vein
PFA	Paraformaldehyde
PGK	Phospho Glycerate Kinase
P-Sp	Para-Aortic Splanchnopleura
PB	Peripheral Blood
PTC	Patched
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
RTK	Receptor Tyrosine Kinase
SAM	Sterile Alpha Motif
Scl	Stem Cell Leukemia
SHh	Sonic Hedgehog
shRNA	Short Hairpin Ribonucleic Acid

SH2	Src-Homology
SMC	Smooth Muscle Cell
ss	Somite Stage
TGFb	Transforming Growth Factor Beta
TMRE	Tetramethylrhodamine Ethyl Ester Perchlorate
TPO	Thyroid Peroxidase
UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor
vWF	Von Willebrand Factor
WT	Wildtype
YS	Yolk Sac

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1 Introduction

1.1 Vascular Development in the Embryo

1.1.1 Vasculogenesis

As the embryo develops in increasing size and complexity, the ability of simple diffusion to deliver nutrients and molecules adequately to all the cells of the embryo reaches its limit. This necessitates the formation of a network of vessels that can connect the developing heart and peripheral organs to deliver blood, oxygen and nutrients after circulation and heartbeat are established. During vascular development, dispersed endothelial/mesoderm precursors begin to coalesce in the process termed vasculogenesis. This forms the first honeycomb-like primitive plexus, which is characterized by a disorganized network of dilated capillaries with homogenous diameters and marker expression patterns. At the beginning, two vascular networks are formed, one in the YS (yolk sac) at E7.5 (0 somite) and the other in the embryo proper later at E7.75 (1 somite).[1] The two networks are connected beginning around E8.0 at which point circulation is established.[2]

Formation of the YS Vascular Plexus

The YS forms beginning around E6.5 during gastrulation as mesoderm-committed cells migrate out of the early posterior primitive streak of the embryo. This sheet of mesoderm cells forms the visceral YS; this structure can be visualized by Flk1-LacZ staining (using a transgenic Flk1-LacZ reporter knock-in line; KDR; VEGFR2) staining where it appears to line the exocoelomic cavity and visceral endoderm.[3] Beginning at E7.0, mesodermal cells in the visceral YS that are precursors to the blood islands begin to proliferate rapidly into mesodermal masses. At E7.75, a network of solid angioblastic cords begins to organize, and

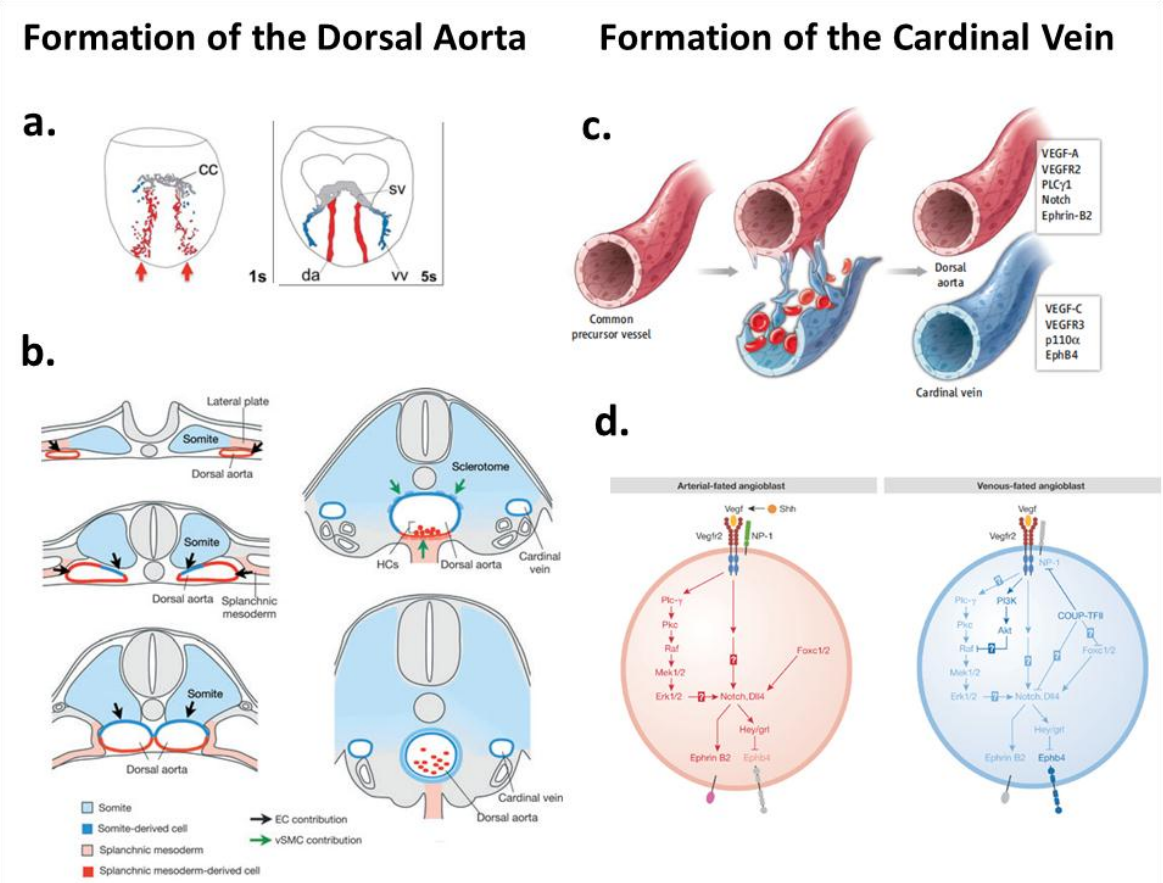

Figure 1. Formation of the Dorsal Aorta and Cardinal Vein During Development

a. Schematic of a 1 somite embryo (left) with Flk1+ angioblasts beginning to align and coalesce into two paired DA (red arrows). cc, cardinal crescent. Schematic of a 5 somite embryo (right) when the paired aortae begin to lumenize (red). vv, vitelline vein. From Chong 2011.[1]

b. Schematic illustrating the contribution of two differentially-derived EC populations to the DA. Lateral plate-derived ECs (red) form the primary DA (top left). As the DA migrates medially, ECs derived from the somites contribute to the roof of the DA (blue; bottom left). After lateral-plate derived hemogenic ECs of the DA floor are exhausted in the generation of HSCs, somite-derived ECs replace them to form the entire mature DA (bottom right). From Sato 2013.[4]

c. Schematic showing the formation of the cardinal vein through selective cell sprouting from the DA. Venous cells (blue) expressing EphB4 sprout ventrally from the EfnB2+ DA (red) to form a lumenized vessel around E9.0. From https://sites.google.com/ site/gofunsite/Home/research-highlights

d. Classic arterial and venous regulation hierarchy. Hedgehog induces VEGF, Notch, and then EfnB2 signaling in arterial cells. From Lin 2007.[11]

by E8.25 differentiate into the ECs surrounding the primitive erythroblasts of the blood islands. The YS primary plexus is laid out by E8.5.[3]

Formation of the Dorsal Aorta

The DA is the first functional intra-embryonic blood vessel to develop, delivering blood from the heart to the rest of the embryo proper by E9.0. It will give rise to the descending aorta in the adult and also serve as the site where definitive HSCs arise. The DA originates de novo from two separate bilateral pre-aortic angioblast cords that first lumenize at E7.75 (3 somite) and then fuse at the midline to a one single DA beginning at E9.5 from the anterior side (Figure 1a).[1] The growth of the DA diameter is almost completely due to the migration of EC precursors from lateral locations in the embryo and not from the proliferation of pre-existing ECs at the midline.[4]

In quail/chick chimera experiments, the primitive DA is formed when somite-derived angioblasts migrate to the midline to form the roof of the vessel while splanchnopleural mesoderm-derived precursors migrate along the endodermal boundary to form the floor (schematized in Figure 1b). The dual lineage of the DA has been confirmed in mouse/avian graft experiments, though the frequency of incorporation of pre-somitic mouse mesoderm into the avian embryos was much lower than in the quail/chick chimeras.[5]

As the splanchnopleural-derived ECs on the DA floor are exhausted in the endothelial to hematopoietic transition (EHT) that gives rise to the HSCs (discussed later), the primitive DA further attracts somite-derived, notch-activated angioblasts to spread over the entire DA, forming the definitive DA.[6]

Formation of the Cardinal Vein

The cardinal vein forms after the development of the DA, through a loose plexus intermediate and not unlumenized cord like the DA.[1] The mechanism of posterior cardinal vein (PCV) formation has been termed "selective cell sprouting" based on studies in zebrafish. During this process, Flk1+ angioblasts sprout ventrally from the DA to form a cardinal vein primordium that is then segregated from the DA (Figure 1c).[7] A similar pattern has been described immunophenotypically in mice, though not in the same molecular detail as in zebrafish. In the mouse, the anterior and posterior cardinal veins are not assembled until E9.0 (13 somite stage) and are not fully formed until E9.5 (20 somites) when EphB4 expression is finally established.[1]

Recent studies in zebrafish, however, have shown that the DA and cardinal veins appear to arise separately from two different angioblast populations: an arterial progenitor that originates earlier and closer to the midline of the embryo that gives rise to the DA, and a venous progenitor that appears later and laterally in the embryo that gives rise to the cardinal vein. The authors selectively labeled the first (arterial-fated) angioblast population red through a pulsed photoconversion of etv2:Kaede cells (Etv2 as an early endothelial marker is described in detail in Section 1.3.1) at the 15-somite stage, while keeping the $Etv2^{-}$ venous-fated angioblasts green. Cell tracing showed that the red cells contributed almost exclusively to the DA and the green cells exclusively to the cardinal vein, suggesting that the two vessels originate directly from distinct angioblast populations. Critically, the authors did not observe labeled Etv2+ cells "sprouting" from the DA to form the cardinal vein. [8, 9]

1.1.2 Arteriogenesis

Arterial and Venous Specification in the Embryo

In the adult circulatory system, the arterial versus venous identity of vessels is defined anatomically and delineated by direction of blood flow and differences in oxygenation, blood pressure and shear forces. However, during development the segregated identity of arteries and veins is pre-programmed by genetic and signaling programs even before heartbeat (E8.25) or circulation (E8.5) are established.[10, 11] While blood flow is not required for arteriovenous specification, it does seem to be required for the maintenance of select arterial markers (Connexin 40 but not Dll4).[1]

Most arteriovenous-specific markers are expressed after vessel formation, implying that arteriogenesis is subsequent to vasculogenesis. Additionally, knock-out of many arterial markers or regulators does not impact the initial formation of the primary vascular network or vessel, but instead downstream angiogenic patterning. The markers and regulators of arterial specification are described below and summarized chronologically in relation to different vasculogenic events in Table 1. A schematic of the arteriogenic cascade is included in Figure 1d.

1.1.3 Angiogenesis

Formation of the Mature Vascular Network

Developmental angiogenesis is the process of remodeling the primary vascular plexus into a hierarchical network of small and large vessels through morphogenic events including sprouting, splitting, and involution and through cellular events such as EC proliferation, apoptosis and migration. In capillaries, angiogenesis refers to the process of sprouting and branching. In arteries and veins, angiogenesis involves the circumferential growth of the

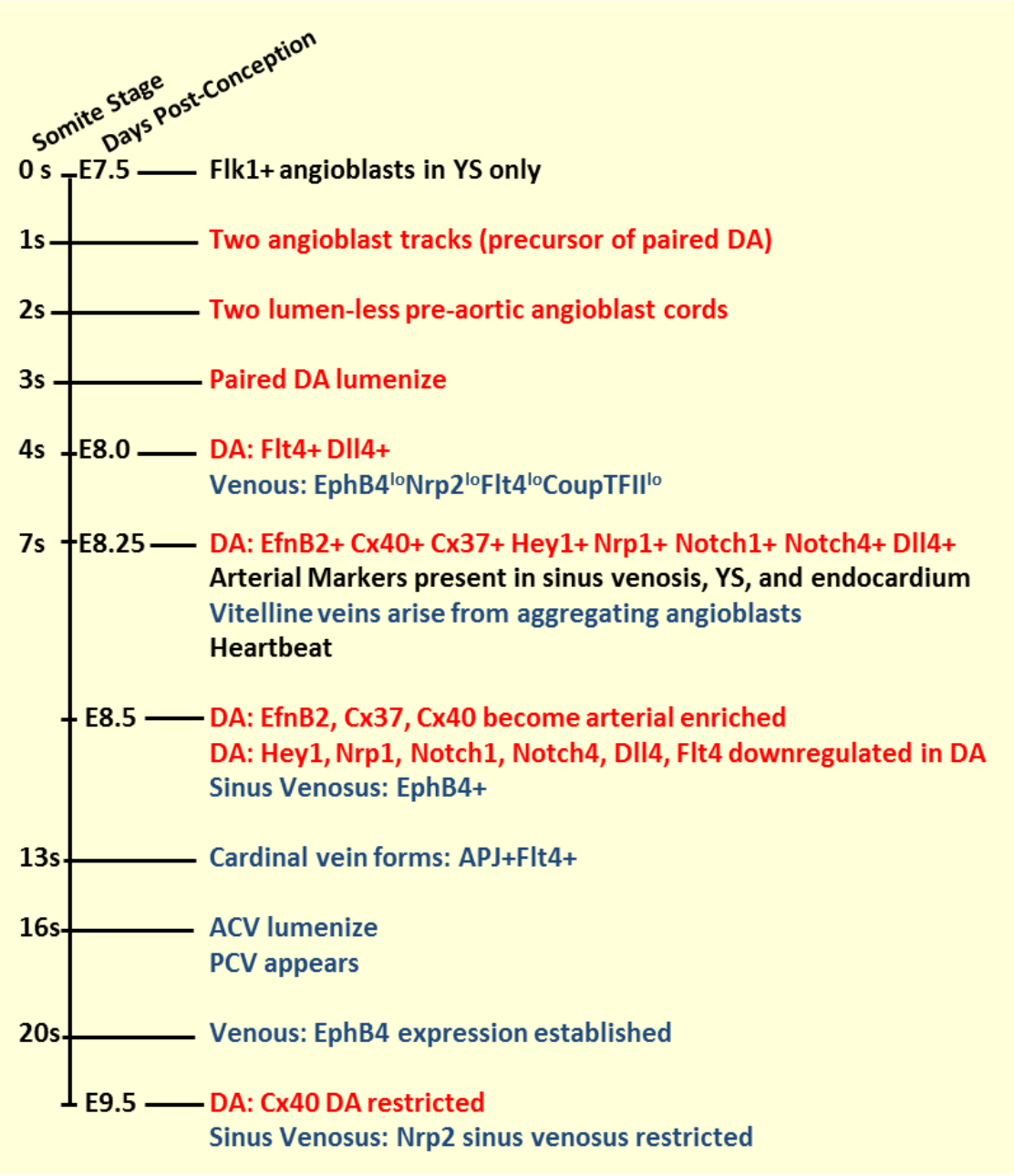

Table 1. Timeline of Mouse Vasculogenesis as Delineated by Arterial Marker Expression
Events and descriptions of arterial vessels are listed in red. Events listed in blue are related to venous vessels. Note that venous markers are established after arterial marker expression and that the arterial/venous identity of veins is at first promiscuous (they inappropriately express arterial markers). The table summarizes data published in Chong 2011.[1] EfnB2 and EphB4 were evaluated by protein expression (LacZ staining) while all other markers were evaluated by mRNA expression (in situ hybridization).

vessel and the recruitment of pericytes and SMCs. The arteriovenous identities of vessels are further enforced by the significantly greater enlargement of arterial vessels and the higher number of surrounding SMCs recruited to the arteries compared to the veins.[12] The process of angiogenesis is most critically regulated by VEGF, which will be discussed in Section 1.2.3.

Angiogenesis has not been extensively studied during embryonic development, in part due to the early mortality of many mutant embryos and difficulties in manipulating the embryonic vasculature. Instead, angiogenesis is often studied postnatally in the retina. The mouse retinal vessels develop after birth after the spontaneous regression of the hyaloid vasculature, which provides a temporary blood supply to the developing eye. Immediately after birth, retinal vessels begin to sprout radially from the optic disc reaching the outer limits of the retina by P7-8 (postnatal day 7-8). The primitive retinal vascular plexus produced through sprouting angiogenesis simultaneously undergoes rapid angiogenic remodeling to generate a hierarchical vascular system comprising of large and small vessels. This process of retinal vascularization is used to study the cellular and molecular processes underlying sprouting angiogenesis and vessel remodelling. Physiologic vessel regression/disappearance is often studied in hyaloid vessels, which spontaneously involute after birth between P1 and P14.[13, 14]

1.2 Signaling Pathways in Vascular Development

1.2.1 Hedgehog

Sonic Hedgehog (Shh) is a member of the Hedgehog family of secreted molecules that signals through the transmembrane receptor Patched (PTC) and the G-Protein Coupled Receptor (GPCR) Smoothened. Inhibition of VEGF and Shh in quail leads to failure of DA

migration and formation.[4] Smoothened null mice fail to form a patent DA but have $Flk1^{+}$ cells present where the vessel should form that do not coalesce into a vessel. The role of Shh in EC tube assembly was confirmed in vitro where addition of Shh induced the formation of tube-like structures in the mouse EC line bEND.3.[15]

During embryonic development, the notochord is a potent source of Shh. In zebrafish, Shh is required for the arterialization of the DA as $Shh^{-/-}$ and cyclopamine-treated embryos (a small molecule inhibitor of hedgehog) fail to form an $EfnB2^{+}$ DA. In contrast, embryos injected with Shh-mRNA exhibit ectopic expression of EfnB2 in the PCV. Mechanistically, the authors showed that Shh from the notochord acted indirectly by inducing VEGF expression in the somite which then activated Notch in the developing aorta. These results first proposed the classical hierarchy of Hedgehog, VEGF, Notch, and EfnB2 signaling during arteriogenesis (Figure 1d).[16] In mice, Shh knock-out did not appear to cause severe vascular defects, but specific expression of arterial markers in the DA was not examined to determine if arteriogenesis was normal.[17]

1.2.2 Bone Morphogenic Protein (BMP)

Bone morphogenic proteins (BMPs) are a group of growth factors that are critical morphogens during development and which establish the polarity of the embryo. During the period of DA formation, the notochord expresses the BMP antagonists Noggin and chordin which are believed to antagonize blood vessel formation. Conversely, the lateral plate mesoderm, endoderm and dorsal edge of the neural plate express BMP1, 2, and 4. $BMP4^{-/-}$ mice exhibit decreased numbers of YS blood islands and lateral plate mesoderm, showing very early mesodermal and vasculogenic defects.[18]

1.2.3 Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factors (VEGF) are principal regulators of vascular development and angiogenesis. The VEGF family of glycoproteins includes five members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and Placental Growth Factor (PlGF), with VEGF-A (the ligand most studied in angiogenesis) commonly referred to simply as VEGF. Each VEGF family protein is also expressed as different isoforms based on differential mRNA splicing. In mice, the main isoforms of VEGF (VEGF-A) are $VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$. These isoforms differ not only in molecular weight, but also in solubility and receptor binding specificities. $VEGF_{188}$ binds heparin sulphate proteoglycan (HSP) proteins in the extracellular matrix (ECM), whereas VEGF120 does not, and is therefore the most diffusible isoform. $VEGF_{165}$, which has intermediate HSP binding properties, is mostly detected on the cell surface or bound to extracellular matrix proteins.[19]

In mammals, there are three receptor tyrosine kinase (RTK) VEGF receptors: VEGFR1 (Flt1), VEGFR2 (Flk1, KDR) and VEGFR3 (Flt4). VEGF (VEGF-A) binds both VEGFR1 and VEGFR2 but not VEGFR3. VEGF-C and VEGF-D bind VEGFR3. Neuropilins (Nrp 1 and 2) are VEGF co-receptors that bind VEGF but lack the catalytic function, and serve to amplify VEGF signaling.[20]

During vascular development, VEGF signaling is hypothesized to be downstream of the BMP pathway.[4] $VEGF^{+/-}$ embryos expressing only one VEGF allele have only a string-like aorta in the anterior part of the embryo at E9.5 with a very small lumen in the posterior part.[21] Somitic VEGFA is important for DA formation. Conditional deletion of just one VEGF-A allele using Collagen2-Cre results in abnormal DA formation in mice.[22]

Flk1 (VEGFR2) is one of the earliest markers of EC/HC commitment and is therefore the classic hemangioblast marker, expressed in E7.5 posterior primitive streak cells. $Flk1^{-/-}$ mice die at around E8.5-9.5 due to hematopoietic and vascular defects.[23] It has been shown that Flk1 is required for the migration of mesoderm cells but not for the differentiation to EC/HCs.[24-26] In agreement with this, VEGF has been hypothesized to direct $Flk1^{+}$ cells to the blood islands in the YS.[27]

Nrp1 is a $VEGF_{165}$ isoform-specific co-receptor. $Nrp1^{-/-}$ mice fail to form a proper DA (only one of the paired aortae is present E9.0) and show significantly lower expression of Connexin40 and EfnB2, but normal levels of Dll4. This defect was shown to be independent of the simultaneous defect in blood flow through the underdeveloped vascular plexus.[28]

Detailed molecular studies in zebrafish show that VEGFA regulates proper formation of the PCV in zebrafish; zebrafish injected with VEGFA morpholino only formed one large vessel instead of separate DA and PCV. This defect was attributed to opposite roles of endogenous VEGFA in promoting angioblast cell sprouting dorsally, but restraining angioblast cell sprouting ventrally. Venous angioblast migration was shown to be PI3K and Notch-dependent using various chemical inhibitors.[7]

In xenopus, the short isoform of VEGFA has been shown to be sufficient for the arterialization of the DA (EfnB2, Dll4, and Notch4 expression).[29] In $VEGF_{188}^{-/-}$ mice, (which maintain expression of the $VEGF_{120}$ and $VEGF_{164}$ isoforms) no EfnB2 expression was found in P5 retinal vessels, showing impaired retinal arterial specification, though the DA was not characterized.[30] When Nrp1 (a co-receptor specific to mouse $VEGF_{164}$ specifically expressed in arterial ECs) is conditionally deleted in the ECs ($Nrp1^{fl/-}$;Tie2-Cre), E15.5 limb vessels show

decreased expression of the arterial marker Cx40 with no effect on the pan-endothelial marker CD31.[31]

VEGF has been shown to induce arterial differentiation in EC precursors in vitro. It induces the expression of Dll4 and EfnB2 and activates Notch signaling in cultured human umbilical vein endothelial cells (HUVEC).[32] High concentrations of VEGF in embryonic stem (ES) cell culture also increases arterial EC differentiation (10 fold more Dll4 mRNA).[33]

1.2.4 Notch

Notch signaling is a highly conserved pathway critical to many aspects of development. Notch ligands and receptors are surface bound; as such, Notch signaling is induced by cell-to-cell interaction and is critical for cell-to-cell communication and boundary formation during development. There are six Notch ligands belonging to two families of transmembrane proteins: Delta (Dll1, Dll2, Dll3, and Dll4) and Jagged (Jagged1 and Jagged2). Structurally, Notch ligands display large extracellular domains containing varying numbers of epidermal growth factor-like repeats and an N-terminal DSL (delta, Serrate and LAG-2) domain that is critical for interaction with the Notch receptor. In mammals, Notch ligands bind selectively to four different transmembrane Notch receptors (Notch 1- 4), which also contain large EGF-like repeat sequences in the extracellular domain.[34]

Upon activation by one of its ligands, the Notch intracellular domain undergoes two proteolytic cleavage events. The first cleavage is catalyzed by ADAM-family metalloproteases, and the second by ɣ-secretase enzymes. The second cleavage reaction by ɣ-secretase releases the Notch intracellular domain (NICD), which translocates to the nucleus to regulate transcription.[35] This core Notch transduction pathway acts in cooperation with the DNA-binding protein CSL/RBPJ (CBF1, Su(H) and LAG-1/Recombining

binding protein suppressor of hairless) and the co-activator Mastermind.[36-38] One target of Notch signaling is the HESR (Hairy and enhancer of split-related) family of helix loop helix (bHLH) transcription factors, which includes Hey 1,2 both induced by Notch signaling.[34]

Notch plays an early role in the formation of the DA beginning with the somitic-derived angioblasts that migrate to the midline to form the primitive aorta. Endogenous activated Notch was shown to be restricted to the posterior half of the somites in chick using an electroporated fluorescent Notch responsive reporter pTP1-Venus (a RBPJk-mediated notch signal). These endogenously-activated cells could be tracked with the reporter as they migrated ventrally to ultimately form the DA, suggesting that cells forming the DA need to have active notch signaling.[6]

Electroporating somitic cells with constitutively active Notch1 induced not just selective migration of the cells to the posterior somite (and cell death of the anterior somatic cells), but also the preferential migration of the cells to the primitive DA compared to controls. Once incorporated in the DA, these cells also expressed more of the endothelial marker VE-Cadh, raising the possibility that Notch plays a role in the differentiation of somite cells into EC, although these cells also differentiated to SMCs more than controls.[6, 39]

Notch family receptors and ligands are very dynamically expressed in the vascular system within the arterial and venous subpopulations. In mice, expression of Dll4 by in situ hybridization is the first to come up in the DA at E8.0 (4-5 somites) before the other arterial markers EfnB2, Cx40, Hey2, Nrp1, Notch1, Notch4, Cx37 (low), and Hey1 (low) at E8.25 (7 – 8 somites).[1] Dll1 expression in the arterial compartment appears last beginning at E13.5.[40]

Dll4 is the most important Notch ligand in arteriogenesis, with even haploinsufficiency of Dll4 leading to a loss of arterial identify in the mouse vasculature. It does not seem to be critical for vasculogenesis as $Dll4^{+/-}$ and $Dll4^{-/-}$ mice are not distinguishable from WT mice up to E8.5. However, by E8.75 in $Dll4^{-/-}$ and E9.0 in $Dll4^{+/-}$ mice, the DA is reduced in size compared to WT (interestingly, the left paired DA is always more affected). In the full $Dll4^{-/-}$ mice, the DA also displays a loss of arterial identity (normal Flk1 and CD31 but no EfnB2 and Connexin37 at E9.0; ectopic expression of EphB4 at ACV-DA fusions). $Dll4^{-/-}$ mice all die at E10.5, as do many $Dll4^{+/-}$ mice, but haploinsufficiency is not fully penetrant as some mice can survive to term and be fertile.[41]

The Notch receptors Notch1 and Notch4 are critical for arterial specification, though with some functional redundancy.[42] Compound Hey1/2 (downstream signaling components of the Notch pathway) knockout mice exhibit arteriovenous malformations (fusion of arteries and veins) and fail to express the arterial markers EfnB2, CD44, and Nrp1 in E9.5 embryos.[43]

Endothelial-specific knockout of Dll1 shows that it is required for the maintenance but not induction of arterial markers (with the exception of Dll4 which is present) by regulating the Nrp1 promoter, which controls VEGF responsiveness to Notch.[40] This requirement for Dll1 to maintain the arterial identity is contrasted with Dll4, which is expressed first and required for induction of the arterial program.

For vascular sprouting, selected ECs within the vessel wall must respond to angiogenic cues. “Tip” cells are non-proliferating cells that probe the environment through filopodia extensions whereas “stalk” cells proliferate extending primitive vessels. Notch signaling plays a role in fine-tuning VEGF signals and regulating endothelial tip-stalk cell interactions. Dll4, induced in response to VEGF in tip cells, signals through the Notch receptors on the

adjacent stalk cells to decrease expression of the ligand Dll4, enforcing the tip-stalk identity. Notch activation in the stalk cells by Dll4 also suppresses the expression of Flk1 which de-sensitizes the cells to further VEGF stimulation. Unlike arterial versus venous specification, it is not known whether tip versus stalk cell identity is genetically controlled or represents a set of stochastic/temporal adaptations to the surrounding microenvironment.

Dll4$^{-/-}$ mice show profound defects in angiogenesis. There is persistence of a primary capillary bed in the YS past E8.5 when remodeling of the YS should begin. In the DA, Dll4-LacZ+ ECs accumulate at the apical portion of the ISV and fail to sprout.[41]

1.2.5 Eph/Ephrin

The Eph receptor family is the largest family of tyrosine kinase receptors in mammals, with 14 members divided into EphA (that bind EphrinA ligands) and EphB classes (which bind promiscuously transmembrane EphrinB ligands; Figure 2b). The Eph receptors include an extracellular portion with a ligand binding domain, a cysteine-rich EGF-like region and two fibronectin-type III repeats while the intracellular region contains a tyrosine kinase domain, a sterile alpha motif (SAM) protein-protein interaction domain, and a PDZ-binding c-terminal motif. The EphrinB ligand class contains an intracellular domain with tyrosines that can be phosphorylated and a PDZ domain (Figure 2a).[44]

Upon binding of the Eph receptor with its cognate Ephrin ligand, the two can signal bi-directionally into both the receptor- (forward signaling) and ligand- (reverse signaling) expressing cells.[45] Binding leads to clustering of the receptor-ligand and oligomerization with other receptor-ligand heterodimers which causes internalization of both molecules into the Eph- and Ephrin-expressing cells. Internalization allows for the termination of receptor-

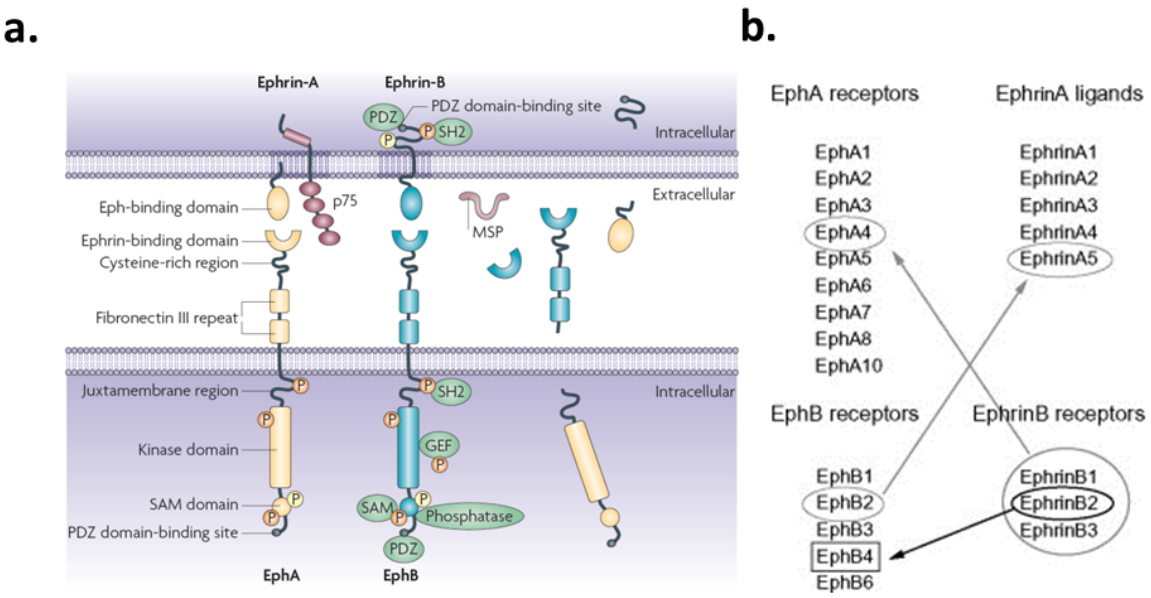

Figure 2. Ephrin Ligand and Eph Receptor Structure and Signaling Interactions

a. Schematic summarizing the domain structures of Eph receptors and Ephrin ligands. Ephrin ligand binding and clustering can trigger Eph receptor forward signaling. Transmembrane EfnB ligands contain in the cytoplasmic tail a PDZ domain binding site and tyrosines that can be phosphorylated, resulting in reverse-signaling in the ligand-expressing cell. From Pasquale 2010.[44]

b. Alternative splicing or proteolysis generates Eph receptors and Ephrin ligands of both A and B classes. Generally, class A receptors and ligands interact separately from class B receptor and ligands, with a few exceptions delineated here. EfnB2 interacts almost exclusively with EphB4. From Salvucci 2012.[45]

ligand adhesive interactions and explains the repulsive role that Eph/Ephrin signaling has during development.[46]

Forward signaling occurs in the Eph receptor-expressing cell when ligand-induced clustering is followed by autophosphorylation and Src family kinase-mediated phosphorylation of the intracellular tyrosine residues of the receptor. This leads to the activation of the tyrosine kinase catalytic domain which includes binding of adaptor proteins containing Src-homology (SH2) domains and downstream phosphorylation. Alternatively, the signaling can also occur through the SAM and PDZ- binding domains of the receptor's intracellular domain.[45]

Reverse signaling occurs in the Ephrin ligand-expressing cell when Src family kinases are recruited to phosphorylate specific tyrosine residues in the intracellular domain of the ligand. Phosphorylated EfnB allows binding of the SH2 domain-containing adaptor protein Grb4 and the transcriptional activator STAT3, which can link Ephrin reverse signaling to transcription.[47] To decouple forward and phosphotyrosine-dependent reverse signaling, EfnB2 proteins with the intracellular phosphorylated tyrosines mutated to phenylalanine can be used as a dominant negative mutant (because of receptor-ligand clustering upon binding) that allows for normal binding and activation of forward signaling in the Eph receptor-expressing cells but no phosphotyrosine-dependent reverse signaling in the EfnB2 ligand-expressing cell (EfnB2-5Y).[48]

In the context of the vascular system, EfnB2 was identified as an arterial marker and EphB4 a venous marker. Their distinct molecular expression early in the developing vessels, before circulation and hemodyamic forces are established, gave rise to the hypothesis that arteriovenous specification is genetically predetermined.[10] In comparison to the other

Class A and B Eph/Ephrins that bind and interact promiscuously, EfnB2 and EphB4 appear very specific in their binding for each other (Figure 2b).[45]

While mammal expression of EfnB2 and EphB4 in the developing vasculature is established only after the embryonic arteries and veins are formed, studies in birds and zebrafish show a dynamic role for Ephrin signaling in the formation of these vessels. Early in DA formation, EfnB2 expression in chick somites overlaps with the Notch-active domain at the posterior edge of the somite. Similar to the effect of over-expressing constitutively-activated Notch in the somitic cells, over-expressing EfnB2 also induced selective migration of these somitic-derived angioblasts to the primitive aorta. In epistatic studies, EfnB2 shRNA completely abrogated the inductive effect that constitutive Notch activation had on increasing somitic cell contribution to the DA. Therefore EfnB2 is hypothesized to be a downstream component of Notch signaling, being both sufficient and required for notch-activated somitic cells to migrate ventrally to the DA.[6]

Despite these roles of Eph/Ephrin signaling during early vascular development in lower vertebrates, EfnB2 does not seem to be absolutely required for mammalian vasculogenesis. $EfnB2^{LacZ/LacZ}$ mice form all the major vessels normally, including the DA, vitelline artery, posterior cardinal and umbilical veins[10]. However, vasculogenic defects in a separately generated $EfnB2^{LacZ/LacZ}$ mice varied, with some more severe mutants having only one or no DA. [49]

The molecular distinction between arteries and veins was first described as the segregated expression of EfnB2 in the arteries versus EphB4 in the veins. Given this specificity, EfnB2 and EphB4 are still used as the classical markers of arterial versus venous fate. In $EfnB2^{+/LacZ}$ mice, EfnB2-LacZ staining is present first in the DA (as well as vitelline and umbilical arteries)

by E8.25 with no staining in the YS vessels (connected to the vitelline artery) until E8.5.[10] This was confirmed by EfnB2 in situ hybridization of WT embryos. Faint expression of the venous marker EphB4 can be seen in the sinus venosus at E8.0, slightly before EfnB2 expression is present in the DA.[1] Other Eph/Ephrin family molecules in the vascular system include EfnB1 expression in both EfnB2^{+} arteries and EphB4^{+} veins and EphB3 expression only on EphB4^{+} veins.[49]

In addition to being specific, antagonistic markers of arterial versus venous fate, EfnB2 and EphB4 appear to be critical functionally for the formation of arterial versus venous vessels. During the formation of the PCV in zebrafish, EfnB2 and EphB4 morpholino knock-down caused excessive ventral sprouting leading to the formation of a single vessel instead of a segregated DA and PCV. This suggests the occurrence of EfnB2/EphB4-mediated repulsion between arterial and venous cells as well as a role of bi-directional signaling by both receptor and ligand for arteriogenesis. Furthermore, EfnB2 morpholino-treated cells injected into WT zebrafish contribute significantly less to the host DA and significantly more to the PCV. Conversely, EfnB2 over-expressing cells contributed significantly less to the PCV compared to controls.[7]

Despite these roles of Eph/Ephrin signaling for proper DA and PCV formation and specification in lower vertebrates, EfnB2 does not seem to be absolutely required for the formation of either the DA, vitelline artery, posterior cardinal and umbilical veins. However, the analyses of the EfnB2$^{-/-}$ mice did not characterize the expression of other "arterial" makers in the DA, notably the Notch pathway receptors/ligands. This analysis could further confirm the classical hierarchy of Hedgehog, VEGF, Notch, and finally EfnB2

being the most downstream marker and effector of arteriogenesis that is supported by the previously discussed work.

EfnB2 signaling is active in the angiogenic vasculature as evidenced by phosphorylated EfnB (reverse signaling) staining in angiogenic vessels.[50] Functionally, the loss of EfnB2 also results in angiogenic defects. In the EfnB2$^{-/-}$ mice, the E9.0 - E9.5 vascular plexus displays a block in remodeling. The capillaries maintain a uniform diameter, fail to grow, and form bidirectional connections while lumenized vessels do not delaminate from the basal endodermal layer.[10]

The ACV, which is formed after the formation of the DA from the fusion of small vessels in the hindbrain and head, does not form in either the full or endothelial-targed EfnB2 knock-out mice.[51] In the EC-targeted knockout of EfnB2, intersomitic vessels (ISVs) sprout normally at E8.5 from the DA but by E9.0 do not remodel to form a mature intersomitic vasculature. This effect is attributable to EC expression of EfnB2 because EfnB2 expression was normally maintained in the caudal half of the somitic mesenchyme of these mice.[51]

As a result of these cardiovascular defects, the EfnB2 knock-out mice are growth-retarded by E10 and die at E11. At death, the mice show enlarged hearts, with little to no blood flow, but no defects in somite polarity or hindbrain segmentation.[10, 49] These cardiovascular defects and eventual embryonic lethality are mirrored in mutant mice with a targeted deletion of EfnB2 to the endothelium, indicating an EC-autonomous role.[51] However, EfnB2 is still required in a non-EC specific manner, as the EfnB2 knock-out mice are not rescued by over-expression of EfnB2 in the Tie-2+ fractions.[52] Of note, EfnB2 dosage seems critical to this phenotype because heterozygous EfnB2$^{+/LacZ}$ mice are born at half the expected frequency.[49]

EfnB2 also plays critical roles in angiogenesis and the post-angiogenic remodeling of the endothelium after midgestation. Inducible knock-out of EfnB2 in the endothelium (with Cdh5-CreERT2) at E15.5 leads to significantly reduced angiogenesis in the skin, associated with oedema and hemorrhaging, and reduced growth of retinal vessels. Conversely, over-expression of EfnB2 in the endothelium (with VE-Cadherin-tTA) at E15.5 leads to formation of a markedly abnormal dermal vessel network with long and unstable vessels.[51] Similarly, expression of a mutant EfnB2 with a targeted mutation in the intracellular PDZ domain (and thus reverse-signaling deficient) markedly impaired angiogenic sprouting in the retina and in tumors.[52] Mechanistic studies have revealed that EfnB2 regulates VEGFR2 and VEGFR3 internalization and signaling in response to VEGFA and VEGFC, and that this regulation underlies the pro-angiogenic function of EfnB2.[53, 54] These studies provide evidence that EfnB2 controls VEGF responses in murine endothelial cells and VEGFR3 internalization in both mouse and zebrafish.[53, 54] More recently, this function of EfnB2 as a promoter of VEGF receptor internalization has been linked to the association of EfnB2 with the sorting protein DAB2 (Disabled2) and the cell polarity regulator PAR3 (Partitioning defective 3 homolog).[55]

Owing to the relative transparency of the embryos and ease of modulating gene expression in vivo by delivering oligomers at defined time-points, vascular development and morphogenesis is often studied in the zebrafish model. Studies in zebrafish treated with EfnB2 and EphB4 morpholinos (which displayed excessive ventral sprouting and fused DA and PCV) led to the conclusion that bi-directional EfnB2/EphB4-signaling was required for arteriogenesis. In this study, cell tracking showed that EfnB2 morpholino-treated cells injected into WT zebrafish contributed significantly less to the host DA and significantly more to the PCV, suggesting that EfnB2 expression directs an arterial cell fate.[7] Since a

number of experimental approaches available in zebrafish are not yet applicable to mice, the zebrafish model continues to inform developmental biologists involved in studies of vascular morphogenesis in vivo.

1.3 Transcriptional Regulation of Vascular Development

1.3.1 ETS Family

Certain transcription factors play major roles in vascular development. Among the earliest transcriptional regulators of vasculogenesis during development is Etv2 (Er71), a member of the ETS ("E twenty-six") family of transcription factors that contains the highly conserved winged helix-turn-helix ETS DNA-binding domain, which binds to the consensus GGAA/T motif.[56]

Etv2$^{-/-}$ mice die early in gestation before E11.0 and the embryos lack all endothelial and hematopoietic cells.[57, 58] Etv2 is only expressed transiently in the early mesoderm between E7 and E11.5. Lineage tracing studies using an Etv2 enhancer have demonstrated that Etv2+ progenitors contribute to mature endothelial and hematopoietic cells (Ter119+ and CD45+ cells). Etv2 appears to directly instruct cell fate decisions, supporting the hemato-endothelial fate over the Nkx2-5-driven cardiac fate during mesoderm differentiation.[59]

Downstream targets of Etv2 include many of the characterized regulators of hemato-endothelial differentiation including Flk1, Scl, Gata2, and Tie2.[56] Consistent with this, a FOX:ETS motif appears to be a specific characteristic of endothelial enhancers, allowing Etv2 to directly modulate (with Fox family transcription factors discussed in Section 1.3.3) the expression of these endothelial genes.[60] In zebrafish, FoxC1a and FoxC1b appear to bind to the enhancer region of Etv2 and induce Etv2 expression in endothelial progenitors,

suggesting that Etv2 expression is regulated by the Fox family of transcription factors.[61] However, other inducers of Etv2 expression remain to be identified, as FoxC1 and FoxC2 knock-out mice still express Etv2, albeit at lower levels.[62]

1.3.2 Sox Family

The three SoxF family member transcription factors, Sox7, 17, and 18, are expressed in the developing vascular system with relative specificity to the arterial network. At E8.0, Sox7 and Sox18 are expressed in the still paired DA, while Sox17 expression picks up around E8.5.[63, 64] Compound Sox17/18$^{-/-}$ mice exhibit some cardiovascular defects, but do not exhibit as severe arteriovenous defects as zebrafish.[63, 65] Recently, it was shown that this was because both SoxF and RBPJ/Notch transcription factors are combinatorially required to activate Dll4 expression in the arterial vasculature (with neither RPBJ nor Sox binding site being sufficient).[66]

1.3.3 Forkhead/Fox Transcription Factors

Two members of the Forkhead/Fox transcription factor family, Foxc1 and Foxc2, have been implicated in arteriogenesis (though Foxc1 and Foxc2 are expressed in both arteries and veins). Compound mutant Foxc1/2$^{-/-}$ mice do not show defects in endothelial Flk1 expression or venous CoupTFII and EphB4 expression. However, they do not express Notch1, Notch4, Dll4, Jagged1, Hey 1, EfnB2 or Nrp1 in E9.0 DA. The authors show that Foxc1 and Foxc2 can directly activate the Dll4 promoter and induce expression of the arterial markers Dll4, Notch1, Notch4, Hey2 and EfnB2 in vitro.[62] In another study, Foxc2 was found to complex with Su(H) and NICD, and to directly bind and activate the Hey2 promoter in ECs. This promoter induction was significantly increased by VEGF.[67] In a

broader context, Foxc2 has been shown to regulate the expression of Dll4, CXCR4, and Angiopotin2 suppressing muscular fate in favor of a vascular fate.[68]

1.3.4 CoupTFII

CoupTFII (Chicken Ovalbumin Upstream Promoter) belongs to the "orphan" nuclear receptor family, but has more recently been identified as a retinoic acid-activated receptor.[69] It is specifically expressed in venous ECs and specifies the venous fate by inducing EphB4 and repressing Nrp1 and Notch expression. Coup-TFII$^{-/-}$ mice show ectopic expression of the arterial markers Nrp1, Notch1, and EfnB2 in the veins and the generation of hematopoietic cell clusters.[70]

1.4 Hematopoietic Development in the Embryo

During embryonic development, the site of hematopoiesis shifts spatially from one organ to another. These successive waves allow production of differentiated hematopoietic cells to support the growing embryo until the more definitive hematopoietic organs and cells are developed. Because circulation connects these different sites beginning around E8.25, however, it is difficult to identify distinctly the different waves. The waves of hematopoiesis are diagrammed in Figure 3 and Figure 4 and are summarized chronologically in Table 2 to complement the text below.

1.4.1 Yolk Sac Hematopoiesis

The first hematopoietic waves occur extra-embryonically in the YS. The very first of these is termed the Primitive wave and occurs around E7.5 to generate nucleated, primitive erythrocytes (with embryonic globin). It begins around E7.25 when homogeneous hemangioblastic cords begin to remodel into YS blood islands that are identified by E8.25 as ECs surrounding inner aggregates of large, nucleated Ery^{p}. Note that while these structures

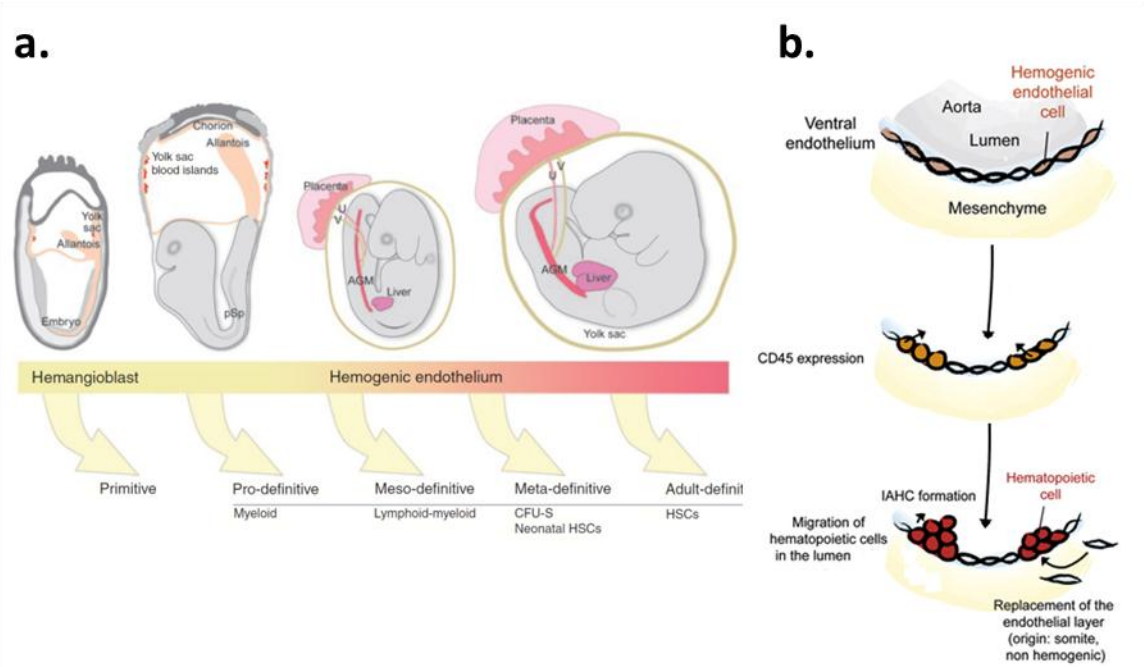

Figure 3. Embryonic Waves of Hematopoiesis in Mouse and the Generation of HSCs in the DA through a Hemogenic Endothelium

a. The five hematopoietic waves listed in Table 2 are schematized under illustrations of the embryo at the different timepoints that the various waves occur (E7.5, E8.25, E9.0 and E10.5 left to right). The two intermediate populations generating hematopoietic cells, hemangioblast and hemogenic endothelium, are listed in this schematic in relation to the five different waves. From Dzierzak 2008.[89]

b. Schematic illustrating the generation of HSCs through a hemogenic endothelium in the DA. HE lines the ventral floor of the DA and co-expresses the EC marker VE-Cadh and the hematopoietic marker CD45 (pre-HSC population). HSCs "bud" from this HE into the lumen of the DA. The exhausted HE is then replaced by non-hemogenic somite-derived ECs as shown in Figure 1b. From Boisset 2012.[147]

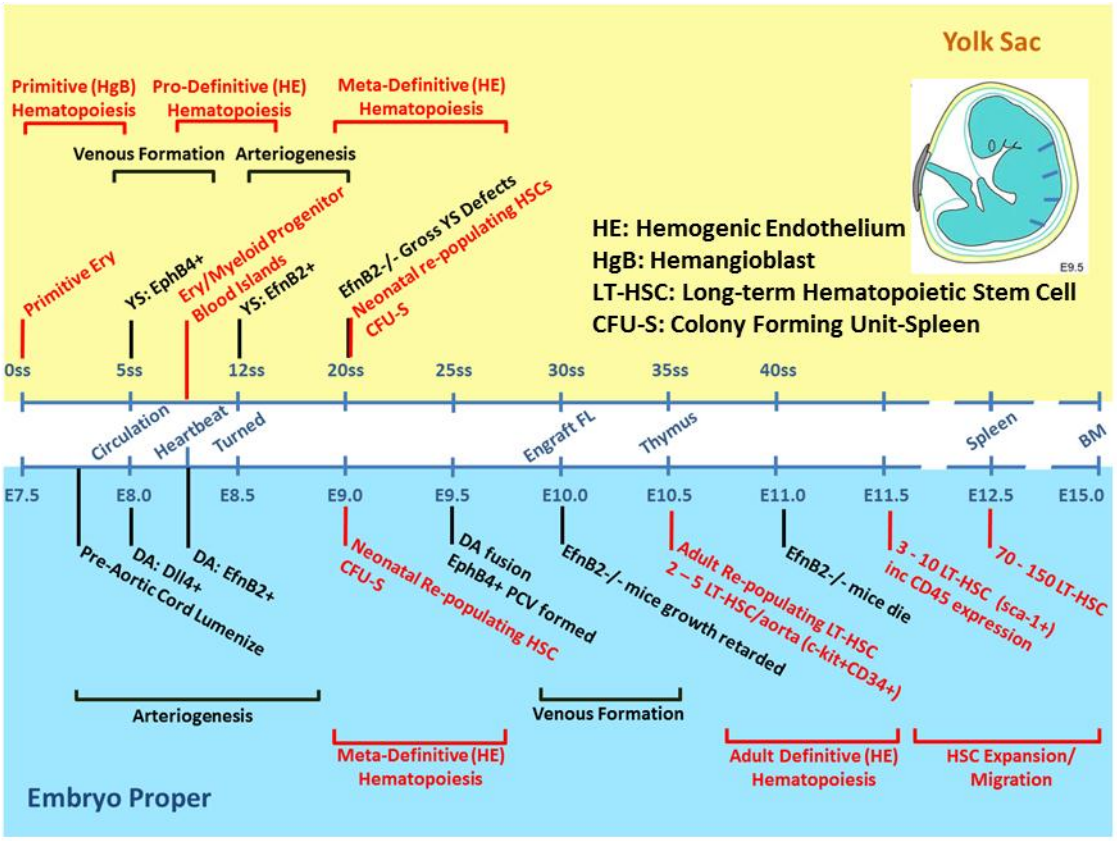

Figure 4. Timeline of Hematopoietic, Vasculogenic and Arteriogenic Programs in Mouse Development

Hematopoietic and vasculogenic programs occurring in the YS are listed at the top half of the figure (yellow shaded box) separated from events occurring in the embryo proper on the bottom (blue shaded box). Vasculogenic/arteriogenic events are listed in black while hematopoietic events (adapted from Table 2) are listed in red.

	Developmental time	Site	Functional activity	Conditions
Primitve	E7.5	Yolk sac[a]	Primitive erythroid	–
	E7.5–E8 (0–4sp)	Yolk sac[b]	Primitive erythroid	*Slc8a1*$^{-/-}$
Pro-definitive	E7.5–E8 (EHF–2sp)	Allantois[a]	Erythroid-myeloid progenitor	Explant preculture
	E7.5–E8 (0–7sp)	Allantois[a]	Erythroid-myeloid progenitor	Explant preculture
	E8.25	Yolk sac[a]	Erythroid-myeloid progenitor	–
	E8.25	Yolk sac[b]	Erythroid-myeloid progenitor	*Slc8a1*$^{-/-}$
	E9.5	Yolk sac[b]	Erythroid-myeloid progenitor	*Cdh5*$^{-/-}$
	E9.0	Placenta	Erythroid-myeloid progenitor	–
Meso-definitive	E7.5–E8 (0–8sp)	pSp[a]	Erythroid-myeloid-lymphoid progenitor	Explant preculture
	E7.5–E 8 (0–5sp)	pSp[a]	Multipotent low-level repopulating progenitor	Explant preculture
Meta-definitive	E9.0	Yolk sac[c]	Neonatal repopulating HSC	–
	E9.0	AGM[c]	Neonatal repopulating HSC	–
	E9.0	Yolk sac[c]	CFU-S	–
	E9.0	AGM[c,d]	CFU-S	–
Adult-definitive	E10.5	AGM[d,e]	Adult repopulating HSC	–
	E10.5	Umbilical and vitelline vessels[d]	Adult repopulating HSC	–
	E10.5-E11	Placenta	Adult repopulating HSC	–

Table 2. Embryonic Waves of Hematopoiesis by Spatial and Temporal Distinction
From Dzierzak 2008.[89]

are termed “blood islands”, the hematopoietic cells are not truly islands in the lumen, but rather maintain physical contact with the surrounding ECs.[71]

Erythro-myeloid progenitors appear in the Pro-Definitive wave in the YS around E8.25, coinciding with the onset of circulation. In vitro erythro-myeloid progenitors (CFU-C) have been reported as early as E7.0 YS, before circulation and indeed before blood islands can be identified morphologically.[72] In vivo CFU-S activity was reported from E8.0 YS, though most other laboratories do not see CFU-S until E9.0 (after circulation).[73] The erythro-myeloid progenitors from this wave are believed to come from the YS, as they are present in E8.25 YS of Slc8a1$^{-/-}$ (coding for a sodium-calcium exchanger required for heartbeat) and VE-Cadh$^{-/-}$ mice, which have no circulation due to the absence of heartbeat and no fully formed vasculature, respectively.[74, 75]

After circulation, a Meta-Definitive wave generates progenitors in the E9.0 YS that when injected directly into the FL can reconstitute all hematopoietic cell lineages in sublethally myeloablated (20 mg/mL Busulfan; 1000 CD34+; 21% CD34+) newborn recipients and can support hematopoietic reconstitution in serial transplants of secondary adults.[76] Engraftment into adult sublethally irradiated Rag2$\gamma_c^{-/-}$ mice (compound mutant mice with severe T, B and NK cell deficiency due to Rag2 and γ_c deletions) showed that E8.0 and E10.0 YS progenitors cultured for four days in organ culture engrafted the myeloid compartment only temporarily and did not reconstitute the lymphoid compartment.[77] While YS c-kit+ cells contain multipotent cells at lower frequency compared to AGM, they contain more myeloid progenitors.[78]

Therefore, while some YS-derived progenitors can give rise to adult myeloid cells, they do not show intrinsic HSC activity. LT-HSC activity after transplant into adult mice is not

detected until E11.0 YS, well after circulation is established and after intra-embryonic-derived cells can engraft adult mice.[79, 80] This lack of full HSC activity, however, may not be cell-intrinsic. Reciprocal grafting of YS into embryonic equivalent sites in xenopus shows that YS-derived cells can contribute to adult definitive hematopoietic cells.[81] Also mouse YS-derived cells can acquire LT-HSC activity if cultured ex vivo on AGM-derived stromal cell lines[82] or HoxB4 (a homeobox DNA-binding transcription factor described in Section 1.7.5) enforced expression combined with stromal cell co-culture.[83]

1.4.2 Intra-Embryonic Hematopoiesis: Dorsal Aorta

A second location of autonomous hematopoietic cell generation is in the embryo proper, first in the para-aortic splanchnopleura (P-Sp), which comprises the developing gut (including what will ultimately be the FL), the paired dorsal aortae, and the splancnic mesoderm. A region termed AGM (aorta-gonad-mesonephros), which comprises of the aorta, genital ridges and mesonephros, can be distinguished by E9.5.

Because intra-embryonic hematopoiesis peaks at E10.5-11.5 after both YS hematopoiesis and circulation is established, it was believed for a long time that multipotent HSCs arose from the YS. However, two early studies showed that grafting E9.0 P-Sp but not YS into the kidney capsule of irradiated mice gives rise to B cells, and that E10.5 AGM contains CFU-S before and at a much higher frequency than the YS. These studies opened the possibility that like the avian models, which had previously been shown to go through a separate intra-embryonic definite wave, mice also have a second site where HSCs arise autonomously.[73, 84]

The Meso-Definitive wave begins in the E7.5 P-Sp and gives rise to the first progenitors that have lymphoid potential (versus YS progenitors).[85] By E8.5, P-Sp cells have lymphoid potential ex vivo at a 10 fold higher frequency than equivalent E8.5 YS cells when cultured

on S17 stromal cell culture.[86] E8.0 intraembryonic progenitors engrafted long term into Rag2γc$^{-/-}$ NK deficient mice, showing the presence of myeloid and lymphoid cells in PB and BM up to 8 months after transplantation in comparison to YS progenitors that only transiently engrafted myeloid but not the lymphoid compartment.[77]

There is also a Meta-definitive wave in the P-Sp when both YS and P-Sp cells from E9.0 embryos injected directly into the FL can reconstitute sublethally myeloablated newborn recipients long term.[76]

The final Adult Definitive wave, which is characterized by the ability to engraft irradiated adult mice (tail vein injection) long term, does not occur until E10.5 in the AGM. Dissociated E10.0 AGM (1.16 embryo equivalents) repopulated adult mice (3/96 mice) in all hematopoietic lineages and could serially reconstitute secondary recipients, whereas YS could not (0/74 mice).[79]

The rate of successful engraftment into irradiated adults increases significantly if E10.0 AGM are cultured ex vivo in organ culture for 2-3 days (on semi-permeable filters at the air-medium interface in myeloid supportive medium) before dissociation and IV injection into the host (24/27 mice; two embryo equivalent per transplant). This procedure does not result in engraftment of E10.0 YS cells (0/16 mice). By E11.0 AGM limiting dilution transplantation, only 0.08 embryo equivalents of AGM cells are required for engraftment. This culture method, however, could not induce engraftment of earlier E9.0 AGM.[80] Addition of IL-3 to the cultures further expanded the HSC population.[87]

Organ culture of explants taken from the YS and P-Sp before circulation is established conclusively showed that not only was HSC activity detectable in the P-Sp at higher levels

than in the YS, but that HSCs arose autonomously intra-embryonically and not from the YS. E7.5 YS and P-Sp were dissected and cultured in toto on S17 stroma with IL-7, KL, and IL-11 for two days before being dissociated and then expanded in the same conditions for three more days. After this five day culture, the cells were re-plated into myeloid/erythryoid/ lymphoid assays. At all stages, myeloid cells were derived from both YS and P-Sp explants, but only P-Sp consistently gave rise to lymphoid progeny (and reconstitute E15 thymic lobes). However, at this early timepoint, no in vivo reconstitution of adult mice was achieved with these organ-cultured cells.[85] At E11.5 it is estimated by limiting dilution that there is a total of 3-10 HSCs in the embryo and by E12.5 the number increases to around 70-150.[88]

1.4.3 Migration of HC/HSCs to Bone Marrow

As the various waves of hematopoiesis generate different progenitors at different sites in the embryo, they also migrate away from their site of generation. Both YS- and DA-derived hematopoietic cells begin seeding the FL at E10.0 and the embryonic thymus at E10.5. There is engraftment to the spleen at E12.5 and finally bone marrow at E15.0.[89]

1.4.4 Surface Markers and Regulators of Developmental Hematopoiesis

LT-HSCs from E11.0 AGM and FL that engraft adults come exclusively from the c-kit$^+$ fraction (~13% of total cells), and are enriched in the c-kit$^+$CD34$^+$ (1.7%) population in AGM (8/26 mice transplanted; 3000-7000 cells). By contrast, the c-kit$^+$CD34$^-$ population only engrafts when 10-fold higher number of cells are injected (2/9 mice transplanted).[90] E10.5 P-Sp/AGM cells also express low levels of CD45 and co-express the endothelial marker CD31.[78] By E11.5, c-kit$^+$ cells increase levels of CD45 expression and by E11.5 all c-kit$^+$ cells are CD45$^+$.[91] While CD45+ cells co-express the endothelial markers CD31 and VE-Cadh, CD41 is

a marker of early hematopoietic cells that is not expressed on ECs. In ES culture, CD45 comes up after CD41.[78] In Ly6A/Sca1-GFP transgenic mice, GFP^+ cells in E11.0 AGM are also c-kit^+ (low at E10.5), which appears to be a good marker for LT-HSCs in the DA, whereas Sca1 is also expressed in mesenchymal and EC (there are GFP^+ cells that do not express c-kit).[92]

Interestingly, while AA.1, CD34, Tie2hi, and c-kit expression are all higher in AGM than in the YS, there is no phenotype that distinguishes between YS and intraembryonic precursors.[93]

1.5 Hematopoietic Differentiation from Embryonic Stem Cells

1.5.1 ES Cell Differentiation Advantages

ES cells are undifferentiated, self-renewing, pluripotent stem cell lines derived from the inner cell mass of the blastocyst embryo with unlimited potential for in vitro expansion in the undifferentiated state. When the factors for maintaining pluripotency are removed, ES cells can differentiate into all the germ layers, dependent on the variety of additional exogenous factors that are added for step-wise directed differentiation. Based on studies in various knock-out ES cell lines, the developmental requirements and kinetics seem to re-capitulate with high fidelity development in vivo.

Because ES cells have high proliferative potential and are differentiated in vitro, they can provide direct access to distinct developmental stages and transitions that may be difficult to access in embryos. This allows easier temporal and dosage control/manipulation of soluble factors and can be advantageous for high-throughput screens of drug compounds and/or mutations.

As an in vitro cell source with the potential of differentiation into many cells types, ES cells are also a promising tool for regenerative medicine to derive desired cells and tissues. In comparison to iPS cells (induced pluripotent stem cells; generated by exogenous delivery of reprogramming factors to fully differentiated cells to enforce a pluripotent "ES-like" phenoypte[94]), ES cells provide the added advantage of lacking epigenetic memory of the previous cell fate.[95]

1.5.2 ES Cell Differentiation Limitations

The main disadvantage of ex vivo differentiation of ES cells is that in vitro culture does not fully recapitulate the 3D structure/gradients in the embryo proper. As a result, it is not a very powerful tool for studying migration, morphogenic events, or processes that are very sensitive to concentration gradients.

While ES differentiation generally recapitulates the temporal patterns of development, in vitro culture results in a more heterogeneous mixture of cells with slightly different levels of maturity that are not temporally synchronized. This disadvantage, however, is usually overcome by the relative ease that in vitro culture allows for cell sorting (ex. FACS) to specifically enrich for certain sub-populations. However, perhaps partially due to disparities in different culture conditions used by different labs, it is not always clear how to correlate sub-populations and specific timepoints identified in the ES system to development in vivo. This can render conclusions drawn from ES cell differentiation hard to interpret in the in vivo context.

A specific disadvantage to developmental hematopoiesis studies using ES cells is that it is still not demonstrated that LT-HSCs can be derived from differentiated ES cells without genetic manipulation. This is a serious concern if studies use ES cells to study adult disease

progression or if the goal is to use the cells as a cell source for hematopoietic reconstitution of patients one day.

1.5.3 Differentiation of Mesoderm Lineages Using ES Cells

Mesoderm Formation: Embryoid Body Differentiation

To recapitulate embryonic development, ES cells are classically differentiated in a liquid 3D suspension culture known as "Embryoid Body (EB) differentiation" with the removal of the pluripotency factor Leukemia Inhibitory Factor (LIF). In this culture, individual pluripotent ES cells begin to proliferate into 3D aggregates, within which cells begin to spontaneously differentiate towards the three different germ layers.[96]

Around Day 3 – 4 of EB culture, cells expressing Flk1 can be isolated from the EBs (around 30% of total live cells). These Flk1+ cells can give rise to all the mesodermal lineages (endothelial, hematopoietic, smooth muscle, and cardiac) in various downstream re-plating cultures. Re-plating into BL-CFC culture, Flk1+ Day 3.5 EB cells have been identified as "hemangioblasts" with bi-potent capacity to form both endothelial and hematopoietic cells at a clonal level.[97]

The marker Flk1 peaks at around Day 3.5 EBs but continues to be expressed in a smaller population of Day 6 EBs, notably in cells that begin to express the EC marker VE-Cadh (6.4% of total live cells). By Day 6, the hematopoietic markers c-kit, CD41 (27.2%), and CD45 (6.8%) also begin to be expressed.[98] And by Day 7 – 8 EB culture, EBs are red due to the differentiation of primitive erythrocytes expressing embryonic globin.

BL-CFC Culture

"Blast Colony Forming Cell (BL-CFC) culture" was the first culture used to identify the putative hemangioblast hypothesized to give rise to both endothelial and hematopoietic lineages. In this culture, $Flk1^+$ "hemangioblasts" from Day 3 – 4 EBs are re-plated into methylcellulose media with cytokines supporting both hematopoietic and endothelial differentiation (IL-6, KL, and VEGF).[97]

In further studies, the HE population was identified in this culture. $Flk1^+$ Day 3.5 EB cells re-plated into BL-CFC gave rise to a $Tie2^{hi}c\text{-}kit^+CD41^-$ HE population (10%) after 48 hours that is also Flk-1^+, $MECA32^+$, VE-$Cadherin^+$, $CD34^+$, $endoglin^+$, claudin 5^+, $CD45^-$, and $CD11b^-$. By CFSE analysis, 40% of the HE cells are hemogenic (ie. give rise to $CD41^+$ hematopoietic cells), though this frequency is much lower (1.2%) by limiting dilution analysis.[99]

OP9 Co-Culture

The HE was also characterized in a different condition, OP9 co-culture, after Day 4 $Flk1^+$E-$Cadh^-$ EB cells were re-plated on OP9 stroma with the hematopoietic cytokine KL. The emergence and differentiation of this HE was imaged using time-lapse microscopy over the course of four days. The HE was identified as consisting of endothelial colonies that were $Flk1^+$, $c\text{-}kit^+$, E-and VE-$cadherin^+$, $CD41^-$, $CD45^-$, $CD11b^-$ and which functionally uptake Ac-LDL. By single cell live imaging, the authors were able to track emerging $CD45^+$ hematopoietic cells to their single cell endothelial precursor. From this, the authors calculated that only 1% of the embryonic ECs at day 4-5 are hemogenic (i.e. become $CD41^+$), and that the first identified "hemogenic endothelial cells" give rise to 1-6 hemogenic endothelial cells by symmetrical division over the course of four days.[100] These results provided in vitro observation of an EHT. A similar HE was observed after re-plating Day 6

Flk1^{+}VE-Cadh^{+} EBs onto OP9 stroma with the cytokines VEGF, TPO and Flt-3.[98] In a study with human ES cells, it was concluded that the HE in OP9 co-culture was a more definitive-like HE (compared the YS-like BL-CFC HE) based on gene profiling.[101]

MethoCult3434 Culture

MethoCult3434 culture is a methycellulose-based culture with the hematopoietic cytokines KL, IL-3, IL-6, and Epo to support the differentiation of definitive hematopoietic colonies (erythroid, granulocyte, macrophage, megakaryocyte, pre-B) from CD34^{+} hematopoietic stem/progenitor cells from mouse bone marrow. It can also be used to re-plate progenitors from ES differentiation culture to read-out definitive erythro-myeloid potential of different pouplations.[98]

Arterial Monolayer Culture

Day 4 Flk1^{+} EBs can be differentiated selectively to "arterial" ECs in monolayer culture when sorted onto Collagen IV-coated glass coverslips and cultured with VEGF at high levels (50 ng/mL). With 50 ng/mL VEGF, CD31^{+} EC cells expressed 10 fold more Dll4 and 40% more Notch4 mRNA than when the VEGF concentration was 10 ng/mL. EfnB2 expression increased slightly (though significantly) as well with increased VEGF concentration.[33]

1.6 Signaling Pathways Regulating Hematopoiesis

1.6.1 VEGF Signaling

VEGF plays critical roles in the developing endothelium but may also affect hematopoiesis in a non-cell autonomous fashion. In xenopus, morpholino knock-down of medium and long isoforms of VEGFA (VEGFA 170 and VEGFA190) preserved vasculognesis and arteriogenesis (Dll4 and EfnB2 expression in the DA). However, the arterial-specified DA was non-hemogenic, with no detectable expression of Notch1, Runx1, SpiB, Scl, or Gfi1 in the DA at

the normal time of HSC emergence. This study also showed the non-cell autonomous requirement of Eto2 required from the somites to induce the hematopoietic program in the DA, which can be rescued by over-expression of medium and long VEGFA isoforms, but not the short secreted isoform (VEGFA 122).[29]

1.6.2 Notch Signaling

Just as many Notch pathway components play roles in the arteriovenous specification of the DA, they also play complex roles in the hemogenicity of the DA. Notch 1, Notch4, Jagged1, Jagged2, Dll4, activated Notch1 intracellular domain and Hes1 are all expressed in E10.5 AGM.[102] Studies in zebrafish suggest that Notch components (deltaC and deltaD) are also expressed in the somites adjacent to the DA and necessary in a non-cell autonomous role to induce hemogenicity and Runx1 expression in the DA.[103]

Functionally, deletion of Notch pathway proteins can lead to specific hematopoietic defects. RBPjκ/CSL mutant mice with no Notch signaling completely lack hematopoietic progenitors in the AGM by CFC assay and died by E10.5 before the peak of HSC emergence.[104] Notch1$^{-/-}$ YS and AGM lack HSCs that can repopulate newborn mice (though the number of progenitors in the YS is similar to that found in WT), and further analysis showed that the defect was at the stage of HE commitment to HSC, as the number of CD34^{+}c-kit^{+} and VE-Cadh^{+}CD45^{-} cells in both YS and AGM were comparable to WT.[105] The complete arterial specification of this non-hemogenic DA was not investigated to see if this non-hemogenic DA was also non-arterial.

E10.5 AGM dissected from Jagged1 (but not Jagged 2) knock-out mice show decreased hematopoietic colony formation, though there is normal arterial specification of the DA (by

EfnB2 staining). This defect could be rescued, however, by ectopic Gata2 expression during ex vivo re-plating.[102]

Dll4+/- mice had pale YS at E10.5, suggesting no YS or embryonic hematopoiesis, though this observation was not fully investigated.[41] Ex vivo culture from up to E8.5 Dll4-/- YS into CFU assays did not show an in vitro hematopoietic defect, though Dll4-/- ES cells showed decreases in hematopoietic colony forming in the same assay.[106]

While Notch is required for the induction of aortic hematopoiesis (and Runx1 expression in the ventral DA) and its various receptors and ligands are expressed in the hemogenic DA, it has also been shown that Notch is not active in the committed intra-aortic clusters in both chick and mouse, and that Notch signaling actually decreases with time in the hematopoietic cells. Indeed, inhibition of Notch signaling increases hematopoietic cell production in the DA.[107] Conversely, constitutive activation of Notch1 (but not Notch4) in the VE-Cadh+ endothelial compartment (with Notch1 intracellular domain) led to a lack of intra-aortic clusters in the DA as well as defects in the fetal liver.[108] Therefore, Notch regulation is dynamic during embryonic hematopoiesis, requiring the precise dose and kinetics of expression for proper differentiation.

1.6.3 Eph/Ephrin Signaling

In the two separately generated EfnB2$^{-/-}$ mice, one had erythrocytes circulating in the embryo at E9.5[10] while the other E9.5 YS was pale with no blood.[49] The hematopoietic defect in the more definitive stages could not be investigated in either EfnB2$^{-/-}$ mice as the mice died beginning at E10.0 before HSC emergence in the DA.

In vitro, EphB4-/- ES have decreased hematopoietic clonogenicity (BFU-E, CFU) and BL-CFC formation (though this may be due to the fact that there was a four-fold decrease in the percentage of Flk1+ cells and the EBs were re-plated without Flk1 sorting), suggesting that EphB4 is required for hematopoiesis. However, EphB4 should not be expressed in the DA around the time of HSC emergence (it has been shown not to be present at E9.5, later timepoints need to be investigated).[10] Therefore, it is not known if the effect of EphB4 knock-out in vitro may have been through EfnB2. Of note, there was a detectable decrease in EfnB2 expression by RT-PCR in late Day 7 – 9 EBs, which could have contributed to the phenotype published in the EphB4-/- ES cells.[109]

1.7 Transcription Factor Regulators of Hematopoiesis

1.7.1 Scl

Scl (Stem Cell Leukemia, or Tal1) is a basic helix-loop-helix (bHLH) transcription factor that has been shown to play several critical roles in the different hematopoietic waves. It does not appear to play an early role in vasculogenesis, as Scl-/- mice form both YS and embryonic vessels, but there are defects in YS vessel remodeling revealing an angiogenic defect.[110] In the YS, Scl is expressed in extraembryonic mesoderm and by E7.5 its expression is enriched in the blood islands.[111, 112]

Scl-/- mice die very early due to the lack of primitive hematopoiesis, before analysis of definitive hematopoiesis can occur.[113] Using a chimeric mouse system, it was shown that Scl is also required for definitive hematopoiesis, including the generation of the HSC, as no Scl-/- ES cells contributed to any of the hematopoietic lineages.[114] Interestingly, the role that Scl plays in the development of the HSC is early, and not in the fully mature HE, as

conditional deletion of Scl in the endothelium (by Tie2-Cre) does not affect the detection of a LT-HSC in the fetal liver.[115]

The in vivo hematopoietic defects in the Scl knock-out mice are also recapitulated in vitro in ES cell differentiation experiments. Scl-/- ES cells fail to make red primitive erythroid cells in EB differentiation (primitive wave) and do not form mature BL-CFC colonies in methycellulose culture, but rather are stuck in a "transitional" colony stage (definitive wave).[116]

1.7.2 Runx1

Runx1 codes for the DNA-binding subunit (alpha) of the heterodimeric core binding factor (CBF) which contains a conserved Runt domain that mediates its dimerization and DNA-binding functions. It is expressed specifically in the ECs of the ventral DA (versus DA roof) where the HSCs bud and in the subaortic mesenchyme adjacent to the DA.[117] Primitive erythropoiesis in the YS does not appear to be affected by Runx1 deletion (both in vitro and in re-plating Runx1-/- YS), though its mRNA is expressed in the hemangioblast precursors in E7.25 extraembryonic mesoderm and E7.75 YS blood islands.[118] Runx1 targeted deletion in ECs decreases the hematopoietic colony formation from E11.5 YS, though this is after circulation and definitive hematopoiesis in the DA occurs.[119]

Runx1 is critically required in the earliest steps of HSC differentiation from the DA. Targeted deletion of Runx1 in the endothelium by VE-Cadh-Cre leads to no detectable engraftment of E11.5 AGM into adult mice compared to controls.[119] As a result, Runx1-/- mice die between E9.5-11.5, probably due to hematopoietic defects.[120-123] However, after the emergence of these HSCs from the DA, Runx1 is no longer needed, as conditional deletion of Runx1 in the

hematopoietic lineages by Vav-Cre does not affect the re-population abilities of the LT-HSC in transplants.[119]

1.7.3 ETS Family

Due to the early embryonic lethality of Etv2-/- embryos (before E11.0), it is not possible to analyze the requirement for Etv2 in the development of hemogenic endothelium using germline knock-out mice. In zebrafish, however, it has been demonstrated that Etv2, Scl-α, and Fli1a specify angioblasts and that Scl-β instructs HSC specification from the hemogenic endothelium derived from these angioblasts.[124] Therefore, the early expression and function of Etv2 has downstream effects regulating the hemogenicity of putative hemogenic endothelium in vivo.

Etv2 also plays roles in HSC maintenance after development is complete. Conditional knock-out of Etv2 in the adult hematopoietic lineages (using Mx1-Cre) leads to a significant decrease in the number of HSCs, which is attributed to a maintenance defect based on colony re-plating assays.[125]

Etv6 and Fli1 are two other ETS family transcription factors that are important in hematopoietic development. Two separate programming circuits involving ETS family transcription factors have been identified in hemangioblasts in the xenopus embryo: an Etv6-VEGFA pathway dependent on VEGFA, and a Fli1/Gata2/Etv2 pathway independent of VEGFA.[126, 127] These pathways work in parallel to activate Scl expression and therefore place ETS factors at the top of the regulatory pathways for hematopoietic differentiation. The relationship between Etv6 and Etv2 has not yet been reported.[128]

1.7.4 Hox Family

Hox (Homeobox) family transcription factors contain a homeodomain that binds DNA and its members include many factors that play important roles in embryonic patterning and development. The expression of the different Hox genes are determined co-linearly along the rostral-caudal axis of the embryo in the order of the genes in the Hox cluster.[129] HoxA3 has been shown to be an apical regulator of HE and regulates many of the previously characterized hematopoietic transcription factors including Runx1, Gata1, and Gfi1B. HoxA3 marks embryonic arterial DA exclusively before Runx1 expression (not in YS HE compared to Runx1) and its over-expression blocks HSC production by repressing Runx1 expression. Furthermore, Runx1 over-expression can reverse the effects of HoxA3 over-expression, suggesting HoxA3 mediates its role through Runx1.[98] Knock-out of HoxA3 was not reported, though it could be interesting to see if loss of HoxA3 would lead to a non-arterial and/or non-hemogenic DA.

HoxB4 has been extensively characterized as a factor that can confer definitive hematopoietic potential to in vitro-derived hematopoietic progenitors from ES differentiation that do not have adult engraftment potential without genetic manipulation.[83] It was suggested by gene expression profiling that “universal” targets of HoxB4 overlapped with FGF signaling and that those factors conferred the infected cells with more self-renewal and survival capacity. Other pathways targeted by HoxB4 include TGF-β, BMP, Wnt, Hedgehog, and Notch.[130]

1.7.5 SoxF Family

SoxF family transcription factors have been studied in the context of hematopoiesis as they are relatively arterial-restricted, particularly in the DA, during embryonic hematopoiesis.

Sox18 expression was characterized in the in vitro ES differentiation system, where it was expressed slightly later (in the CD41+ hematopoietic cells) than Sox7 (in the Flk1+ hemangioblasts).[131] Sox17 was identified as a marker during ES differentiation (using Sox17-eGFP knock-in ES cells) in second-wave Flk1+ EBs that were characterized as containing more definitive hematopoietic progenitors (compared to first wave Flk1 EBs).[132]

Indeed, Sox17+ cells in the second Flk1+ wave of ES differentiation were more enriched for CD45 expression and erthro-myeloid colony formation in vitro than the Sox17- fraction. In Sox17 knock-out ES cells, however, there was neither a block in CD45 expression nor any effect on myeloid or erythroid colony formation, but there was a drastic decrease in T lymphocyte differentiation, suggesting that a bona fide HSC could not differentiate without Sox17. This was supported by analysis of both Sox17 knock-out and Sox17 EC-conditional knockout (VE-Cadh-Cre) mice which did not contain HSCs at E11.5 that could reconstitute an adult mouse. It appeared by gene expression analysis of the Sox17-/- ES cells that Sox17 was downstream of both Runx1 and HoxA3, but upstream of EfnB2 and Notch1.[133]

1.7.6 GATA

The GATA family of transcription factors includes six members, GATA1 – 6, which all contain a zinc-finger motif that binds the consensus sequence (A/T)GATA(A/G). GATA1 – 3 are mainly expressed in the hematopoietic lineages.[134] GATA1 is expressed in primitive and definitive erythroid cells, megakaryocytes, eosinophils and mast cells, and is critical for normal erythropoiesis beyond the proerythroblast stage.[134] Mechanistically, GATA1 activates erythroid-specific genes and represses genes associated with proliferation. Not surprisingly, $GATA1^{-/-}$ embryos die at E10.5 due to severe anemia.[135]

GATA2 is also critical for erythropoiesis. During erythroid differentiation GATA2 is expressed before GATA1 (note that GATA2 binds and activates its own transcription), followed by a switch to almost exclusive GATA1 expression (GATA1 suppresses GATA2 expression). This "GATA switch" requires the transcriptional co-factor FOG1 (Friend of GATA).[136]

GATA2 also plays roles in HSC emergence from the AGM and in the proliferation and survival of adult HSCs. During developmental hematopoiesis, it appears that GATA2 can bind to the *Scl* promoter contributing to HSC specification by the *GATA2/Fli1/Scl* triad.[137] Additionally, GATA2 is a target of Notch1 signaling, which could be an inducer of this pathway.[138]

1.8 Hemangioblast and Hemogenic Endothelium

1.8.1 Evidence for Hemangioblast

The hemangioblast is commonly defined as a bipotent precursor cell that can give rise to blood and endothelial cells. The concept was first proposed when it was observed that endothelial and hematopoietic cells are proximal to each other in the YS blood islands. This led to the hypothesis that a precursor cell exiting the primitive streak, referred to as a "hemangioblast", clonally gave rise to endothelial and hematopoietic cells in each blood island.[139] Co-expression of endothelial and hematopoietic genes in the early mesodermal precursors also suggested the existence of a hemangioblast.[140]

Genetic evidence for the existence of a hemangioblast included studies in which knock-out of *Flk1* or *Scl* disrupted both endothelial and hematopoietic differentiation during mouse embryonic development.[110, 141] Additionally, $Flk1^+$ cells sorted from the embryo clonally differentiated into ECs and hematopoietic cells when cultured *in vitro* in BL-CFC culture, suggesting that endothelial and hematopoietic bipotency could be observed in a single cell

from the embryo.[97] However, vascular smooth muscle cells could also differentiate from embryo-derived Flk1^{+} cells, suggesting that this population was not strictly bipotent, but more likely to correspond to a multipotent mesodermal precursor.[142]

Additional data challenges the existence of the conceptualized hemangioblast that clonally gives rise to blood islands containing both endothelial and blood cells, and instead propose that blood islands are actually aggregates of already committed endothelial and hematopoietic progenitors that have migrated separately from the primitive streak rather than arising from a single, true hemangioblast.[71] This hypothesis is supported by the observation that while blood islands cannot be identified morphologically in the YS until around E8.25, *in vitro* Ery^{p} activity (EryP-CFC colonies expressing embryonic globin) can be detected as early as E7.25 (and peaking at E8.25). Therefore, at least some hemangioblasts or Ery^{P} precursors arise prior to blood island formation.[3]

Blood islands are also not isolated "islands" in the mouse YS, but rather a band of interconnected vessels containing blood. The high level of connectivity between these "islands" does not support the hypothesis that blood islands are clonally-derived from individual hemangioblasts.[71]

Lineage tracing experiments have also challenged the concept of a hemangioblast. Using mutant mice with an inducible Runx1-Cre activated at E7.5 of development, this early Runx1^{+} mesoderm was shown to give rise to hematopoietic progeny (myeloid and erythroid cells in the E12.5 fetal liver) but no endothelial progeny (VE-Cadh^{+} cells from E12.5 YS), providing no evidence of a bipotent Runx1^{+} hemangioblast after E7.5 of development.[143] In a separate lineage tracing analysis using tetrachimeric embryos with fluorescently-labelled ES cells (Rosa-eGFP, -eCFP, and mRFP) injected into a developing blastocyst, individual blood

islands were observed to contain combinations of different fluorescently-labelled and non-fluorescently-labelled cells suggesting a polyclonal origin of the blood islands.[144] Together, these lineage tracing experiments suggest against the notion of a clonal hemangioblast in the YS blood islands, though the most stringent lineage tracing experiments of mesodermal/hemangioblast cells exiting the primitive streak into the YS have not been done.

Evidence for a hemangioblast *in vitro* was reported using $Flk1^+$ cells sorted from Day 3 – 4 of EB differentiation into BL-CFC culture. These experiments showed both endothelial and hematopoietic differentiation from a single $Flk1^+$ cell, suggesting it was the *in vitro* equivalent of the primitive streak hemangioblast.[97] However, this $Flk1^+$ ES-derived population can also form smooth muscle and cardiac cells *in vitro*, suggesting that it is not strictly bipotent, but rather an early mesoderm precursor.[145] Therefore, whilst the concept of a hemangioblast giving rise to the first endothelial and blood lineages in the embryo remains widely accepted, there is no conclusive experimental evidence to support this model.

1.8.2 Evidence for Hemogenic Endothelium

The observation that intra-aortic cell clusters bud from the ECs of the DA, coupled with the striking number of cell surface markers that ECs and HSCs share has given rise to the hypothesis that the endothelium can give rise to hematopoietic ells through an endothelial to hematopoietic transition (EHT). This endothelium, termed hemogenic endothelium (HE), is defined as a fully differentiated endothelial cell which expresses endothelial markers and gap junctions to form part of a mature vessel that can give rise to hematopoietic cells in very

specific embryonic locations (DA, umbilical and vitelline arteries) and in a very restricted timepoint during development (E10.5 – 11.5).[140]

Elegant genetic labelling and lineage tracing experiments have demonstrated an endothelial cell origin of HSCs. Temporal and selective labeling of E11.0 AGM endothelium in mouse embryos carrying an inducible VE-Cadherin-Cre allele and a Rosa26R:LacZ reporter allele showed that 2-24% of adult bone marrow cells carried the β-gal signal indicative of an endothelial origin (50% when constitutive VE-Cadh marking the progeny of all endothelial cells was used). These β-gal$^+$ hematopoietic cells persisted in the adult thymus, spleen and vessels, demonstrating an ability to sustain definitive hematopoiesis. Marking the subaortic mesenchyme but not the endothelium using Myocardin-Cre did not produce any labeling in the adult hematopoietic cells, showing that HSCs are derived exclusively from an endothelial intermediate.[146]

The EHT transition of HE cells in the ventral wall of the AGM into hematopoietic intra-aortic clusters was visualized *ex vivo* using AGM from mice carrying a Ly-6A-GFP transgene combined with CD41 and c-kit antibody staining. In these experiments, E10.0 embryo slices or whole dorsal aorta organs were maintained in culture and imaged for Ly-6A-GFP$^+$ hematopoietic cells budding from CD31$^+$ (antibody stained) ECs of the DA. This convincingly showed that a fraction of ECs are capable of "transdifferentiating" into hematopoietic cells, supporting the existence of an endothelial population capable of producing hematopoietic cells, and thus an HE population.[147]

The number of HSCs generated by the E11.5 DA is low. It is estimated that there are only 70 VE-Cadh$^+$CD45$^+$ cells existing in the 2 – 4 intra-aortic clusters in the DA. By limiting dilution transplantation, only one of these 70 VE-Cadh$^+$CD45$^+$ cells is a *bona fide* HSC. The

hematopoietic potential of this VE-Cadh^{+}CD45^{+} "pre-HSC" population could be increased *ex vivo* by re-aggregating it with CD45^{-}GFP^{-} supporting cells and culture for 96 hours, which increased the number of transplantable HSCs to 148. In these experiments, VE-Cadh^{-}CD45^{+} cells (mature hematopoietic cells) did not give rise to transplantable HSCs. Together, these data suggest that HSCs have to go through, or are at least be enriched in, an endothelial VE-Cadh^{+}CD45^{+} pre-HSC intermediate.[148]

Recently, the timing of hematopoietic specification from the HE was defined at E9.5, much earlier than previously predicted. Using mouse embryos carrying a +23 Runx1 enhancer reporter gene expressing GFP in the putative HE of the DA, single cell transcriptional analysis of GFP^{+} cells sorted from the DA established hematopoietic specification by E9.5 (from gene array and principal component analysis), the peak of *Runx1* gene expression in the HE. The work also restricted the definition of HE to endothelial cells of the DA that can only give rise to hematopoietic cells and not endothelial cells upon *ex vivo* re-plating onto OP9 stroma. This was because no +23 Runx1-GFP^{+} endothelial cell between E8.5 and E10.5 could give rise clonally to both endothelial and hematopoietic cells.[149]

Evidence for the existence of a HE has also been established *in vitro* using ES cell differentiation. Flk1^{+} Day 3.5 EB cells re-plated into BL-CFC gives rise to a Tie2^{hi}c-kit^{+}CD41^{-} HE population after 48 hours of culture that is also Flk-1^{+}, MECA32^{+}, VE-Cadherin^{+}, CD34^{+}, endoglin^{+}, claudin 5^{+}, CD45^{-}, and CD11b^{-}. By CFSE analysis, 40% of the HE cells are hemogenic (ie. give rise to CD41^{+} hematopoietic cells), though this frequency is much lower (1.2%) by limiting dilution analysis.[99]

The HE was also characterized in OP9 co-culture where Day 4 Flk1^{+}E-Cadh^{-} EB cells were re-plated with the hematopoietic cytokine KL. The emergence and differentiation of this HE

was imaged using time-lapse microscopy over the course of four days. Here, the HE was identified as consisting of endothelial colonies that were Flk1^{+}, c-kit^{+}, E-cadherin^{+} and VE-cadherin^{+}, CD41^{-}, CD45^{-}, CD11b^{-} and which functionally uptake Ac-LDL. By single cell live imaging, the authors were able to track emerging CD45^{+} hematopoietic cells to their single cell endothelial precursor. From this, the authors calculated that only 1% of the embryonic ECs at day 4-5 are hemogenic (i.e. become CD41^{+}), and that the first identified "hemogenic endothelial cell" gives rise to 1-6 hemogenic endothelial cells by symmetrical division over the course of four days.[100] These *in vitro* results provided evidence for EHT. The emergence of a similar HE was observed after re-plating Day 6 Flk1^{+}VE-Cadh^{+} EBs onto OP9 stroma with the cytokines VEGF, TPO and Flt-3.[98]

1.8.3 Evidence for YS versus Intra-aortic Mesoderm Precursors *in vitro*

The sections above have included a discussion of hemangioblast and HE populations identified *in vitro* during ES cell differentiation. Due to the nature of ES differentiation in cultures, it has been difficult to distinguish YS- from DA-like precursors of hematopoietic cells. This section discusses *in vitro* work with ES cells that has sought to separate YS versus DA-like waves of hematopoiesis.

During EB differentiation in serum-free culture, two waves of Flk1^{+} cells were identified. The early Flk1^{+} cells, sorted from Day 3.25 EBs, were identified as precursors of the YS-like hematopoietic program while the second Flk1^{+} wave, sorted from Day 5.25 EBs, was identified as reflecting intra-aortic hematopoiesis. This distinction was based on data showing a) that Day 3.25 EB-derived Flk1^{+} cells had greater "primitive" erythroid potential (based on high embryonic hemoglobin production) than Day 5.25 EB-derived Flk1^{+} cells and

b) that Day 5.25 EB-derived Flk1$^+$ cells had increased lympho/myeloid differentiation potential over Day 3.25 EB-derived Flk1$^+$ cells.[132]

More recently, studies with human ES cells (re-plating Day 4 mesodermal cells) showed that BL-CFC cultures model YS-like hematopoiesis with high embryonic globin expression and low *Runx1* expression. Conversely, co-cultures of ES cells onto OP9 stroma model more DA-like hematopoiesis with low embryonic globin expression expression but high *Runx1* expression.[101]

Neither study, however, demonstrated that the HSCs derived in culture from the DA-like wave could reconstitute hematopoiesis after transplantation.[101, 132]

1.8.4 Hemogenic Endothelium and YS/DA Hematopoietic Precursor Definitions for this Work

In this work, Flk1$^+$ cells isolated from Day 3 – 4 EBs will be defined as an early mesodermal population containing precursors for blood and endothelial lineages, with the understanding that others have previously identified it as the hemangioblast.[99] This Day 3 – 4 EB Flk1$^+$ mesodermal precursor correlates temporally with the first Flk1$^+$ wave in the work of Irion et al, and also the BL-CFC re-plating of human ES cells by Choi et al, both of which were identified as modeling YS-like hematopoietic programs.[101, 132] Therefore, in this work, **Day 3 – 4 Flk1$^+$ cells in BL-CFC culture** are considered as modeling a YS-like hematopoietic program, and defined as **YS-like mesodermal precursors**. In addition, the data from these experiments will be correlated with results from re-plating **E9.5 YS** into hematopoietic colony assays with MethoCult3434.

In the current work, DA-like hematopoiesis is modeled with Flk1$^+$VE-Cadh$^+$CD41$^-$ cells sorted from Day 6 of EB differentiation into OP9 co-culture and Methocult3434 re-plating. This Day

6 EB population correlates temporally with the second Flk1$^+$ wave in the work of Irion et al, and also the OP9 co-culture re-platings of Choi et al that were shown to model more DA-like hematopoietic programs.[101, 132] Day 6 EB re-platings into OP9 co-culture have also been used in other studies to model DA-like hematopoietic programs.[98] Therefore, in the current study, DA-like hematopoiesis is investigated with Day 6 Flk1$^+$VE-Cadh$^+$CD41$^-$ cells cultured onto OP9 monolayers and will be compared to re-plating **E9.5 DA** into OP9 and Methocult3434 cultures. Thus Day 6 Flk1$^+$VE-Cadh$^+$CD41$^-$ will be defined as an endothelial cell population that more closely models the DA-like versus the YS-like hematopoietic progenitor.

In the current work, the HE will be defined most stringently as an adherent endothelial cell that differentiates into non-adherent CD41$^+$/CD45$^+$ hematopoietic cells in OP9 co-culture. Because the current work does not include time-lapse imaging documenting this EHT transition, the **Day 6 EB Flk1$^+$VE-Cadh$^+$CD41$^-$ population** will be defined as a **DA-like endothelial population** that contains the **functional HE** that gives rise to the **hematopoietic CD41$^+$/CD45$^+$ cells** within the five days of co-culture on OP9 stroma.

1.9 Project Motivation

Developmental hematopoiesis in vivo occurs in several waves that are spatially and temporally distinct, as described above. The distinction between these waves, however, is not as clear in the ES system where, while there is no circulation which confounds the in vivo results, there is little or no spatial distinction in the differentiating EB aggregates. In addition, the adult definitive wave has not been convincingly identified in the ES/EB system, as there is still no endogenously derived (ie. no genetic manipulation) LT-HSC capable of reconstituting the adult bone marrow.[83] An extensive characterization of the different

hematopoietic progenitors in the ES/EB system by correlating them to the different embryonic waves may allow for the enrichment of a potential definitive HSC.

It is hypothesized that enriching for the most "DA-like" endothelium in ES/EB culture would help to identify an HSC-equivalent in the ES system. In vivo, it appears that an arterial endothelium is established before hematopoiesis, suggesting that the endothelium is arterial before it is hemogenic. Studies of mice with ectopic arterial or venous identity confirm that hematopoiesis is strongly correlated and subsequent to arterial commitment.[70]

To identify a "DA-like" hemogenic endothelium, the surface protein EfnB2 was selected. Previous studies have not only shown the specific arterial expression of EfnB2 during development, but have also shown that it is functionally critical for the arteriovenous segregation of the DA and PCV.[7] A role for EfnB2 in hematopoiesis has not been previously investigated. Through all vertebrate studies, EfnB2 appears to be a robust arterial (and DA marker) and the goal was to extend this observation to in vitro ES differentiation.

Work has shown that sorting for arterial associated genes (Sox17-eGFP) can enrich for hematopoietic progenitors.[133] In this case, EfnB2 has the advantage of being a cell surface protein that can be used for cell sorting based on endogenous expression without genetic knock-in.

Investigating EfnB2 during in vitro differentiation was also hypothesized to be another strategy to study the potential interaction between the arteriogeneic and hematopoietic programs that have yet to be fully clarified. This is because given the vast number of mutant vertebrates studied, the classical hierarchy of Shh, VEGF, Notch, and EfnB2 (most downstream) appears to still hold true (Figure 1d).[11] Studies have shown that arteriogenesis

can be uncoupled from hematopoiesis when components of these signaling pathways (specifically VEGFA medium and long form isoform morphant zebrafish, and Jagged1 knockout mice) are manipulated and EfnB2 expression is preserved in the DA but not HSC emergence.[29, 102] These studies show that EfnB2 is not sufficient for the "arterial" endothelium to be hemogenic. However, as the most downstream marker/regulator of the arterial endothelium, it would be interesting to study if EfnB2 was absolutely necessary for hematopoiesis, and if so how, as it could potentially be the most direct inducer of the hematopoietic program in the arterial cascade.

In studying EfnB2, there appeared to also be the potential to clarify distinctions among different HE populations in vitro. The focus has mostly been on the DA HE that gives rise to the LT-HSC. However, there is almost certainly a YS-like HE (identified in the human ES culture). EfnB2 is expressed in the YS arterial vessels (versus EphB4 in the YS venous vessels). Closer investigation of the expression kinetics reveals that while EfnB2 expression in the DA precedes HSC emergence (ie. arterial endothelium before hemogenic endothelium), EfnB2 is expressed later in the YS after the first two YS waves of hematopoiesis.

More specifically, EfnB2 is expressed beginning at E8.5 in the YS. The is after both Primitive (E7.5; Ery^{p}) and Pro-Definitive (E8.25; Ery-myeloid) waves of hematopoiesis, and 12 hours before the Meta-Definitive (E9.0;Ery-myeloid-lymphoid) wave.[10] This would lead to the hypothesis that the first two YS waves do not require EfnB2 (or an arterial HE). Manipulation of EfnB2 expression could help identify EfnB2-independent waves in vitro that could correlate with these YS-like HE waves of hematopoiesis. This could also correlate

EfnB2 expression with only the multilineage hematopoietic wave (YS Meta-Definitive, AGM Meta-Definitive, AGM Adult-Definitive).

1.10 Project Aims

Aim 1: Identify and characterize an HE during ES cell differentiation to test if EfnB2 expression can enrich for a more definitive DA-like HE in vitro

- Characterize the HE transition in BL-CFC differentiation in relation to endothelial, arterial, and hematopoietic markers by FACs and gene expression profiling [Chapter 3.1.1]
- Identify an "arterial HE" using EfnB2 surface expression and other "arterial" markers [Chapter 3.1.2 – 3.1.3]
- Characterize the expression of EfnB2 and its pathway components in the embryo during HSC emergence [Chapter 4.1]
- Test if endogenous EfnB2 expression during HE differentiation in vitro enriches for "definitive" hematopoietic progenitors [Chapter 3.3.4]

Aim 2: Evaluate the requirement for EfnB2 expression during ES cell differentiation and in vivo development to see if it can decouple arteriogenesis and hematopoiesis as well as "YS-like" versus "DA-like" HE populations

- Knock-down EfnB2 using shRNA during ES differentiation in vitro to evaluate the effect on hemangioblast, endothelial, arteriogenic, and hematopoietic commitment [Chapter 3.2.1 – 3.2.5]
- Use the genetic manipulation of EfnB2 to identify a potentially different YS-like versus DA-like non-hemogenic endothelium [Chapter 3.3.1-3.3.2]
- Characterize the different YS and DA putative HE populations from EfnB2 knock-out and EC-specific conditional knock-out mice by re-plating ex vivo [Chapter 4.2 – 4.4]

Aim 3: Explore the role of EfnB2 expression and signaling in the HE and the mechanism by which it can link the arteriogenic and hematopoietic programs

- Characterize the perturbations in gene expression profile of the putative HE after EfnB2 knock-down in vitro [Chapter 3.3.3]
- Characterize the effect of blocking EfnB2 interaction with its receptor EphB4 using peptide blockers in vitro [Chapter 3.3.5]
- Characterize the effect of using signaling deficient EfnB2 protein to decouple forward versus reverse signaling roles of EfnB2 during hematopoietic differentiation in vitro [Chapter 5.5]
- Characterize the potential niche requirement for EfnB2 in the stromal cell layer [Chapter 4.5]
- Characterize the effect of over-expressing EfnB2 on hematopoietic differentiation in vitro [Chapter 5.1]
- Test if enforced expression of EfnB2 or Notch signaling inhibition in hematopoietic deficient Scl-/- ES cells can rescue hematopoiesis [Chapter 5.2 – 5.3]

2 Materials and Methods

2.1 Cell Culture Techniques

2.1.1 In vitro Differentiation of ES Cells

ES Cell Maintenance

J1 and $Scl^{-/-}$ mouse ES cells were maintained and differentiated as previously described.[150] Cells were maintained on 0.1% gelatin in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 20% pre-tested fetal calf serum (FCS), 2% LIF conditioned medium, 1% penicillin-streptomycin and 1.5 x 10^{-4} monothioglycerol (MTG; Sigma). The cells were passed every 48 hours at 1:6 dilution, not more than 10 times.

Embryoid Body Differentiation

24 hours prior to the onset of differentiation, ES cells were re-plated on gelatin in IMDM media with the same maintenance media supplements. ES cells were differentiated in suspension culture into embryoid bodies (EBs) by culturing in IMDM supplemented with 15% pre-tested FCS, 2 mM L-glutamine, 200 ug/mL transferrin (Roche), 0.5 mM ascorbic acid, 1% penicillin-streptomycin and 4.5 x 10^{-4} MTG. Cells were plated at 1 x 10^4 cells/mL density.

EBs were grown for 3.5 days before FACS sorting for Flk1+ hemangioblasts. In re-aggregated EB cultures, Day 4 EBs were harvested, trypsinized and re-plated at 2.5 x 10^5 cells/mL as previously described.[98]

Blast Colony Differentiation

Flk1+ cells were re-plated in BL-CFC methycellulose as previously described.[150] The medium contained 1% methylcellulose, 10% FCS, 2 mM L-glutamine, 1% penicillin-streptomycin, 300

ug/mL transferrin, 25 ug/mL ascorbic acid, 4.5 x 10-4 MTG, 5 ng/mL VEGF, 5 ng/mL IL-6, and 100 ng/mL KL (instead of 20% D4T conditioned media) in IMDM. Cells were plated at a density of 4.25 x 10^4 cells/mL. After culture, cells were harvested by diluting the cultures in warm PBS before centrifugation at 1200 rpm for 10 minutes.

OP9 Differentiation

Day 6 EBs were harvested and/or sorted depending on the experiment and re-plated into OP9 co-culture. [98] OP9 stromal cell layers were plated onto the appropriate dishes/plates 24 hours prior to re-plating the EBs. 7,000 cells were plated onto Ibidi channel slides and 50,000 were plated per 6 well. The sorted EBs were plated as single cells (25,000 per Ibidi channel or 200,000 per 6 well) in media containing 10% FCS, 1% penicillin-streptomycin, 2 mM L-glutamine, 5 ng/mL VEGF, 40 ng/mL TPO, and 40 ng/mL Flt3 in IMDM.

Methocult Differentiation

Cells sorted from Day 6 EBs were plated into commercially available MethoCult3434 methylcellulose media from Stem Cell Technologies on Corning Ultra Low Adhesion plates (50,000 cells/6 well or 10,400 per 24 well). Colonies were scored (with conditions blinded) between Days 9 -12 of differentiation, as noted in the text.

Arterial Monolayer Differentiation

$Flk1^+$ cells were sorted from Day 3.25 – 4 EBs and re-plated on Collagen-IV coated glass coverslips in medium containing 15% pre-tested FCS, 1% penicillin-streptomycin, 4.5 x 10^{-4} MTG, 2 mM L-glutamine, 25 ug/mL ascorbic acid, and 50 ng/mL VEGF in IMDM. Cells were analyzed by FACS on Days 3-4 of differentiation.[33]

2.1.2 Lentiviral Infection

shRNA Constructs

Third-generation HIV-1 based lentiviral short hairpin RNA (shRNA) constructs against EphrinB2 with the shRNA driven by the human U6 promoter and the puromycin resistence selection marker driven by the human phospho glycerate kinase (PGK) promoter were used. The constructs were purchased from Sigma-Aldrich Mission. The first sequence (TRCN0000058427) was CCGGCTGGTACTATACCCACAGATACTCGAGTATCTGTGGGTATAGTAC CAGTTTTTG (the actual target sequence in the hairpin structure is underlined) which targeted the coding domain of EfnB2 (both human and mouse) and was cloned by restriction digestion with the *Bam*HI and *Kpn*I enzymes into the plko.1-eGFP backbone to re-place the puromycin resistence selection marker with enhanced green fluorescent protein (eGFP), under the PGK promoter. The same cloning was done for the empty vector plasmid to generate a PGK-eGFP control lentivirus.

The second sequence (TRCN0000066493) was CCGGCGGGTGTTACAGTAGCCTTATCTCGAG ATAAGGCTACTGTAACACCCGTTTTTG (the actual target sequence is underlined) which targeted the 3' UTR of mouse EfnB2. This construct was used as purchased, with the puromycin selection marker under the PGK promoter intact. Control plko.1-puromycin was used as the control.

Overexpression Constructs

The overexpression constructs for WT EfnB2 and mutant 5Y-EfnB2 were generated by cloning the EfnB2 coding regions from the previously published YFP lentiviral vectors (WT and 5Y, gift from T. Makinen) into the 277.pCCLsin.PPT.hPGK.EGFP.pre backbone (gift from A Follenzi).

Lentivirus Production

Lentivirus was generated by cotransfection of 293T cells with three plasmids: a cytomegalovirus (CMV) promoter-driven packaging construct expressing the *gag* and *pol* genes, a Rous sarcoma virus promoter-driven construct expressing *rev*, a CMV promoter-driven construct expressing the VSV-g envelope, in addition to the shRNA/control constructs described above. Transfection was done using the Lipofectamine2000 reagent (Invitrogen). Virus-containing supernatant was collected 72 hours after transfection and concentrated by ultracentrifugation before use. Each shRNA (puromycin and eGFP) virus was transfected separately and pooled after concentrating the virus. The same was done for pooled puromycin and eGFP PGK control empty vector virus.

2.1.3 Blocking Peptide Treatment

Blocking Peptide Culture

The sequences of the TNYL-RAW and SNEW EfnB2 blocking peptides have been characterized in the literature previously (TNYL-RAW: TNYLFSPNGPIARAW; SNEW: SNEWIQPRLPQH).[151] These peptide sequences were custom ordered from Anaspec. All peptides (TNYL-RAW, TNYL-RAW scrambled, and SNEW) were used at a final concentration of 225 uM diluted in the appropriate differentiation media.

2.1.4 Immunophenotypic Analysis and Cell Sorting

Antibody Staining

Cells were harvested and pelleted by centrifugation before dissociation using 0.05% trypsin for three minutes. After straining out cell clumps, DNA, etc. using a 40 uM mesh, the cell suspension was incubated at 37°C for one hour to allow re-expression of specific surface

markers that were sensitive to trypsin digestion. Staining was done on ice for one hour using the antibody clones and concentrations listed in Table 3.

Cell Apoptosis Analysis

Cells were stained in a final concentration of 50 nM tetramethylrhodamine, ethyl ester, perchlorate (TMRE; Invitrogen), 1:20 dilution of Annexin V-eFluor450 (as directed by kit; eBioscience), and 1:1000 dilution of propidium iodide (1 mg/mL stock; Invitrogen).

Flow Cytometry

Cells were analyzed on a Cyan (Beckman Coulter), Fortessa (BD), or ARIA II (BD) and plots were generated in either Summit v4.3.02 (Beckman Coulter) or FACS DIVA (BD). Compensation was calculated either with single stains or with unstained cells spiked with compensation beads when cell numbers were insufficient. FACS gates were determined empirically in each experiment using the appropriate controls.

Cell Sorting

For FACS sorting, cells were stained with PE conjugated rat anti-mouse Flk-1 mAb (Table 5) and sorted on a MoFlo/ARIA II machine. For MACS sorting, cells were stained with either purified rat anti-mouse Flk1 or biotinylated anti-mouse Flk1 followed by incubation with either anti-Rat or streptavidin microbeads (in buffer containing 2 mM EDTA and 0.5% BSA in PBS) and then passed manually through a MACS™ Selection (LS) or depletion (LD) column (Miltenyi Biotec). Sort purity was checked by FACS on a Cyan (Beckman Coulter).

		Flow Cytometry		Immunofluorescence	
Antibody	Clone	ug/100 uL Staining Volume	Fluorophore	ug/1 mL Staining Volume	Fluorophore
Flk1	Avas12a1	0.2	PE	--	--
EfnB2	Goat Polyclonal	0.25	SaV-AF647	2.5	SaV-AF568
EphB4	Goat Polyclonal	0.5	aGoat-AF647	--	--
CD41	MWReg30	0.125	eFluor450	--	--
VE-Cadh	BV13	0.3	PE	2	AF488
c-kit	2B8	0.017	PE-Cy7	--	--
Notch1	HMN1-12	0.1	PE	--	--
Notch4	HMN4-14	4	PE	--	--
Dll4	HMD4-1	0.5	APC	--	--
CD150	TC15-12F12.2	0.3	PE	--	--
Sca-1	D7	0.2	PE-Cy7	--	--
CD45	30-F11	0.2	PE-Cy5	--	--
pEfnB	Cell Signaling	1:100	aRab-AF488	1:100	aRab-AF568

Table 3. Antibodies for Flow Cytometry and Immunofluorescence

	Forward Primer	Reverse Primer
GAPDH	TGT AGA CCA TGT AGT TGA GGT CA	AGG TCG GTG TGA ACG GAT TTG
Flk-1	CAC CTG GCA CTC TCC ACC TTC	GAT TTC ATC CCA CTA CCG AA
VE-Cadh	CCA CTG CTT TGG GAG CCT T	GGC AGG TAG CAT GTT GGG G
FoxC1	CAA GAC GGA GAA CGG TAC GTG	GGC TCT CGA TTT TGG GCA CT
FoxC2	AAC CCA ACA GCA AAC TTT CCC	GCG TAG CTC GAT AGG GCA G
Sox17	TCC ATG AGG TGA CAT GCT GAG GTT	AGC TCC AGA AAC TGC AGA CCA GAA
Hey1	GCG CGG ACG AGA ATG GAA A	TCA GGT GAT CCA CAG TCA TCT G
Hey2	AAG CGC CCT TGT GAG GAA AC	GGT AGT TGT CGG TGA ATT GGA C
Notch4	CTC TTG CCA CTC AAT TTC CCT	TTG CAG AGT TGG GTA TCC CTG
CoupTFII	TCA ACT GCC ACT CGT ACC TG	CCA TGA TGT TGT TAG GCT GCA T
EphB4	TGGCTGATCACGAACTTGACCTAC	AGG CAG GAC TCG TCT CCT ATT T
Scl	CAC TAG GCA GTG GGT TCT TTG	GGT GTG AGG ACC ATC AGA AAT CT
mRunx1	GCA ACT TGT GGC GGA TTT GTA	GCA GGC AAC GAT GAA AAC TAC T
hRunx1	TCT TCA CAA ACC CAC CGC AA	CTG CCG ATG TCT TCG AGG TTC
HoxA3	TCA GCG ATC TAC GGT GGC TA	GAG GCA AAG GTG GTT CAC CC
CD150	GGT GTG ATG GAT TGC CCA GT	CGA GGA TGC GGA CAC TTT TGT
TaqMan	EfnB2, Dll4, Sca-1, CD41, CD45	

Table 4. qPCR Primers for Gene Expression

	Forward Primer	Reverse Primer	
Tie2-Cre	CGC ATA ACC AGT GAA ACA GCA TTG C	CCC TGT GCT CAG ACA GAA ATG AGA	55°C
iVE-Cadh-Cre	GAA AAT GCT TCT GTC CGT TTG C	ATT GCT GTC ACT TGG TCG TGG C	55°C
EfnB2 Floxed	AAG TTA TAA GCT TCA ACG CGT CC	GAG CCC CAG GTT CTA GAA TAA	62°C
EfnB2 No Flox	GCT GCC CGC GGC CGG TCC CAA CG	CCG TTA GTG GCA ACG TCC TCC GTC CTC	69°C
EfnB2 KO	GGC TCT GAA ACC TCT GAT GC	GTC TTC CAG GTT GGA CGT GT	69°C
EfnB2 No KO	CGG TTA TTT AAG CAG TGC TAT CC	GCT TTT CCC ATG CAA TGT TT	60°C

Table 5. Primers and Conditions for Mouse Genotyping

2.1.5 Cell Imaging

Live Cell Surface Staining

Media from the plates was carefully aspirated to prevent the loss of as many non-adherent hematopoietic cells in suspension as possible. Media containing the antibodies listed in Table 3 were all used at a dilution of 1:500 for live cell surface staining. The plates were stained for 30 minutes at 37°C and the media was replaced with 10% FCS/PBS buffer before imaging (to decrease potential background caused by phenol red in the media).

Cell Apoptosis Analysis

Cells were incubated with tetramethylrhodamine, ethyl ester, perchlorate (TMRE; Invitrogen) at a final concentration of 50 nM and with a 1:100 dilution of Annexin V-eFluor450 (eBioscience) for 15 minutes at 37°C before washing the plates with media three times. The cells were imaged at room temperature immediately following staining.

Intracellular Fixed Staining

Cells were washed with PBS three times before fixing in 4% paraformaldehyde (PFA/PBS) for 10 minutes on ice. PFA was quenched by washing the plates two times in 10 mM Glycine/PBS (PBSG) and PBS one time. The cells were permeabilized using 0.5% TritonX/PBS (PBST) for 15 minutes, blocked with 5% serum/1% BSA/0.2% PBST for one hour and stained with the primary antibodies overnight in blocking buffer as listed in Table 3. After washing the plates two times in 0.2% PBST, secondary antibodies were used at a dilution of 1:1000. After washing with PBS three times, the cells were fixed again in 4% PFA/PBS before final washes in PBSG (two times) and PBS (one time).

Cytospins and May Grunswald Giemsa Staining

Cells were cytospun onto glass slides at 400 rpm for 5 minutes and allowed to dry overnight before staining. Staining by May Grunwald was done by dunking the slide into the solution five times before a PBS wash. Then the slide was dipped into 5% Giemsa/PBS five times before a gentle wash under water.

Light and Confocal Microscopy

For light microscopy, plates and slides were imaged on an Olympus IX51 inverted scope. Images were acquired using IPLab v4.08. For confocal microscopy, the channel slides were imaged on a Zeiss LSM 710 NLO or LSM 780 (Zeiss) and acquired using Zen 2009 (Zeiss).

2.1.6 Gene Expression Analysis

RNA Isolation

RNA was extracted with a Qiagen RNeasy Micro kit for cultures predicted with fewer than 5 x 10^5 cells and Qiagen RNeasy Mini kit for samples larger than that. For FACS sorted populations, the cells were sorted directly into RLT lysis buffer (Qiagen kit). RNA concentration was determined after isolation using a Nanodrop before cDNA synthesis or storage at -80°C.

cDNA Synthesis

cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) to synthesize up to 2 ug of cDNA in a 20 uL reaction volume. cDNA was stored at -20°C until use for qPCR.

qPCR

qPCR was performed using both SYBR green and TaqMan primers. All primers used with SYBR Green (FastStart Universal SYBR Green Master Mix; Roche) are listed Table 4. The dissociation curve of each primer was analyzed to experimentally confirm only one qPCR product was produced with each primer pair, and water controls were always run to confirm no background. TaqMan primers were purchased from Applied Biosystems/Invitrogen and used with TaqMan Universal PCR Master Mix (Roche). qPCR was run on a 7900HT Fast RT PCR System (Applied Biosystems) for 40 cycles with an annealing temperature of 60°C. Results were analyzed using SDS 2.4 (Applied Biosystems).

2.2 Ex Vivo Techniques

2.2.1 Mouse Strains

All animals used in this study were maintained in accordance with the NIH Care and Use Committee. EfnB2 knock-out mice [10, 49] were previously generated and characterized in the literature; the animals used here were gifts from Raft, S. and Mukouyama, Y. C57/BL6 EfnB2 floxed mice[51] were also generated previously and bred with either tamoxifen-inducible VE-Cadh-Cre[152] or constitutive Tie2-Cre[153] to generate endothelial-specific knock-out of EfnB2.

Timed pregnancies were obtained by breeding two females with one male in late afternoon and checking every day for plugs in the morning. The presence of a plug was considered E0.5.

2.2.2 Genotyping

Mice were tagged and tailed before weaning at 21 days after birth and tail samples were used to extract DNA for genotyping. For the EfnB2 floxed mouse line, DNA extraction was

performed using a Qiagen Blood and Tissue DNA Extraction Kit, while DNA was extracted from all other lines using 50 mM NaOH (1 hour; 95°C) and quenched with 1 M Tris-HCl.

The primer sequences and annealing temperatures for each genotyping reaction are included in Table 5. All PCR products were run on a 1% agarose gel for evaluation.

2.2.3 Embryo Dissections

Pregnant mothers were sacrificed by cervical dislocation at the appropriate day of litter development (between E8.0 – 10.0) before removing the embryos. The embryos were dissected under a tissue culture hood and dissection scope to prevent contamination and all embryos/tissues were kept on ice when not being dissected. Whole DA and YS removed from each embryo were dissociated with 0.12% Collagenase I for between 20 – 40 minutes (with pipetting every 10 minutes) until there was minimal visible tissue chunks (all samples were quenched at the same time to keep uniformity). All body remnants from each embryo were frozen for future DNA extraction to determine genotype.

2.2.4 Immunofluorescence of Embryo Sections

Embryos used for immunofluorescence were dissected into cold PBS and fixed in 4% paraformaldehyde/PBS at 4°C for 3 hours with gentle rocking. The embryos were washed two times in PBS before incubation in 10% sucrose/PBS while rocking until the embryo sank to the bottom of the tube. This was repeated with 20% sucrose/PBS, 30% sucrose/PBS, and finally 15% sucrose/50% OCT/PBS. The embryo was then carefully transferred into a mold with OCT and then flash frozen by dropping into methyl butane on dry ice. The embryo blocks were then sectioned onto glass slides using a cryostat and stored at -80°C until use for staining.

The sections were first fixed onto the slides with 4% paraformaldehyde (PFA/PBS) for 10 minutes. PFA was quenched by washing the slides two times in 10 mM Glycine/PBS (PBSG) and PBS one time. The sections were then permeabilized using 0.5% TritonX/PBS (PBST) for 15 minutes, blocked with 5% serum/1% BSA/0.2% PBST for one hour and stained with the primary antibodies overnight in blocking buffer at the concentrations listed in Table 5. After washing the slides two times in 0.2% PBST, secondary antibodies were used at a dilution of 1:1000. After washing with PBS three times, the slides were fixed again in 4% PFA/PBS before final washes in PBSG (two times) and PBS (one time).

2.2.5 Re-Plating Assays

OP9 Co-Culture

7,000 OP9 stromal cells were plated onto Ibidi channel slides (Ibidi) 24 hours prior to embryo dissections to form a confluent layer of stromal cells for the co-culture. One embryo equivalent of DA or 0.2 embryo equivalent of dissociated YS tissue was re-plated onto each channel slide in media containing: 10% FCS, 1% penicillin-streptomycin, 1% fungizone, 50 ug/mL ascorbic acid, 300 ug/mL transferrin, 1.5 x 10^{-4} monothioglycerol (MTG; Sigma), 1 ng/mL IL-7, 5 ng/mL IL-11, 100 ng/mL KL, 5 ng/mL VEGF, 40 ng/mL TPO, and 40 ng/mL Flt3 in IMDM media. Cultures were scored and harvested for FACS at Day 4 – 5 of culture.

MethoCult3434

0.2 embryo equivalent of dissociated YS cells were re-plated into one 6 well (ultra low adhesion plate; Corning) with 2 mL of MethoCult3434 media (Stem Cell Technologies) with additional 1% penicillin-streptomycin to counter the additional risk of contamination due to dissections. Cultures were scored between Day 9 – 12 of culture, as indicated in the data.

2.2.6 Statistical Analysis

Mean and standard deviations were calculated using the functions in Microsoft Excel. Pooled variance was calculated for the fold-change experiments manually in Excel. The variance (s_i^2) in each biological replicate was calculated between the technical replicates (square of the formula standard deviation). The pooled variance (s_p^2) was calculated using the formula: $s_p^2 = \frac{\sum_{i=1}^{k}(n_i - 1)s_i^2}{\sum_{i=1}^{k}(n_i - 1)}$ and graphed with the mean.

In the ex vivo analysis, dot plots were generated in GraphPad. Standard error and student's one sided T test scores were calculated in the program as well.

3 Results Chapter 1: The Role of EfnB2 in the Differentiation of an Arterial-like Hemogenic Endothelium in vitro

3.1 EfnB2 is Expressed in the Hemogenic Endothelium

3.1.1 Characterization of the Hemogenic Endothelial Transition in vitro

Lancrin et al. defined the HE immunophenotypically as the $Tie2^{hi}c\text{-}kit^{+}CD41^{-}$ population emerging in Day 2.0 BL-CFC culture.[99] To study this HE population in our culture system, it was therefore necessary to first confirm its presence. A schematic of the experiment is shown in Figure 5a. Briefly, ES cells were differentiated in suspension culture for 3.5 days before early mesoderm $Flk1^{+}$ cells (around 15-30% of total live cells; Figure 7b) were sorted and re-plated into BL-CFC culture for 4 days. In this BL-CFC culture system, the single $Flk1^{+}$ early mesoderm progenitor cells grow "suspended" in the methylcellulose. At Day 1.25 of BL-CFC differentiation, small, compact colonies appear, which have the morphologic characteristics of HE colonies. These tight HE clusters are largest at Day 1.75 before non-adherent hematopoietic cells begin to "bud" from these clusters at Day 2.0, forming blast colonies. The HE and blast colonies described in the literature were both observed morphologically in this culture (images not shown), suggesting that the same HE emergence and transition published by Lancrin et al. was occurring in this culture system.[99]

To document the emergence and differentiation of this HE, Figure 5b graphs gene expression of the endothelial marker VE-Cadh (red line) and the hematopoietic marker CD41 (green line) across differentiation. As expected, VE-Cadh expression was induced rapidly upon BL-CFC re-plating (Day 3.5 EB) and peaked at Day 2, around the time of emergence of the HE population described by Lancrin et al.[99] The hematopoietic marker CD41 was detected after peak expression of VE-Cadh at Day 2, and markedly increased between Day 3

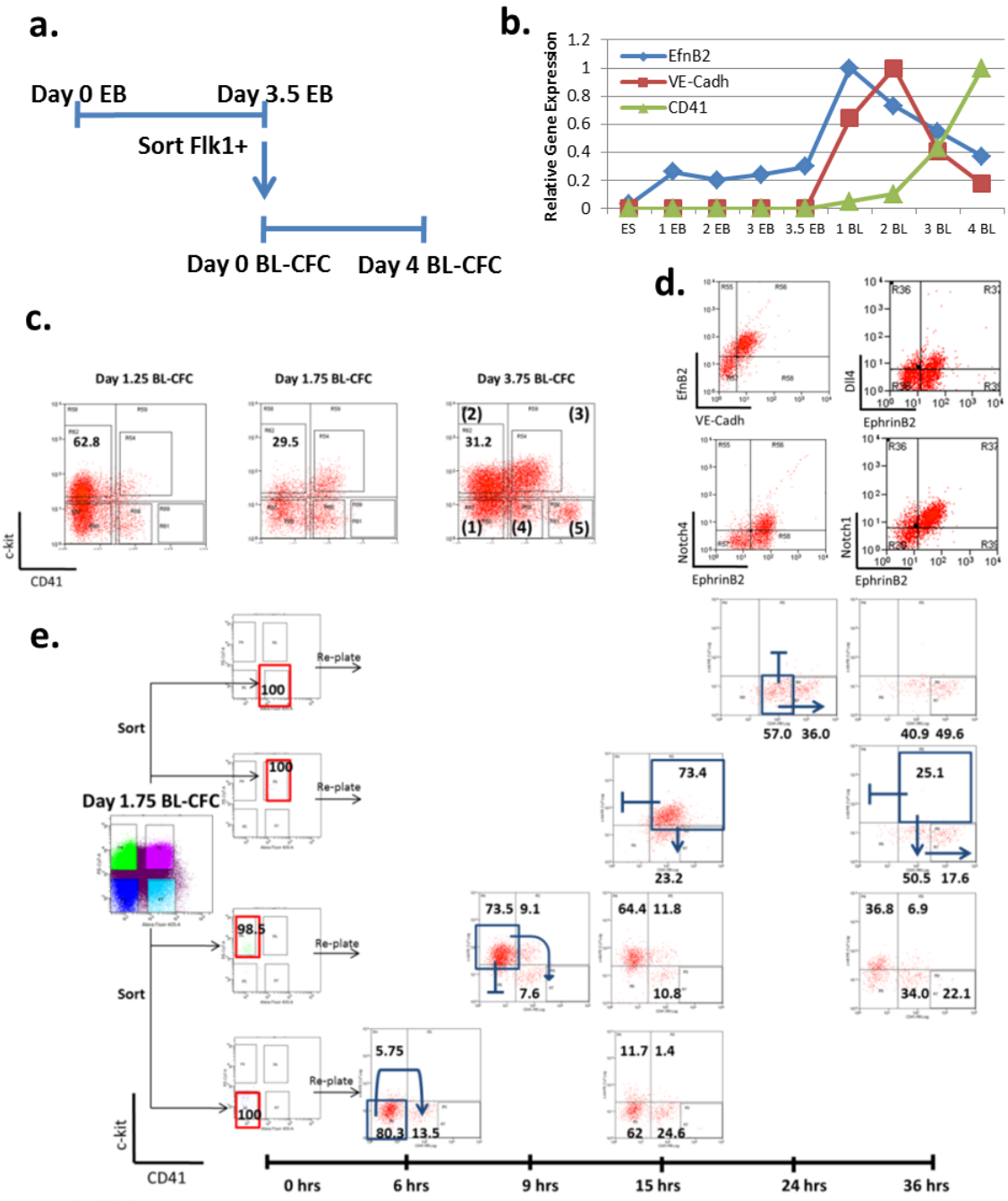

Figure 5. EfnB2 is Temporally Expressed in the Hemogenic Endothelium During BL-CFC Differentiation

a. Schematic of experiment

b. Gene expression graphed across EB and BL-CFC differentiation shows the kinetics of EfnB2 expression against endothelial (VE-Cadh) and hematopoietic (CD41) differentiation. Each gene was normalized to GAPDH expression and then normalized over the different timepoints to make the maximum expression equal 1.

c. c-kit and CD41 staining delineates five different populations in Day 1.25, 1.75, and 3.75 BL-CFC that appear sequentially across BL-CFC differentiation (numbered 1 -5).

d. Day 1.75 BL-CFC co-express EfnB2 protein in conjunction with other endothelial/arterial-enriched markers VE-Cadh, Dll4, Notch4, and Notch1.

e. The four c-kit/CD41 populations in Day 1.75 BL-CFC were sorted and re-plated as single cells back into BL-CFC media before being re-analyzed 6 – 36 hours later to show the lineage relationship between the five populations.

and 4 of BL-CFC culture, suggesting that hematopoietic differentiation followed endothelial commitment.

These results confirmed the sequential endothelial, HE and hematopoietic differentiation occurring during BL-CFC differentiation, and suggested that VE-Cadh was a good endothelial marker. Lancrin et al. used $Tie2^{hi}$ as a marker for endothelial commitment, but Tie2 expression was detected in EB differentiation in these cultures even before re-plating into BL-CFC (data not shown), and Tie2 is known as a shared endothelial and hematopoietic marker. In addition, in the experimental system of Lancrin et al, Tie2 expression was not as rapidly downregulated as VE-Cadh upon hematopoietic differentiation.[99] Therefore, we concluded that VE-Cadh seemed to be a more specific and robust marker for endothelial commitment than Tie2, and we used Ve-Cadh in combination with CD41 to define the HE as $VE\text{-}Cadh^{+}CD41^{-}$.

The early hematopoietic marker, c-kit, that Lancrin et al. used in the HE definition was added to the analysis to confirm the emergence and differentiation of the HE.[99] Figure 5c shows c-kit/CD41 expression between Day 1.25 and Day 3.75 of BL-CFC differentiation. From this time course, c-kit appears to be an early hematopoietic marker that marks hematopoietic competence, as its expression is detected before the expression of CD41.

Our analysis identifies five populations (delineated in the Day 3.75 BL-CFC plot in Figure 5c) beginning with a c-kit/CD41 double negative population (Population 1), a c-kit single positive population (Population 2), a c-kit/CD41 double positive population (Population 3), a c-kit (lo) single positive population (Population 4) and finally a c-kit (hi) single positive population. It appears that there is a sequential acquisition of c-kit expression first detected at Day 1.25 (Population 2) before the emergence of a $CD41^{+}$ population by Day 1.75 (Population 3). We

find a subsequent loss of c-kit expression, giving rise to a CD41 single positive population (Population 4) beginning on Day 1.75 BL-CFC culture. This CD41 single positive (lo) population (Population 4) appears more mature than the c-kit/CD41 double positive population (Population 3) as only these cells co-express the definitive hematopoietic marker CD45 (data not shown). At later time points (Day 3.75), there emerges a population that expresses high CD41 but not c-kit (Population 5). These results show that c-kit and CD41 expression defines the kinetic emergence of five distinct cell populations.

They also suggest a lineage relationship among the five populations, but to more definitively test this relationship, the populations were sorted, re-plated, and analyzed by flow cytometry. BL-CFC cultures at Day 1.75 (the peak of HE emergence in this culture; Figure 5e) were harvested, trypsinized, stained and sorted for the four c-kit/CD41 populations that were present (Populations 1 – 4). These sorted populations were individually re-plated into BL-CFC culture and re-analyzed for c-kit/CD41 expression 6 to 36 hours later (Figure 5e). The plots at 0 hours show that the four populations were sorted to virtually 100% purity before re-plating.

Six hours after re-plating, the c-kit/CD41 double-negative population (Population 1) had given rise to both c-kit single positive and CD41 single-positive populations (Population 2 and 4). By 15 hours, Population 1 had given rise to cells in all four c-kit/CD41 populations, suggesting that the double-negative c-kit/CD41 population (Population 1) contained an early endothelial/hematopoietic progenitor (mesoderm).

Cells sorted from the c-kit single positive population (Population 2) gave rise to both CD41+ populations (Populations 3 and 4) after 9 and 15 hours. Note that at these time points, Population 2 did not give rise to double negative cells (Population 1). This suggests that c-kit

single-positive cells reflect a more committed hematopoietic progenitor compared to Population 1 as it can give rise to all CD41+ populations, but not immediately the early c-kit/CD41 double negative progenitor (Population 1; mesoderm). This c-kit+CD41- population correlates with the population defined as HE (Tie2^{hi}c-kit^{+}CD41^{-}) by Lancrin et al.[99] These results suggest that the acquisition of c-kit expression first, before CD41, marks hematopoietic competence before hematopoietic commitment (CD41).

Upon re-plating, the c-kit/CD41 double positive population (Population 3) only gave rise to Populations 3 – 5, all expressing CD41. This suggests that acquisition of CD41 is an irreversible commitment to hematopoiesis. Similarly, Population 4 only gave rise to Populations 4 and 5.

This experiment suggests that there is a lineage relationship between the five populations that are delineated by c-kit/CD41 expression. In all cases, the populations did not give rise to populations numbered before them. The identification of these populations provides a framework for evaluating cells at different levels of maturation in BL-CFC culture, and could help define “arterial” marker expression in the context of different levels of HE maturation. The model summarizing the identification and relationships among these five populations (including the HE population) is schematized in Figure 6a.

To fully identify the HE, the endothelial marker VE-Cadh was added to the two hematopoietic markers c-kit and CD41. Figure 6b shows VE-Cadh staining as it correlates with the five compartments defined by c-kit and CD41 (Figure 6a). VE-Cadh is expressed heterogeneously (57.1%) in Population 1; this pattern is consistent with this population representing the earliest of the five populations, and including both hematopoietic and endothelial precursors (mesoderm). 90% of Population 2 (HE: c-kit^{+}CD41^{-}) expresses VE-

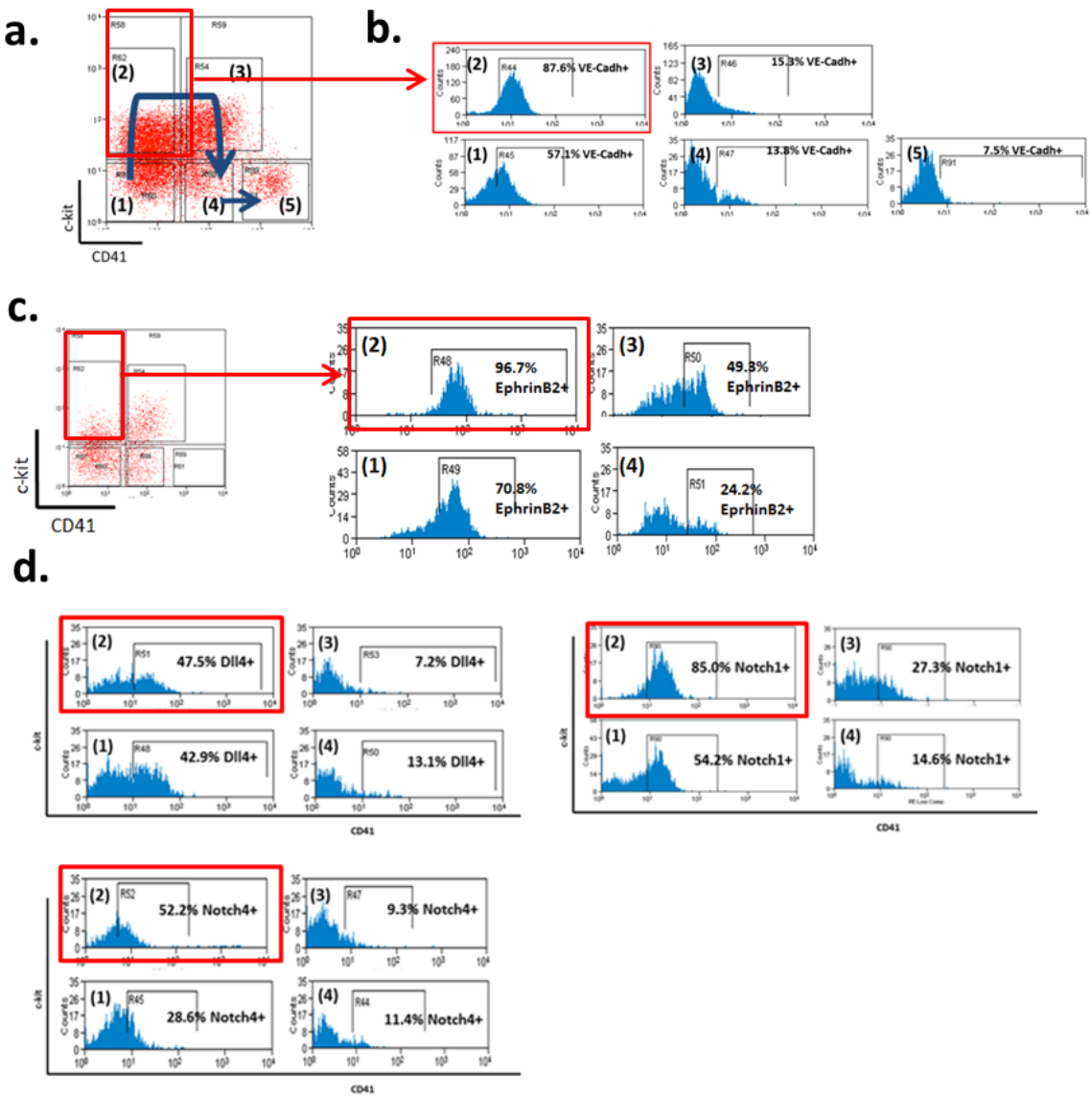

Figure 6. Hemogenic Endothelium in BL-CFC Differentiation Shows "Arterial" Gene Expression

a. Schematic summarizing the pattern of differentiation through the five populations delineated by c-kit/CD41 in Day 1.75 BL-CFC based on the results of Figure 5e.

b. VE-Cadh expression in Day 1.75 BL-CFC gated to the five populations in Figure 6a shows selective VE-Cadh expression in the c-kit+CD41- HE population (red box).

c. EfnB2 expression in Day 1.75 BL-CFC gated to the four c-kit/CD41 populations shows homogenous expression of EfnB2 in the c-kit+CD41- HE population (red box).

d. Dll4, Notch4, and Notch1 expression in Day 1.75 BL-CFC gated to the four c-kit/CD41 populations show heterogenous expression of these arterial-enriched markers in the c-kit+CD41- HE population (red box).

Cadh, further confirming the endothelial identity of this HE population, in correspondence with the Tie2^{hi} HE defined by Lancrin et al.[99] As the cells acquire CD41 (Population 3), there is an irreversible commitment to hematopoiesis (Figure 5e), marked by a massive reduction of the endothelial marker VE-Cadh, with only 15.3% of the cells in Population 3 expressing VE-Cadh. VE-Cadh is further reduced in the mature hematopoietic CD41 single positive populations, representing 13.8% and 7.5% in Populations 4 and 5; respectively.

From this analysis, we define the HE as a c-kit^{+}CD41^{-}VE-Cadh^{+} cell population. This population is most abundant (by absolute numbers) at Day 1.75-Day 2.0 of BL-CFC differentiation, when 20-30% of the live cells fall into this HE gate (Population 2; Figure 5c). This percentage is higher than the 10% Tie2^{+}c-kit^{+}CD41^{-} HE cell population reported by Lancrin et al.[99] This difference may be due in part to our use of purified kit ligand (KL), the ligand for c-kit, instead of the KL-containing conditioned media used by Lancrin et al.[99]

In summary, the emergence of CD41^{+} hematopoietic cells was dissected from early mesoderm progenitors (Population 1), which first acquire c-kit expression (Population 2; HE hematopoietic "competence") followed by CD41 expression (Population 3; hematopoietic "commitment"), and which subsequently lose c-kit expression (Population 4; hematopoietic maturation) and finally acquire CD41 expression (Population 5). Population 2, c-kit^{+}CD41^{-}VE-Cadh^{+}, is the HE population that correlates with the previously published HE population by Lancrin et al.[99] The model of the HE emergence and transition is summarized in Figure 6a, and will be used as the framework for all subsequent analyses of arterial marker expression.

3.1.2 EfnB2 is Temporally Expressed During the Hemogenic Endothelial Transition

Once the HE compartment was defined as c-kit^{+}CD41^{-}VE-Cadh^{+} in this culture, we examined whether this HE population expressed various "arterial" markers, including Dll4, Notch1, Notch4, EfnB2, Neuropilin1, and CXCR4, with a focus on EfnB2.

To gain an overall assessment of the expression kinetics of EfnB2, its expression across differentiation from unsorted EB and BL-CFC cultures was analyzed and compared to expression of VE-Cadh and CD41 (Figure 5b). EfnB2 (blue line) was expressed at low levels during EB differentiation when VE-Cadh and CD41 were both undetectable, but levels increased drastically upon BL-CFC re-plating. Interestingly, the expression of EfnB2 peaked one day before the endothelial marker VE-Cadh in BL-CFC culture. After its peak at Day 1, EfnB2 expression declined throughout the rest of differentiation, as hematopoietic commitment (CD41 expression) was induced.

For a more detailed analysis, surface expression of EfnB2 in relation to the different c-kit/CD41 populations was analyzed by flow cytometry. The four blue histograms in Figure 6c represent the percent cells that express EfnB2 in the four different c-kit/CD41 gates. In this analysis, EfnB2 is highly expressed in the double negative population (Population 1; early mesodermal precursor). Indeed, 70.8% of the double negative cells express EfnB2, a higher percentage than that of VE-Cadh^{+} cells (57.1%), corroborating the early gene expression kinetics of EfnB2 (Figure 5b).

Interestingly, 96.7% of the HE population (c-kit+CD41+) expressed EfnB2. Subsequently, surface expression of EfnB2 (similar to that of VE-Cadh) is massively reduced once CD41

expression is acquired, decreasing from 49.3% in Population 3 to 24.2% in Population 4. However, this reduction of EfnB2 is lower than the reduction of VE-Cadh.

The observation that 96% of the HE population (Population 2) expresses EfnB2, which a putative marker for arterial endothelium, suggests the possibility that the HE is arterial. These results also raise the possibility for a functional role for EfnB2, as it is expressed quite homogenously in the HE population and is dynamically regulated during differentiation. The downregulation of EfnB2 upon hematopoietic commitment suggests that expression of this arterial-related gene precedes hematopoiesis.

3.1.3 EfnB2 Expression in the Hemogenic Endothelium Correlates with Other Putative Markers for Arterial Endothelium

There is no generally accepted definition for what an arterial endothelium is outside of an anatomically preserved vascular system. EfnB2 has been considered a classic "arterial" marker based on its expression and functional requirement in vivo. We therefore consider "arterial" gene expression to signify expression of EfnB2 in addition to broad expression of a panel of different arterial-enriched markers and transcriptions factors. To this end, Day 1.75 BL-CFC cultures (at the peak of HE in BL-CFC differentiation) were stained with these arterial-enriched markers, and compared to EfnB2 expression (Figure 5d). These plots generally show co-expression of EfnB2 with the other arterial-enriched markers Dll4, Notch1, and Notch4; however, in general EfnB2 was the more broadly expressed marker. Other markers tested included CXCR4, which was not detected, and Neuropilin 1, which was very broadly expressed (data not shown).

To establish the kinetics of expression of the different arterial-related markers, we measured Dll4, Notch1, and Notch4 in the different compartments delineated by c-

kit/CD41, as previously performed for EfnB2 in Figure 6c. The results are shown in Figure 6d, where the four blue histograms represent the percentage of cells that express the different "arterial" markers in the four different c-kit/CD41 gates.

In this analysis, only 47.5% of the HE population (Population 2) expresses Dll4 (Figure 5i) whereas over 96% of the HE population expresses EfnB2 (Figure 5h). This correlates with the broader staining of EfnB2 in Day 1.75 BL-CFCs in Figure 5d. Therefore, Dll4 expression marks only a subset of the HE population compared to EfnB2, which marks almost the entire HE population. It is possible that Dll4 expression delineates an arterial ($Dll4^{+}$c-$kit^{+}CD41^{-}$VE-$Cadh^{+}$; 47.5%) versus non-arterial ($Dll4^{-}$c-$kit^{+}CD41^{-}$VE-$Cadh^{+}$) HE, and that the $Dll4^{+}$ HE is more enriched for hematopoiesis, but this hypothesis remains to be tested. By analyzing the pattern of Dll4 expression in relation to Populations 1 – 4 defined by c-kit/CD41 staining, it emerges that Dll4 is down regulated similarly to EfnB2 as the cells mature and acquire CD41 expression, with only 7.2% of the c-$kit^{+}CD41^{+}$ (Population 3) and 13.1% of Population 4 cells expressing Dll4. This down regulation of Dll4 upon hematopoietic commitment and maturation is more pronounced than the down regulation of EfnB2.

Notch4 and its ligand Dll4 display almost identical expression patterns (Figure 5d). Notch4 is heterogeneously expressed (52.2%) in the HE compartment (comparably to Dll4, but not EfnB2), and is markedly reduced after CD41 expression is induced (9.3% and 11.4% in Populations 3 and 4 respectively).

We have also examined Notch1 expression in the different c-kit/CD41 hematopoietic compartments. In the c-kit/CD41 double negative cells (Population 1), almost all the HE cells express Notch1 (85.0%), more similarly to EfnB2 than to the other Notch ligands and receptors. While Notch1 is commonly considered an early hematopoietic marker,

CD41^{+} cells display a reduced expression of Notch1 (27.3% and 14.6% in Populations 3 and 4 respectively) in this system. Overall, Notch1 generally marked a wider subset of cells throughout ES differentiation, and unlike Dll4, which was not present in the Flk1^{+} early mesoderm, Notch1 was expressed early, marking about 70% of the cells at the beginning of BL-CFC culture and remaining at 60-70% of total live cells throughout culture, though it was generally expressed more abundantly in the VE-Cadh^{+} endothelial compartment at the time of HE analysis (Day 1.75 BL-CFC; data not shown)

Finally, while we find that the HE population in BL-CFC culture expresses EfnB2, a putative marker of arterial endothelium, EphB4, a putative marker for venous endothelium was also expressed (data not shown). This could suggest that these cells have not yet fully committed to the arterial fate. In zebrafish, EfnB2 and EphB4 are co-expressed by cells of the common precursor vessel, which will ultimately form both the EfnB2^{+} DA and the EphB4^{+} posterior cardinal vein. Thus EphB4 expression can be promiscuous, especially early in arteriovenous specification.[4]

This immunophenotypic analysis of EfnB2 suggests that while it may be arbitrary to select one single marker to establish the arterial identity of the HE, the co-expression of a panel of other “arterial” markers and correlation with EfnB2 more strongly suggest an arterial identity of the HE. This population could delineate a potentially interesting arterial-like HE that more closely re-capitulates the DA.

The results correlating EfnB2 expression to c-kit/CD41 staining also suggest a model in which arterial gene expression preceeds hematopoiesis, as the markers are induced before and down-regulated upon CD41-defined hematopoietic commitment.

3.2 EfnB2 Helps Specify Hematopoietic Cells from the Hemogenic Endothelium

3.2.1 EfnB2 Silencing in BL-CFC Culture Affects Mesoderm but not Hematopoietic Specification

Identification of an EfnB2+ HE population during BL-CFC differentiation prompted experiments to explore the hypothesis that EfnB2 is required for the HE to be hematopoietic. To test this hypothesis, shRNA constructs to knock down EfnB2 were used.

To successfully knock down EfnB2, a pool of two different shRNA constructs (one directed at the coding region and one at the 3' UTR) was used after preliminary trials showed that the two constructs each individually knocked down EfnB2 expression by 30-40% and additively knocked down EfnB2 by around 80% when pooled (data not shown; RNA levels). The shRNA construct that was directed at the coding region was cloned to replace the puromycin selection marker with eGFP, while the 3' UTR construct retained the puromycin selection marker, to make each individual shRNA contain a different selection marker. In all the experiments, eGFP was used as a marker for successfully knocked-down cells, while puromycin was never used owing to the disruptive effects of adding puromycin to the ES cells during differentiation.

Therefore, it is critical to note that when eGFP+ silenced cells are sorted and analyzed in all experiments, it contains cells that have either only the coding region-directed shRNA (eGFP+) or cells that have both coding region and 3' UTR successfully integrated into the genome (eGFP+ and Puromycin-resistant). eGFP- cells (that are sometimes mentioned in the text) likely contain cells where EfnB2 expression is knocked down by the puromycin resistant construct (3' UTR shRNA) but not the coding region construct (eGFP). A pool of

puromycin and eGFP PGK empty constructs (at similar titers) were used for the PGK controls, which were also sorted based only on eGFP expression.

The schematic for the experiment to evaluate the effect of knocking down EfnB2 in BL-CFC differentiation is diagramed in Figure 7a. In this experiment, the ES cells were re-plated into EB suspension culture as single cells with shRNA or PGK control virus in the media. Therefore, EfnB2 was knocked down before mesoderm commitment (infection at Day 0 EB) and Figure 7b shows the efficiency of infection after four days. In Day 4 EBs, 44.5% of the shRNA infected cultures were GFP+, while 82.7% of PGK (empty vector) control infected cultures were GFP+.

In Figure 7b, GFP is plotted against Flk1 expression, and shows that EfnB2 silencing may have a detrimental effect on mesoderm specification. Only 8.3% of GFP+ cells in Day 4 EBs express the mesoderm marker Flk1, which is lower than that observed in the GFP+ PGK infected cells (12.7% Flk1+). This selection against Flk1 expression after EfnB2 knock down is also suggested by the gene expression results in Figure 7c analyzing the expression of EfnB2 in the Flk1+ versus Flk1- cells sorted from Day 4 EBs. First note that EfnB2 expression is always higher in the Flk1+ sorted cells versus the Flk1- sorted cells, suggesting that mesoderm committed cells may up-regulate expression of EfnB2, or that EfnB2 may play a role in mesoderm specification. More important, however, is the finding that the knock down of EfnB2 appears consistently more potent (-72.2%) in the Flk1- population than in the Flk1+ (-40.9%) population.

This finding, combined with the results in Figure 7b showing that there were fewer Flk1+ cells in the shRNA culture, suggest that successful knock down of EfnB2 may hamper mesdoerm specification (Flk1 expression). There is not a full block of mesoderm

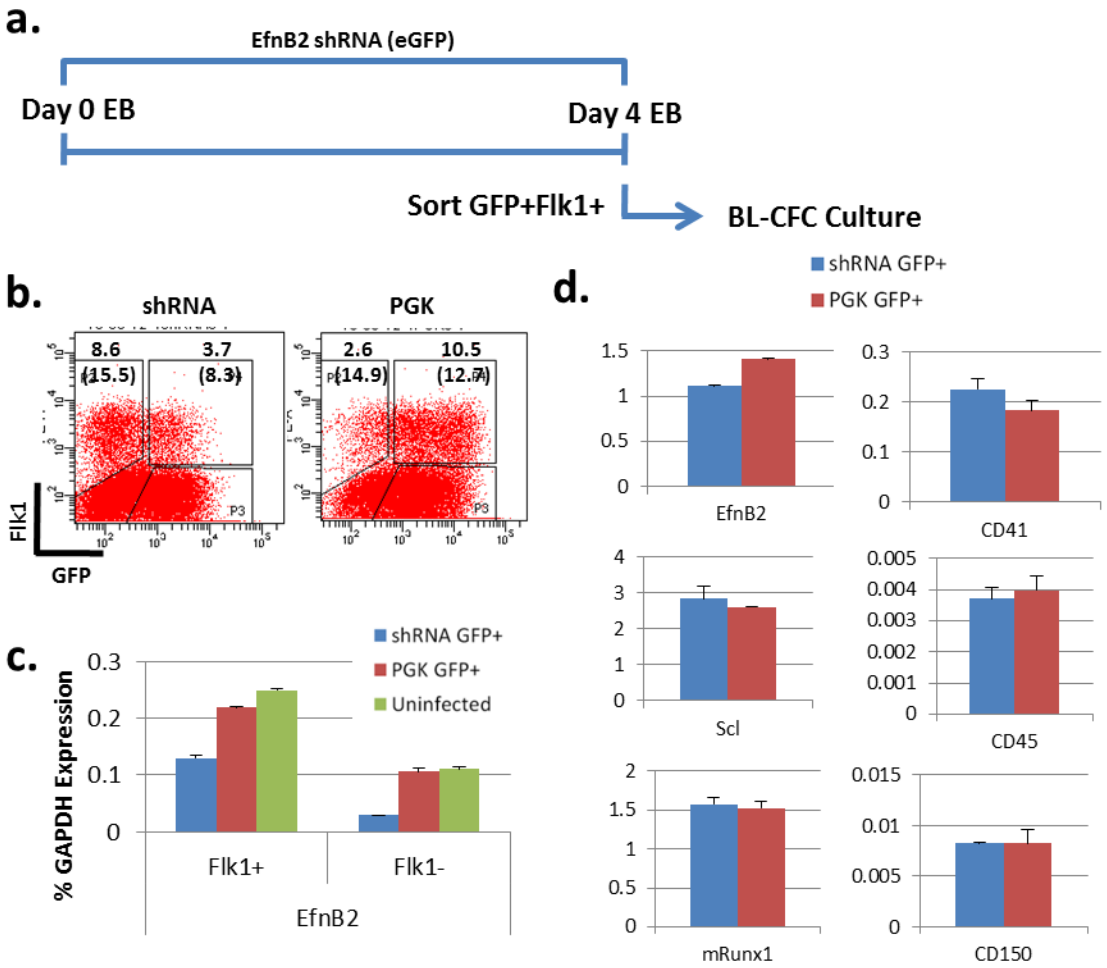

Figure 7. EfnB2 Silencing Affects Mesoderm Commitment but not Hematopoietic Differentiation in BL-CFC Culture

a. Schematic of experiment

b. GFP and Flk1 expression in Day 4 EBs show decreased Flk1+ percentages in the shRNA GFP+ population compared to PGK GFP+ controls. Numbers on the plot show the percentage of total live cells in each gate and numbers in parentheses are the percentages of the GFP- or GFP+ population that are Flk1+.

c. EfnB2 gene expression evaluated by qPCR from Day 4 EBs (GFP+ from shRNA/PGK infected, GFP- cells in uninfected controls) show the level of EfnB2 knock-down after four days of infection. Gene expression is normalized to GAPDH and graphed as Mean + SD.

d. Hematopoietic markers in shRNA/PGK/uninfected Day 4 BL-CFC cultures shows comparable gene expression levels. Gene expression is normalized to GAPDH and graphed as Mean + SD.

specification after EfnB2 knock down, however, as there was still GFP+ shRNA infected cells that were Flk1+ (albeit knocked down at lower levels). Note in this figure that PGK control infected cells and Uninfected control cells express relatively similar levels of EfnB2 (red versus green bars respectively) suggesting that infection did not modulate endogenous EfnB2 expression levels.

As there were sufficient numbers of GFP+Flk1+ cells from the shRNA and PGK Day 4 EB cultures, they were sorted and re-plated into BL-CFC culture (with no additional virus in the BL-CFC media). Morphologically, there was no difference between the shRNA and PGK infected Flk1+ cells re-plated into BL-CFC culture for four days (images not shown). To analyze molecularly if the EfnB2-silenced cells differentiated to hematopoietic cells, RNA was collected from Day 4 BL-CFC culture from both shRNA and PGK infected cultures and evaluated for various endothelial and hematopoietic markers by qPCR. As shown in Figure 7d, all markers including the hematopoietic "master regulators" Scl and Runx1 and the surface markers CD41, CD45, and CD150 were comparable in knock down and control cells. This suggests that while EfnB2 silencing may perturb mesoderm commitment (Flk1 expression; Figure 7b), it does not prevent the subsequent differentiation of residual mesoderm precursors to hematopoietic cells during BL-CFC differentiation.

The experiment was modified slightly to sort for Flk1+ mesoderm cells from uninfected Day 3.5 EBs and re-plated into BL-CFC culture media with shRNA or PGK virus to allow normal hematopoietic differentiation. However, there was also no effect of EfnB2 knockdown in this delayed infection protocol (data not shown).

There are two possible explanations for the failure of EfnB2 knockdown to modulate hematopoiesis in BL-CFC culture. The first explanation is technical, and relates to the

incomplete knock down and potential selection against fully knocked down cells in the shRNA cultures. As shown in Figure 7c, the cells that specify to mesoderm and re-plated into the BL-CFC cultures were less knocked down for EfnB2 than the cells that were Flk1-. Therefore, Flk1 selection for re-plating into BL-CFC culture may have selected for cells that had either lower but sufficient levels of EfnB2 to differentiate properly or for those cells that had successfully overcome the knock down of EfnB2 by unidentified mechanisms.

Another explanation could be biological and related to the culture conditions used. BL-CFC culture supports relatively early hematopoietic development from mesoderm (Flk1+) rather than from a further specified HE. This is consistent with the observation that EfnB2-/- mice form primitive and early waves of blood (albeit at lower levels than littermates as evidenced by their pale YS).[10] If so, it could be that the results reflect a relatively early hematopoietic wave of differentiation that does not require EfnB2. All together, these results suggest that EfnB2 plays an early role in mesdoerm specification but no role in the commitment to early hematopoietic cells.

3.2.2 EfnB2 Silencing After Mesoderm Commitment Does not Affect Endothelial Commitment

Given that knockdown of EfnB2 had an effect on mesoderm specification but not early hematopoietic differentiation in BL-CFC culture, a different strategy was designed so that it was possible to (1) knock down EfnB2 after mesoderm specification and (2) evaluate later "definitive-like" hematopoietic differentiation. Others in the literature have plated Day 6 EBs (which have already specified to early endothelial or hematopoietic phenotypes by VE-Cadh and CD41 expression) into more definitive-like hematopoietic culture conditions that were designed to support HSCs from adult bone marrow.[98]

One such assay involves co-culture of the sorted Day 6 EBs with OP9 stromal cells derived from mouse bone marrow. In this culture, differentiated ES cells form an adherent monolayer of endothelial colonies that give rise to non-adherent hematopoietic cells in the liquid medium above, similar to the "budding" of HSCs from the ventral DA into the lumen of the vessel. The other re-plating assay is similar to the methylcellulose based BL-CFC culture used previously, but with a media commercially known as MethoCult3434, which contains more definitive cytokines that support the differentiation of human CD34+ HPC/HSCs from bone marrow rather than the cytokines in BL-CFC media that support very early hematopoietic differentiation from the hemangioblast.

It was hypothesized that re-plating Day 6 EBs into these cultures would not only give a readout of hematopoietic specification of a more "mature" HE compared to BL-CFC culture from Day 3.5 EBs, but that this experimental setup would also extend the window of time for the shRNA/PGK virus to knock down EfnB2. To increase the efficiency of knockdown later in EB differentiation when the EBs were already formed, it was necessary to address diffusion kinetics and to derive a strategy to get the virus to infect all cells and not just the ones on the surface of the EBs most accessible to the virus. Therefore, the protocol was modified to dissociate the EBs at Day 3.5 – 4, add the virus to the dissociated cells and re-aggregate them at high densities (starting from single cells) in the presence of the virus until Day 6 similarly to protocols generating second wave Flk1+ cells.[132] It was hoped that infecting the EBs in this re-aggregation step between Day 3.5 and Day 6 of EB differentiation would allow both normal mesoderm specification and higher infection efficiency.

To evaluate this strategy of differentiation and infection, expression of EfnB2, VE-Cadh, and CD41 was monitored through EB differentiation and graphed in Figure 8a. Encouragingly,

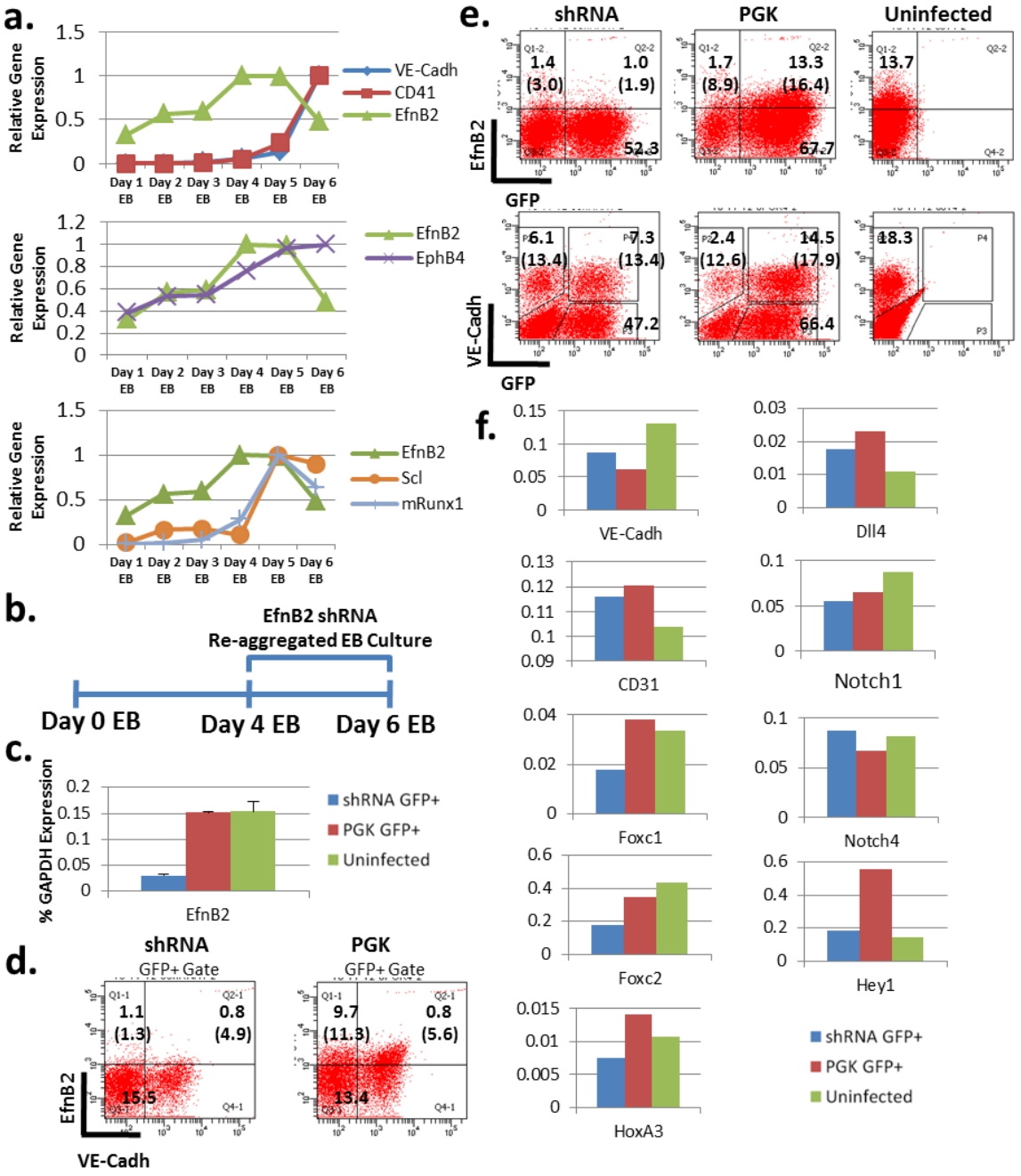

Figure 8. EfnB2 Silencing After Mesoderm Commitment Does Not Affect Endothelial Commitment but Perturbs "Arterial" Gene Expression

a. Gene expression graphed across EB differentiation shows the kinetics of EfnB2 expression against endothelial and hematopoietic markers. EBs were trypsinized and re-aggregated at Day 3.5. Each gene was normalized to GAPDH and then normalized over all timepoints to make the maximum expression equal 1.

b. Schematic of experiment

c. EfnB2 gene expression evaluated by qPCR from Day 6 EBs shows the level of EfnB2 knock-down after 48 hours of infection. Gene expression is normalized to GAPDH and graphed as Mean + SD.

d. EfnB2 staining in Day 6 EBs (gated on GFP+ cells) shows the level of protein knock-down.

e. EfnB2 and VE-Cadh staining in Day 6 EBs plotted against GFP shows the effect of EfnB2 knock-down on VE-Cadh expression . Numbers on the plot show the percentage of total live cells in each gate and numbers in parentheses are the percentages of Marker+ population that are GFP+ or GFP-.

f. Gene expression of arterial-enriched markers in shRNA/PGK/uninfected cultures from Day 6 EBs.

both endothelial and hematopoietic differentiation proceeded as expected, with VE-Cadh and CD41 expression increasing at Day 4 EB differentiation (12 hours after re-aggregation). The kinetics of hematopoietic specification was also confirmed by analysis of the master regulators Scl and Runx1, whose expression increased rapidly between Day 4 and 5 of EB differentiation before decreasing at Day 6.

Almost coincidentally was the pattern of EfnB2 expression which picked up the most rapidly between Day 3 and Day 4 of EB differentiation before stabilizing and decreasing slightly before Day 6 (Figure 8a). This pattern supported the proposed experimental setup of knocking down EfnB2 between Day 4 and 6 of EB differentiation, as this period coincides with the peak of EfnB2 expression. As an additional note, the pattern of EfnB2 expression was almost perfectly mirrored by the expression of its receptor (and the classical venous marker) EphB4, with the only difference being the persistence of EphB4 expression into Day 6.

Therefore, the expression kinetics of CD41, VE-Cadh, and EfnB2 across the six days of EB differentiation with a re-aggregation step at Day 4 presented an opportunity to knockdown EfnB2 efficiently after mesoderm specification (but before endothelial or hematopoietic specification) and to analyze its effects on more definitive hematopoiesis. The schematic for the proposed experiment is diagramed in Figure 8b. In this experiment, the shRNA and PGK virus are added to the media at Day 4 when the EBs are re-aggregated.

Figure 8e shows that the efficiency of infection (measured by the percent of GFP+ cells in shRNA culture) was comparable to the previous protocol of infection when the virus added at Day 0 of EB differentiation (Figure 7b). In this re-aggregation protocol, 53.3% of cells in shRNA infection culture were GFP+ and 80.0% were GFP+ in PGK cultures.

Figure 8c shows that the level of EfnB2 knockdown by qPCR in GFP+ cells sorted from Day 6 EBs re-aggregated in shRNA versus PGK/no virus for 48 hours is 80.0%. Note that PGK and uninfected controls had almost identical levels of EfnB2 expression, suggesting that viral infection did not have an effect on EfnB2 in this re-aggregation culture.

In comparing these results (Figure 8c) to infection at Day 0 of EB differentiation (Figure 7b), note first that the relative level of EfnB2 expression in Day 6 EBs is intermediate between the Flk1+ (0.2) and Flk1- (0.1) cells. Next, note that the level of knockdown in the re-aggregated culture is more comparable to the levels seen in the GFP+Flk1- populations in the previous experiment and more potent than that observed in the GFP+Flk1+ mesoderm (Figure 7c). Therefore, this protocol to knock down EfnB2 after mesoderm specification overcame the issue in the previous experiment that EfnB2 knock down was selecting against mesoderm precursors.

To confirm efficient knock down of EfnB2, its expression was also evaluated at the protein level by antibody staining for flow cytometry (Figure 8e; top row). In this analysis, only 1.9% of GFP+ shRNA infected cells expressed detectable EfnB2 protein compared to 16.4% of GFP+ PGK infected cells and 13.7% GFP- of uninfected cells. This equates to an 80% reduction of cells in the knock down cultures that express detectable staining of the EfnB2 surface protein. The knockdown of EfnB2 was also seen (at lower levels) in the GFP- population in the shRNA cultures, which was expected given that the GFP- shRNA infected cells contained cells that were infected with only the 3' UTR puromycin shRNA construct.

With the successful knockdown of EfnB2 in the Day 6 EBs, the first question was if this knockdown had any effect on endothelial commitment, as EfnB2 is predominantly expressed in VE-Cadh+ endothelial cells. Figure 8e plots VE-Cadh expression against GFP in

Day 6 EBs, 48 hours after infection, and shows that VE-Cadh expression is comparable not only between shRNA GFP+, PGK GFP+ and uninfected GFP- populations (13.4%, 17.9%, and 18.3% respectively) but that it is also comparable between the GFP+ versus GFP- populations in shRNA infected cultures (13.4% both).

To further confirm that successful EfnB2 knock down did not prevent VE-Cadh expression, EfnB2 expression (or lack of EfnB2 expression) was evaluated against VE-Cadh in Figure 8d. It appears that there is a slight increase in the number of VE-Cadh+ cells that express EfnB2 (4.9%) compared to VE-Cadh- cells (1.3%) in the shRNA cultures, and these populations were both reduced in comparison to PGK infected cultures. Therefore, EfnB2 silencing was still slightly more successful in the VE-Cadh- population than in the VE-Cadh+ population, but there was no block in endothelial commitment in the EfnB2- population in the shRNA cultures.

Figure 8d also confirms the earlier finding that EfnB2 expression occurs early relative to endothelial commitment. During BL-CFC differentiation, EfnB2 was expressed in c-kit/CD41 double negative mesoderm that were not yet endothelial (Population1; Figure 6c). Here in Figure 8d, there is a distinct non-EC (VE-Cadh-) population in both shRNA and PGK (though lower in shRNA cultures) that expresses EfnB2 (1.3% in shRNA and 11.3% in PGK). It is not known if these VE-Cadh-EfnB2+ are the Population 1 mesoderm and presumed precursors to the HE.

The expression of a panel of endothelial and hematopoietic markers in Day 6 EBs after shRNA/PGK/no infection were analyzed by qPCR (Figure 8f). The results in this figure are from only one experiment, however, they will confirmed in further experiments re-plating VE-Cadh+ GFP+ cells (versus total GFP+ in this experiment) in Figure 14. Here, within the

GFP+ cells, the gene expression of endothelial markers VE-Cadh and CD31 were similar between shRNA and PGK. However, GFP+ shRNA cells expressed lower levels of the arterial-associated transcription factors Foxc1, Foxc2, HoxA3, and Hey1 (though Hey1 seems elevated compared to uninfected controls). Sox17 expression levels were similar in all cultures. Expression of the arterial-enriched surface markers Dll4 and Notch1 were similar, if slightly lower in shRNA compared to PGK controls (though with elevated Dll4 levels in the PGK compared to uninfected controls). Therefore, EfnB2 silenced endothelium appears to express lower levels of the arterial-enriched transcription factors but similar levels of Dll4 and Notch1 mRNA.

All together, these results suggest that EfnB2 silencing after mesoderm commitment does not affect endothelial commitment but perturbs "arterial" gene expression. This result correlates with the characterization of the EfnB2-/- mice in which the primary vasculature is normal up to E9.5 showing no defect in vaculogenesis, but which show subsequent defects in arterial gene expression and angiogenic remodeling of the existing embryonic vasculature.[10, 49]

3.2.3 EfnB2 Silencing Affects Only Late Hematopoietic Development but not Early Specification

Since it is established in the literature that an endothelial intermediate is necessary for definitive hematopoietic differentiation (see Introduction), it was encouraging that the silencing of EfnB2 did not impact the specification of endothelium from the mesoderm. Indeed, if endothelial commitment had been detrimentally affected by EfnB2 knock down, any subsequent hematopoietic defects could be attributed to this earlier endothelial defect and not to a specific hematopoietic defect. However, because the endothelial program was

not affected by EfnB2 knock down, the next question was to evaluate if this enodthelium silenced for EfnB2 is hematopoietic.

Based on the gene expression profile shown in Figure 8a, the hematopoietic regulators and markers Scl, Runx1, and CD41 begin to rapidly increase between Day 4 and Day 6 of EB differentiation. This suggested that it was possible to analyze the effect of EfnB2 knock down on hematopoiesis in as early as Day 6 EBs, 48 hours after infection.

The early hematopoietic markers c-kit and CD41 were analyzed in Day 6 EBs by flow cytometry (Figure 9b). In this analysis, it is evident that the early wave of c-kit/CD41 expression during EB differentiation is unaffected by EfnB2 silencing. The percentages of all c-kit/CD41 populations were similar across all conditions (shRNA, PGK, and uninfected).

There could be two explanations for this. First, the silencing of EfnB2 could have been too late to block this early wave of hematopoiesis, which may have been primed before EfnB2 silencing was enforced. This will be tested later in experiments presented in Figure 11. The second explanation could be that this wave was not affected because it was derived directly from a hemangioblast and not HE intermediate. This is supported by the kinetics of VE-Cadh and CD41 expression in Figure 8a where VE-Cadh and CD41 expression appear almost synchronously (indeed VE-Cadh protein is not detectable by flow cytometry at Day 4 EB differentiation; data not shown), suggesting that it is unlikely the CD41 expression observed in Day 4 – 6 EBs is derived from a VE-Cadh+ endothelium. This result also agrees with the results of knocking down EfnB2 in BL-CFC culture where EfnB2 had no effect on hematopoietic commitment from the Flk1+ mesoderm (Figure 7).

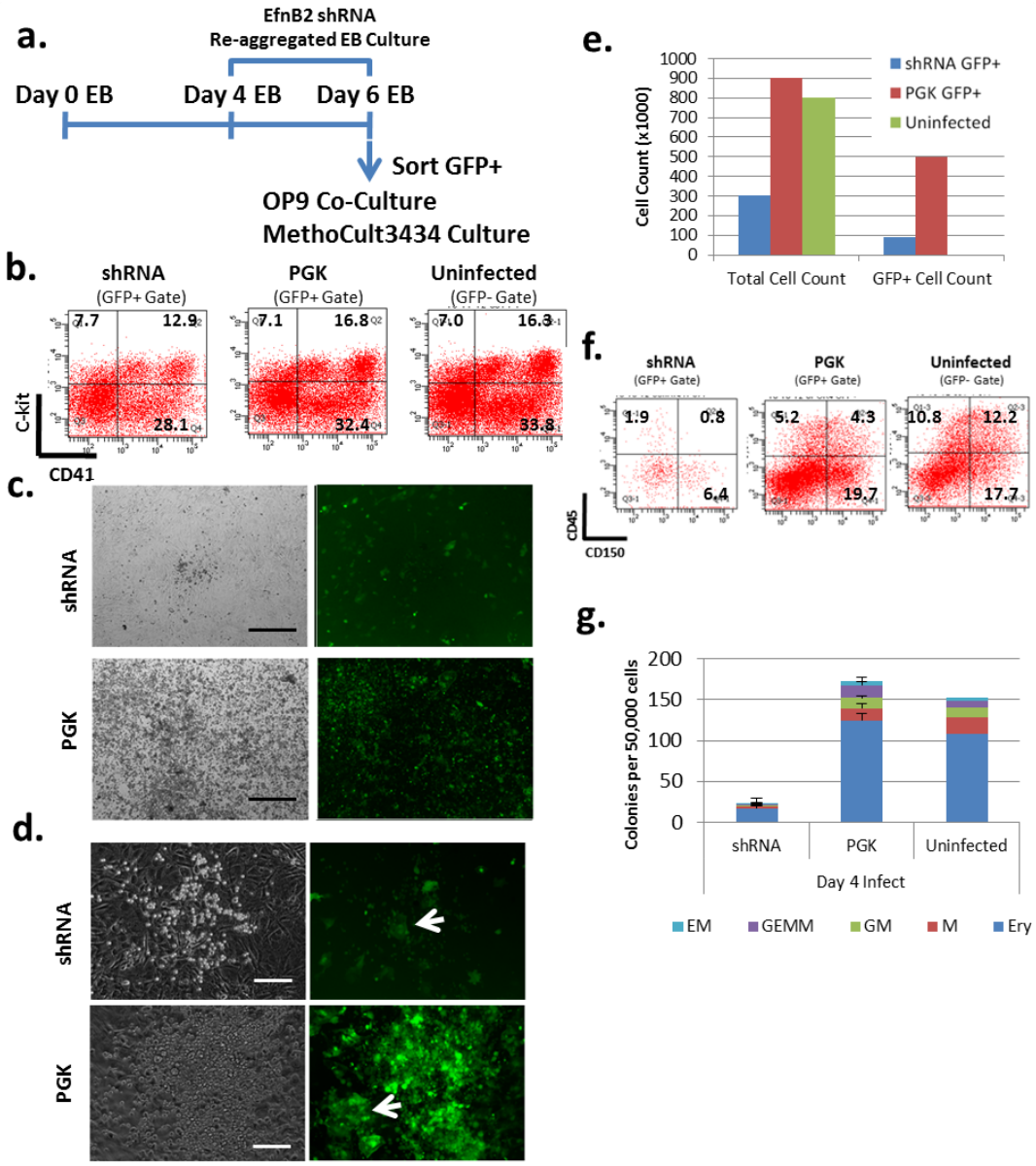

Figure 9. EfnB2 Silencing After Mesoderm Commitment Affects Only Late Hematopoietic Development but not Early Specification

a. Schematic of experiment
b. c-kit/CD41 staining in Day 6 EBs re-aggregated with shRNA/PGK virus for 48 hours do not show perturbed expression of these early hematopoietic markers.
c. Brightfield and GFP images of Day 5 OP9 co-cultures show fewer hematopoietic colonies in the shRNA cultures compared to PGK control. Scalebar: 500 uM
d. Higher magnification images of Day 5 OP9 co-cultures show adherent GFP+ endothelial colonies (white arrows) in both shRNA and PGK cultures. Scalebar: 200 uM
e. Total number of live cells harvested from Day 5 OP9 co-cultures is lower in shRNA versus PGK/uninfected controls (cell counts from re-plating 50,000 cells).
f. Day 5 OP9 co-cultures infected with shRNA (versus PGK/uninfected controls) show decreased expression of the definitive hematopoietic markers CD45 and CD150.
g. shRNA infected Day 6 EBs form fewer colonies in MethoCult3434. Colonies were scored at Day 9 and graphed as Mean + SD. (Representative of 3 experiments)

To test if later hematopoiesis is defective in the EfnB2 silenced cells, the GFP+ cells were sorted from Day 6 EBs and re-plated into the previously described OP9 and MethoCult3434 cultures. The schematic for this experiment is outlined in Figure 9a. Figure 9c and d show bright field and GFP images taken of the shRNA and PGK infected cells re-plated (without further virus) into OP9 co-culture for 5 days. It is critical to note here that the images of colonies in the shRNA cultures are an over-representation of the colony formation, as the images reflect the few available hematopoietic colonies. Most fields of the shRNA culture plates were empty (data not shown).

In Figure 9c, the dark cells in the brightfield images are the non-adherent hematopoietic cells formed from the infected ES cells, which express GFP (green channel images) compared to the GFP- OP9 stromal cells. As is evident from the images, the PGK infected cells not only form more hematopoietic colonies, but also larger colonies that run into each other. The shRNA cultures form very few hematopoietic colonies and the ones that do form contain fewer cells. Figure 9d includes images of the colonies at higher magnification to show GFP+ adherent cells (presumably endothelial cells; marked by the white arrows in the figures) that are present in both shRNA and PGK cultures. The hematopoietic cells in both cultures also express GFP, confirming that the cells are derived from the infected and sorted Day 6 EBs and not the GFP- OP9. The continued expression of GFP in both cultures also confirm that the silencing and control vectors have integrated into the genome and were stably replicated/expressed, seven days after the initial infection at Day 4 of EB differentiation.

For a more quantitative evaluation of endothelial/hematopoietic differentiation and proliferation in the two cultures, total cell number and the number of GFP+ cells harvested from each of the cultures at Day 5 of OP9 co-culture are graphed in Figure 9e. The “Total

Cell Count" graphed includes OP9 cells present in the co-culture, while the "GFP+ Cell Count" reflects only the ES cells that have maintained GFP expression. The data shows a more than 80% decrease in the number of cells harvested from shRNA versus PGK-infected plates (in the GFP+ count), suggesting that the knockdown of EfnB2 not only prevents hematopoietic differentiation, but also cell expansion and proliferation.

To characterize the hematopoietic cells from both cultures, the cells were harvested at Day 5 and analyzed by flow cytometry for expression of the late hematopoietic markers CD45 and CD150. Expression of both CD45 and CD150 were decreased within the GFP+ cells (CD45: 2.7% versus 9.5% and CD150: 7.2% vs. 24.0%; Figure 9f). The decrease in the absolute numbers of CD45 or CD150 cells is calculated to be 17 and 16 fold, respectively, when corrected for the total number of GFP+ cells collected from the plates (Figure 9e and Figure 9f).

To confirm the hematopoietic defect in a different assay, the GFP+ cells were sorted from Day 6 EBs, re-plated as single cells into the MethoCult3434, and allowed to form hematopoietic colonies in the presence of definitive cytokines before scoring at Day 9. The results are graphed in Figure 9g and show that the silencing of EfnB2, but not PGK control infection, reduces the number of colonies formed in all definitive hematopoietic lineages (EM, GEMM, GM, M and E).

Therefore, in two distinct definitive hematopoietic re-plating assays (OP9 co-culture and MethoCult3434), it was shown that EfnB2 deficiency disrupts late hematopoietic differentiation and potentially also proliferation. Since knock down of EfnB2 did not affect early hematopoietic commitment in either BL-CFC culture or EB differentiation, nor did it

affect earlier endothelial commitment from the mesoderm, it can be concluded that EfnB2 plays a time-restricted and cell-specific role in control of hematopoiesis from ES cells.

3.2.4 EfnB2 Silencing Does not Affect Endothelial Re-Plating or Cell Survival

From the images in Figure 9d, it is clear that while there are decreased numbers of hematopoietic cells in the OP9 co-cultures at Day 5, there were adherent GFP+ colonies that were presumably endothelial based on morphology. To confirm the endothelial identity of these adherent cells, the cultures were stained with VE-Cadh and CD41. The images in Figure 10a show that GFP+ adherent colonies express the endothelial marker VE-Cadh. These VE-Cadh+ colonies are present in the shRNA cultures as well. Note that the GFP expression is lower, but still detectable, in the shRNA cultures compared to PGK control. The non-adherent cells were confirmed as hematopoietic based on the CD41 staining in these images, which is not present in the shRNA cultures. Note that there is no co-expression of the endothelial marker VE-Cadh and the hematopoietic marker CD41 in control cultures. This is in line with the current use of fluorescent reporters under the control of VE-Cadh and CD41 regulatory elements to track the EHT from HE to hematopoietic cells (reviewed by Gordon Keller).[154, 155]

These results confirm that endothelial colonies form upon re-plating of both PGK control and shRNA infected cells sorted from Day 6 EBs, indicating that endothelial colony formation is not affected by EfnB2 knock down. The number and frequency of VE-Cadh+ endothelial cells was not quantified here, but in later confirmatory experiments (Figure 12d).

Based on cell counts from Day 5 OP9 co-cultures in Figure 9e in which there were much fewer cells in the shRNA cultures compared to control PGK, it was necessary to determine if this was due to a lack of differentiation/proliferation or cell death caused by the EfnB2

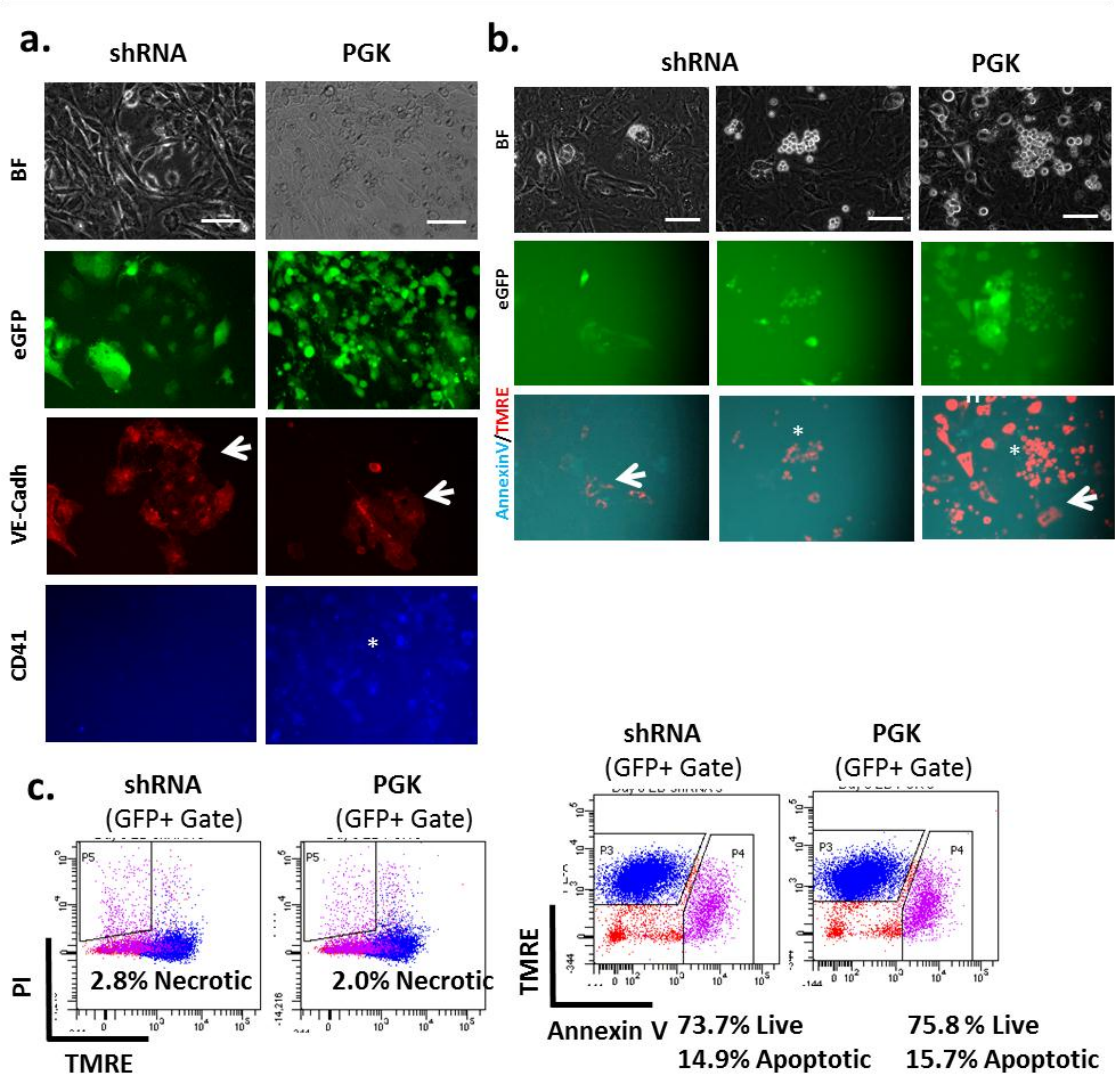

Figure 10. EfnB2 Silencing After Mesoderm Specification Does not Affect Endothelial Colony Re-Plating or Cell Survival

a. Day 5 OP9 co-cultures show normal VE-Cadh+ endothelial colony formation (white arrows) but no CD41+ hematopoietic cells (white asterisk) in shRNA versus PGK control infected cultures. (Representative of 3 experiments). Scalebar: 100 uM

b. Day 5 OP9 co-cultures show TMRE+Annexin- live GFP+ endothelial (white arrows) and hematopoieic (white asterisks) cells in shRNA and PGK infected cultures. Scalebar: 100 uM

c. Day 6 EBs (72 hours after shRNA/PGK infection in re-aggregation culture) stained with TMRE (live cells), Annexin V (apoptotic cells) and PI (necrotic cells) show comparable levels of viability/death. (n = 1)

knock-down. To test this, Day 5 OP9 co-cultures were stained with TMRE (tetramethylrhodamine ethyl ester perchlorate) and Annexin V. TMRE is a cell permeable cationic dye that is readily sequestered by active mitochondria and that stains live cells red.[156] Annexin V was used to stain apoptotic cells. From the images in Figure 10b, it is evident that both endothelial cells (marked by the white arrow) and hematopoietic cells (marked by the white asterisk) in the shRNA cultures are live, just as they are in the PGK cultures. The number of AnnexinV+ dead cells was expected to be low and thus not observably different between shRNA versus PGK-infected ECs, but the critical observation here is the confirmation that the shRNA-infected ECs present in these cultures are not dead, albeit non-hemogenic.

It is interesting that there is a built-in "dead cell" control in these cultures as the OP9 cells (which form a confluent monolayer of cells in all the fields of view) that are GFP- are actually also TMRE-. While the OP9 cells were not assumed to be dead, by TMRE staining they were not metabolically active enough to sequester the TMRE. This negative staining confirms some specificity of this dye to marking live cells. It is interesting to note also that all shRNA cells stain less brightly for TMRE compared to the PGK infected cells. It is not known if different levels of TMRE staining correlate directly with different levels of metabolic activity.

There was the possibility that shRNA infection had a cytotoxic effect earlier, immediately after knock-down and that with the Day 6 GFP+ sort, there was selection against the cells that had died upon EfnB2 knock down. Therefore the viability of the cells at Day 6 EBs was evaluated quantitatively by flow cytometric staining with TMRE, AnnexinV, and propidium iodide. As shown in Figure 10c, EfnB2 silencing only had a slight effect on cell viability. 73.5% of the shRNA versus 75.8% of the PGK cells were TMRE+AnnexinV- live cells. 14.9% of

the shRNA were AnnexinV+ apoptotic and 2.8% TMRE-PI+ necrotic compared to 15.7% and 2.0% respectively in the PGK cultures. In conclusion, EfnB2 knock down does not seem to have a significant effect on cell viability early after knockdown.

All together, the TMRE/AnnexinV/PI co-stainings of Day 6 EBs show a slight increase in apoptotic/necrotic cells, but it does not appear that EfnB2 silencing induces immediate and widespread cell death. Also, in sorting the Day 6 EBs, only live cells are re-plated into the OP9 and MethoCult3434 cultures, and therefore any small differences in cell viability are then compensated for after the sort into the re-plating assays. After the re-platings of these live cells from both shRNA and PGK infected cells, it appears that they remain alive and metabolically active in culture by the images taken of the Day 5 OP9 co-cultures. Therefore, cell death cannot explain the decrease in the number of hematopoietic cells from these re-plated cultures between shRNA and PGK infections.

The presence of endothelial colonies in the shRNA cultures despite the absence of hematopoiesis also suggests that indiscriminate cell death cannot explain the effect of knocking down EfnB2 on hematopoiesis, as these endothelial cells must proliferate to form the colonies. Indeed, it shows that EfnB2 knockdown has very specific effects and does not affect all vasculogenic and hematopoietic programs indiscriminately. With no observed effect of EfnB2 knock down on cell survival or endothelial re-plating, it appears that EfnB2 may have a very distinct role in hematopoietic differentiation and/or proliferation.

3.2.5 EfnB2 Silencing Before Mesoderm Specification Re-capitulates the Effect of Later Infection

Given that early hematopoietic commitment marked by c-kit/CD41 expression in Day 6 EBs (48 hours after silencing) was not affected by EfnB2 knockdown while the later

hematopoietic differentiation in OP9 co-culture and MethoCult3434 re-plating was so severely affected (Figure 9), it was possible that knocking down EfnB2 after mesoderm commitment in Day 4 re-aggregated EBs was too late to affect the earliest waves of hematopoiesis that were already primed (based on the detectable expression of Scl, Runx1, and CD41 in Day 4 EBs at the RNA transcript level; Figure 8a). Therefore, Day 0 EBs were infected to see if earlier knock down of EfnB2 would prevent early hematopoietic commitment. The schematic for this experiment is illustrated in Figure 11a. Note that the virus was added at Day 0 EB when the ES cells were single cells, so there was no need to dissociate the EBs at Day 4, and so they were left to differentiate until Day 6 EB without re-aggregation.

The level of knock down after infection beginning at Day 0 of EB differentiation is graphed in Figure 11b. qPCR shows that EfnB2 was knocked down in the shRNA-infected cells by 54.4% compared to PGK, with PGK not significantly affecting EfnB2 expression compared to uninfected controls. The knockdown of EfnB2 was confirmed at the protein level by flow cytometric staining in Figure 11c. In the shRNA cultures, only 2.0% of GFP+ cells expressed detectable EfnB2 while 8.9% expressed EfnB2 in PGK controls. Both samples expressed slightly more EfnB2 in the GFP- population (3.7% in shRNA and 10.0% in PGK).

In agreement with the experiments infecting Day 4 EBs, knocking down EfnB2 beginning at Day 0 of EB differentiation did not affect endothelial specification despite the previously characterized effect of EfnB2 silencing on mesoderm commitment in Day 3.5 EBs (Figure 7b). As shown in Figure 11d, 5.5% of GFP+ cells in the shRNA cultures expressed VE-Cadh compared to 4.9% in the PGK GFP+ population, which suggests an actually slight increase in endothelial commitment. Both shRNA and PGK cultures had almost identical levels of VE-

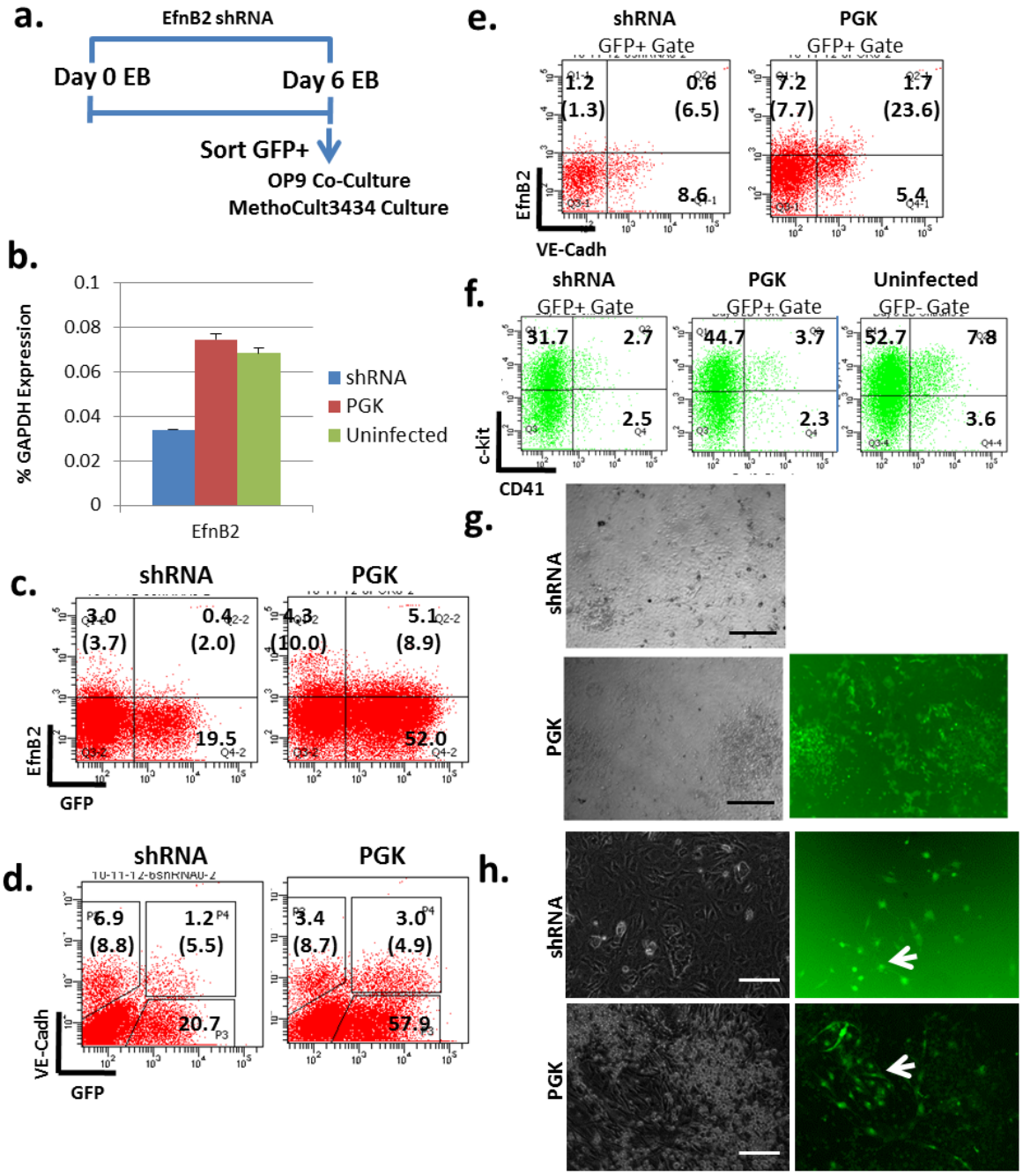

Figure 11. EfnB2 Silencing Before Mesoderm Specification Recapitulates the Effect of Later Infection

a. Schematic of experiment
b. EfnB2 gene expression evaluated by qPCR shows the level of knock-down (Mean + SD; GFP+ cells from shRNA/PGK infected cells; GFP- from uninfected)
c. EfnB2 staining of Day 6 EBs plotted against GFP shows the level of protein knock-down. Numbers on the plot show the percentage of total live cells in each gate and numbers in parentheses are the percentages of the GFP- or GFP+ population that are EfnB2+.
d. VE-Cadh staining of Day 6 EBs plotted against GFP shows no effect of EfnB2 knock-down on VE-Cadh expression.
e. EfnB2/VE-Cadh staining of Day 6 EBs shows fewer EfnB2+VE-Cadh+ endothelial cells in shRNA cultures.
f. c-kit/CD41 staining of Day 6 EBs shows no effect of EfnB2 knockdown on early hematopoietic markers.
g. Brightfield and GFP images of Day 5 OP9 co-cultures show fewer hematopoietic cells in shRNA infected cultures versus PGK controls. Scalebar: 500 uM
h. Higher magnification brightfield and GFP images of Day 5 OP9 co-cultures show presence of GFP+ endothelial colonies (white arrows) in shRNA infected cultures. (n = 1) Scalebar: 200 uM

Cadh expression in the GFP- population (8.8% and 8.7% respectively). Within the shRNA culture, there were fewer VE-Cadh+ cells in the GFP+ versus GFP- population (5.5% versus 8.8%), but this decrease was higher than within the PGK cultures (4.9% versus 8.8%).

Therefore, the knockdown of EfnB2 before mesoderm specification does not have an impact on the subsequent endothelial cell commitment in Day 6 EBs. It is assumed that this was the case because while there was an observable decrease (around 50%) in mesoderm commitment in the previous experiment (Figure 7b), it was not a complete block, and therefore those successfully committed mesoderm were able to continue to specify to endothelial cells normally.

Figure 11e includes plots of EfnB2 staining against VE-Cadh and shows that EfnB2 is more successfully knocked down in the VE-Cadh- compared to the VE-Cadh+ population (6.5% versus 1.3%), but that overall, fewer cells in both compartments express EfnB2 compared to PGK controls (23.6% and 7.7% in GFP+ and GFP- respectively). This result is similar to the previous results showing more successful knockdown of EfnB2 in the Flk1- versus Flk1+ mesoderm. This pattern may suggest that endothelial differentiation also slightly selects against cells successfully knocked down for EfnB2, however, this needs to be further tested.

The analysis of if this earlier knock down of EfnB2 would lead to an early hematopoietic defect in Day 6 EBs is shown in Figure 11f. From this plot, it is evident that early hematopoietic commitment evaluated by c-kit/CD41 staining is comparable between shRNA and PGK control cultures, much like in the cultures silenced for EfnB2 after mesoderm specification. In this experiment, 5.2% of shRNA GFP+ Day 6 EBs express CD41 compared to 6.0% in PGK controls and 10.4% in uninfected controls. Note that these percentages of CD41+ cells are significantly lower compared to the previous experiments, attributable to

the fact that no re-aggregation step was performed. Re-aggregation (at Day 4 EB differentiation) consistently increases the percentage of CD41+ cells, potentially by allowing early, primed hematopoietic cells to aggregate with other primed cells and proliferate/differentiate more quickly given paracrine interactions.

After analyzing the early hematopoietic progression in Day 6 EBs following early knock down, the late hematopoietic re-plating of these cells into OP9 and Methocult3434 culture was also investigated. Figure 11g shows large GFP+ hematopoietic colonies in the PGK cultures and much fewer, smaller colonies in the shRNA cultures. Note that as before, the level of hematopoiesis is over-represented in the shRNA culture images, as they were specifically selected to show the few hematopoietic colonies that were present. Thus infection at Day 0 of EB differentiation recapitulates the effects seen in the experiments infecting at Day 4 of EB differentiation (Figure 9). In the higher magnification images of Figure 11h, GFP+ adherent endothelial colonies can be identified in both shRNA and PGK cultures (highlighted by the white arrows), suggesting that endothelial cell re-plating is intact in the EfnB2 silenced cells, but that there is a defect in hematopoietic differentiation or proliferation.

All together, these results suggest that the timing of EfnB2 silencing does not affect the result that silencing only affects late hematopoietic re-plating but not early hematopoietic specification in the EBs. Despite the lower percentage of CD41+ cells in the Day 6 non-re-aggregated EBs infected at Day 0 of EB differentiation before mesdoerm specification, it was shown that EfnB2 knocked down cells commit normally to early hematopoiesis marked by c-kit/CD41 expression in Day 6 EBs. Therefore EfnB2 seems absolutely dispensable for this early wave of hematopoiesis.

This result is similar to the results in Figure 7d showing the dispensability of EfnB2 expression during BL-CFC differentiation. The lack of effect of EfnB2 knockdown in both of these cultures seems to corroborate each other. Together, they suggest that the mechanisms for early hematopoietic differentiation from Day 4 to Day 6 of EB differentiation and between Day 0 to Day 4 of BL-CFC differentiation are perhaps similar. Note that Day 4 – 6 EB differentiation temporally correlates precisely with Day 0 to Day 2 BL-CFC differentiation.

3.3 Dissecting the Mechanism of EfnB2's Role in Hematopoiesis from the Hemogenic Endothelium

3.3.1 EfnB2 Silencing Makes Putative Hemogenic Endothelium Non-Hemogenic

Given that cell death does not explain the reduction of hematopoietic cells generated by EfnB2 silenced cells re-plated into OP9 or Methocult3434 culture, it was tested if it was instead due to a failure or block in differentiation of the HE into hematopoietic cells. The previous knock down experiments showed that there was normal development of both VE-Cadh+ endothelial and CD41+ hematopoietic cell populations in the Day 6 EBs, 48 hours after shRNA infection (Figure 8e and Figure 9b). The experiments also showed that when total Day 6 EBs (sorted for GFP+) were re-plated into OP9 and Methocult 3434 cultures, there were drastic decreases in hematopoietic output. However, it was not clear if the hematopoietic defect of the EfnB2 silenced cells was in the VE-Cadh+(CD41-) endothelium containing the putative HE population or in the CD41+ early committed hematopoietic cells. Therefore, it was tested if by sorting and re-plating these populations separately, it would be possible to dissect the role of EfnB2 in these two different populations.

Figure 12 shows the results of sorting the VE-Cadh+CD41- endothelial cells from Day 6 shRNA, PGK and uninfected EBs, with the schematic of the experiment laid out in Figure 12a. Based on previous work in the literature (summarized in Sections 1.8.3 and 1.8.4) and the results presented in Figure 9 and Figure 10, these VE-Cadh+CD41- cells are defined to contain the putative HE that gives rise to non-adherent hematopoietic cells, similar to the HE in the embryo. By sorting this population, the presence of this putative HE can be evaluated without the background of hematopoiesis occurring from the already specified CD41+ cells in the Day 6 EBs.

Images of VE-Cadh+CD41- endothelial cells from shRNA and PGK infection re-plated into OP9 co-culture for five days are shown in Figure 12b. In this figure, tiled higher magnification images were compiled showing that there was essentially no hematopoiesis from the EfnB2 knocked down endothelium compared to control infected cultures (uninfected cultures are not shown here because there was no GFP marker to clearly distinguish ES cells from the OP9 stromal cell layer, but they were run as controls in this experiment). Only one sparse hematopoietic colony was visualized in the shRNA culture (marked by the white asterisk), whereas several very large hematopoietic colonies were visualized in the PGK culture. Additionally, all non-adherent elements in the shRNA cultures were dead cells or debris, which was evident in the full size images before they were compressed into this tiled image.

In contrast to the reduction of GFP+ hematopoietic cells in the shRNA cultures on OP9 stroma, many GFP+ adherent endothelial colonies were present in both the shRNA and PGK re-plated cells (one is highlighted by the white arrow in Figure 12b). The endothelial and hematopoietic identity of these colonies is confirmed in Figure 12c where the same plates

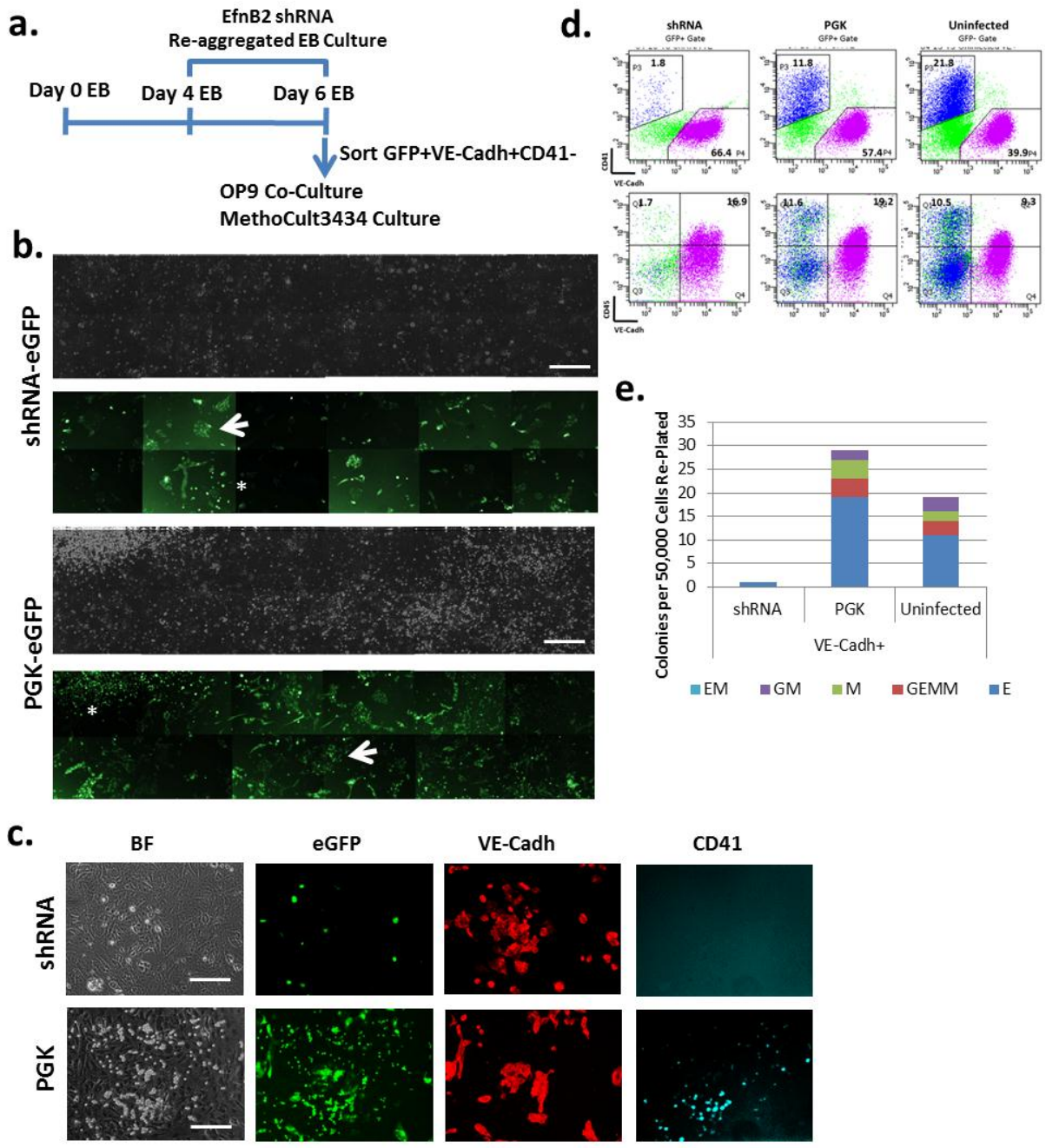

Figure 12. EfnB2 Silencing Renders Putative Hemogenic Endothelium Non-Hemogenic

a. Schematic of experiment
b. Tiled brightfield and GFP images of Day 5 OP9 plates show the presence of adherent GFP+ endothelial colonies (white arrows) but only sparse non-adherent GFP+ hematopoietic cells (white asterisks) in shRNA versus PGK control infected cultures. Scalebar: 500 uM
c. Day 5 OP9 plates stained for VE-Cadh and CD41 show GFP+VE-Cadh+ endothelial colonies but no GFP+CD41+ hematopoietic cells in shRNA versus PGK control infected cultures. (Representative of 3 experiments) Scalebar: 100 uM
d. Day 5 OP9 plates stained for VE-Cadh/CD41/CD45 show comparable VE-Cadh+ percentages but decreased CD41+/CD45+ percentages in shRNA versus PGK/uninfected controls. (n = 1)
e. shRNA infected cells replated into MethoCult3434 (50,000 GFP+VE-Cadh+CD41- Day 6 EBs) gives rise to fewer definitive colonies compared to PGK/uninfected controls. (Representative of 3 experiments)

were stained with VE-Cadh and CD41. Distinct surface staining of VE-Cadh+GFP+ endothelial cells was observed in both shRNA and PGK cultures while CD41 staining was only present in the non-adherent cells in the PGK cultures.

To quantify the hematopoietic defect in the shRNA infected cells compared to the PGK and uninfected controls, the Day 5 OP9 cultures were harvested for flow cytometry and plotted in Figure 12d. It is evident from these plots that endothelial differentiation is not compromised by EfnB2 knock down upon re-plating: 66.4% of the GFP+ cells in the shRNA cultures are VE-Cadh+ compared to 57.4% and 39.9% in the PGK and uninfected (GFP-) controls respectively. Note that it was not possible to gate on GFP expression for the uninfected cultures to mark the ES cells (that were only gated loosely by forward/side scatter), so the percentages of marker+ cells in the uninfected samples will be artificially low, because it does not gate out all the marker- OP9 stromal cells).

Therefore the knockdown of EfnB2 in the endothelial population containing the putative HE does not affect endothelial cell re-plating on OP9 co-culture, similar to the results presented when re-plating whole Day 6 EBs (Figure 9). It is important to note, however, that though the percentages of GFP+ cells expressing VE-Cadh are comparable in the shRNA and PGK cells (66% versus 57%), the absolute number of VE-Cadh+ cells is lower in the shRNA cultures, as assessed by the cell yields from the shRNA cultures (quantified previously in whole EB re-platings in Figure 9e). However, despite lower absolute numbers, the endothelium containing the putative HE silenced for EfnB2 forms endothelial colonies at a relatively comparable frequency based on the percentage of VE-Cadh+ cells in the shRNA re-platings.

In contrast to the similar percentages of VE-Cadh+ cells in the different infection conditions, there is a drastic loss of CD41+ staining in the shRNA re-plated HE, as shown in Figure 12d. Only 1.8% of the GFP+ shRNA cells are CD41+ compared to 11.8% and 21.8% in PGK and uninfected controls respectively. The reduction in absolute number of CD41+ cells is even more drastic, given that there are far fewer GFP+ cells in the shRNA population by Day 5 of OP9 co-culture, despite plating equal numbers beginning at Day 0 of OP9 co-culture. These results provide striking evidence that knock down of EfnB2 renders the endothelium containing the putative HE (VE-Cadh+CD41- cells sorted from Day 6 EBs) non-hemogenic, and that the effect is limited to hematopoietic differentiation and not endothelial differentiation, thereby suggesting that EfnB2 can decouple these two processes.

It is worth noting in Figure 12d that expression of CD41 and VE-Cadh is mutually exclusive at this timepoint, similar to the pattern during BL-CFC differentiation (Figure 6b). This is in contrast with CD41/VE-Cadh staining in Day 6 EBs where there is consistently a distinct VE-Cadh/CD41 double positive population (5-10%; data not shown). This strengthens the idea that hematopoietic differentiation proceeds stepwise through an HE in the OP9 re-platings compared to a more HE-independent mechanism in the EBs. Also it is interesting to ponder what the VE-Cadh/CD41 double negative cells are in these OP9 co-culture, especially given that it is not a rare population (around 30% in both shRNA and PGK cultures; Figure 12d).

The bottom row of plots in Figure 12d show the same cultures stained for the definitive hematopoietic marker CD45. Given that there was such a decrease in the percentage of CD41+ hematopoietic cells in the shRNA cultures, it was surprising that the percentage of CD45+ cells was not as markedly different from PGK controls (though still lower): 18.6% in shRNA compared to 30.8% and 19.8% in PGK and Uninfected respectively. When plotted

against VE-Cadh staining, however, there is a distinct population that co-expresses both VE-Cadh and CD45 in all infection conditions. This VE-Cadh+CD45+ population has been characterized previously in the literature with cells re-plated ex vivo from the embryo as a pre-HSC population.[148]

In this system, it can be hypothesized that the VE-Cadh single positive endothelium goes through this VE-Cadh+CD45+ population co-expressing VE-Cadh and CD45 before maturing into a CD45 single positive hematopoietic population, though this remains to be tested. It is surprising that the VE-Cadh+CD45+ population is relatively intact in the shRNA infected cultures: 16.9% in shRNA cultures compared to 19.2% and 9.3% in the PGK and uninfected controls respectively (Figure 12d). Therefore, not only is the VE-Cadh single positive endothelium comparable by percent positive cells in the shRNA infected cultures, but this endothelium co-expresses CD45. The immunophenotypic identification of this VE-Cadh+CD45+ population in the shRNA culture that contains no non-adherent cells suggests this population is an adherent cell population that morphologically is more endothelial than hematopoietic.

While the VE-Cadh+CD45+ population appears to be intact in the shRNA infected cultures, it appears that there is no maturation from the VE-Cadh/CD45 double positive population to the CD45 single positive hematopoietic population in the shRNA cultures. Only 1.7% of the GFP+ cells in the shRNA cultures are CD45 single positive compared to 11.6% in PGK cultures. Coincidentally, these numbers are almost identical to the percent CD41+ cells in the plots above (1.8% and 11.8% respectively). It can be concluded from these correlations that the mature non-adherent hematopoietic cells expressing CD41 and CD45 but not VE-

Cadh do not form from the EfnB2 knocked down endothelium containing the putative HE (VE-Cadh+CD41-) in OP9 co-culture.

The finding that EfnB2 silencing can render a endothelium containing the putative HE (VE-Cadh+CD41-) non-hemogenic (ie. producing non-adherent CD41+ or CD45-single positive cells) in the OP9 co-culture was confirmed when the same Day 6 EB cells were sorted into MethoCult3434 culture. In this experiment (Figure 12e), only one erythroid colony was formed from the 50,000 putative HE cells re-plated from the Day 6 EBs. This is compared to the colonies that formed in all the different categories (EM, GM, M, GEMM and mostly E) from the PGK and uninfected cultures.

Altogether, these data suggest that shRNA infected endothelium (VE-Cadh+CD41-) can re-plate as endothelial cell colonies, and indeed even acquire mixed VE-Cadh+CD45+ phenotype (reminiscent of the pre-HSC population sorted from embryos[148]) but cannot mature into hematopoietic cells that express CD41 or CD45 and down-regulate VE-Cadh. Therefore, EfnB2 silencing makes the putative HE non-hemogenic.

3.3.2 EfnB2 Silencing Affects Early Committed Hematopoietic Cell Hemogenicity

In addition to sorting the VE-Cadh+CD41- endothelium from infected Day 6 EBs, the early CD41+(VE-Cadh-) hematopoietic progenitors (hypothesized to come directly from a hemangioblast rather than an HE) were also sorted and re-plated into the definitive Methocult3434 cultures, as shown in the schematic in Figure 13a. The question was if these EfnB2 silenced CD41+(VE-Cadh-) early hematopoietic cells would display downstream defects in later hematopoietic differentiation or maturation (Figure 12).

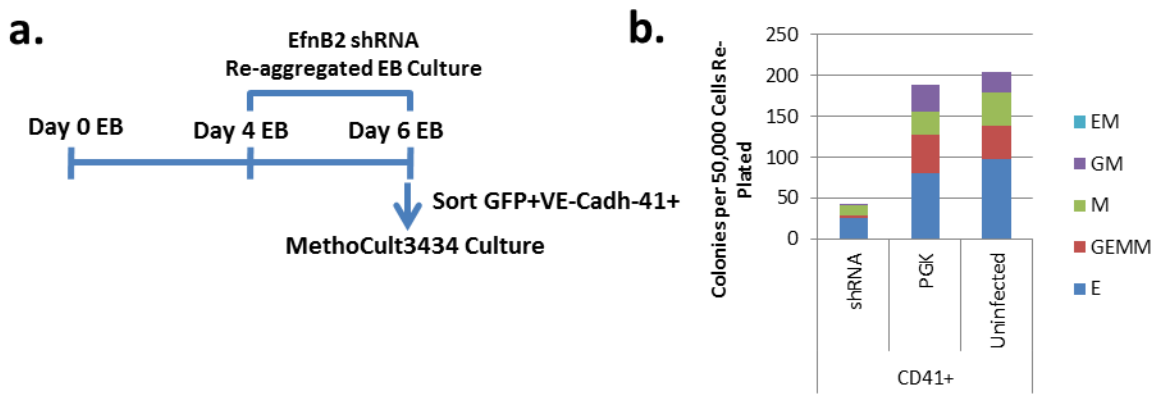

Figure 13. EfnB2 Silencing Affects Hematopoietic Maturation/Expansion from Early Hematopoietic Cells

a. Schematic of experiment

b. shRNA infected cells replated into MethoCult3434 (50,000 GFP+VE-Cadh+CD41- Day 6 EBs) gives rise to fewer definitive colonies after 9 days of culture compared to PGK/unifected controls. (Representative of 3 experiments)

The results from re-plating EfnB2-silenced early committed hematopoietic cells (CD41+VE-Cadh- cells) from Day 6 EBs into Methocult3434 culture strongly support the idea that while these committed hematopoietic cells form at normal frequency relative to controls (by CD41+ percentages in Day 6 EBs), there is a defect in the hemogenicity of these early hematopoietic progenitors that leads to lower clonogenicity and hematopoietic proliferation upon re-plating (Figure 13b).

3.3.3 EfnB2 Silencing Affects the Transcriptional Profiles of Both ECs and Early Committed Hematopoietic Cells

Given the striking effects of EfnB2 knockdown in both the endothelium containing the putative HE and the early committed hematopoietic populations in Day 6 EBs (48 hours after infection), the next question was how EfnB2 could potentially exert these drastic effects during differentiation. To investigate the role of EfnB2 during this process, both endothelium (VE-Cadh+CD41-) and early hematopoietic populations (VE-Cadh-CD41+) were sorted 48 hours after infection (GFP+) or control (GFP-) re-aggregation for gene expression analysis by qPCR, at the time that they were also re-plated into the definitive hematopoietic assays (Figure 12 and Figure 13).

The results are graphed in Figure 14 as fold change over PGK and uninfected controls, with Figure 14a graphing the results of sorted endothelium (VE-Cadh+CD41-) and Figure 14b graphing the results of sorted hematopoietic cells (CD41+VE-Cadh-). The expression level of each gene in each cell population was first normalized to its individual GAPDH expression. After GAPDH normalization, gene expression in the EfnB2 silenced cells was calculated as fold change compared to PGK (blue bars) or uninfected controls (red bars). If control PGK

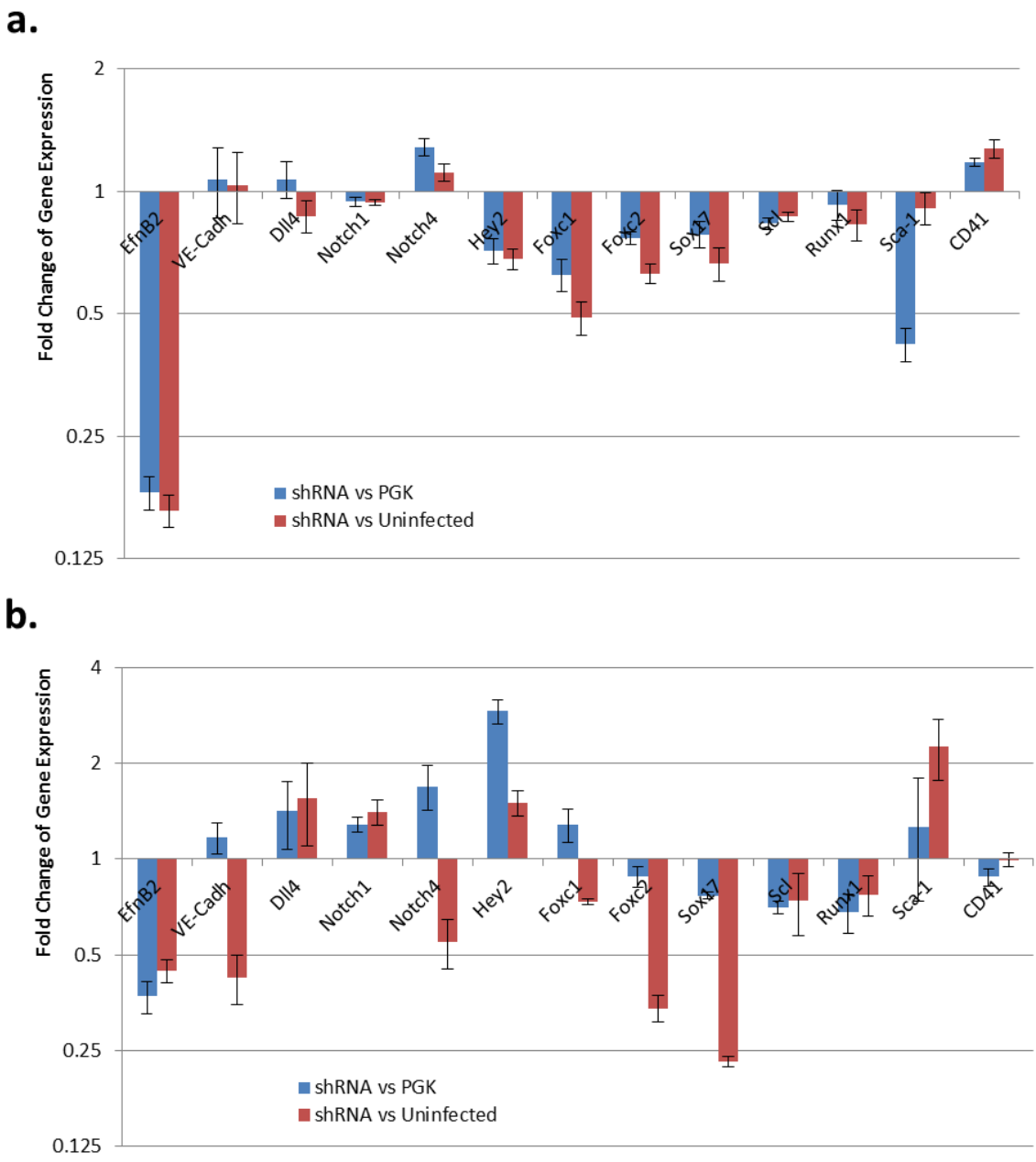

Figure 14. EfnB2 Silencing Affects the Transcriptional Profiles of Endothelial and Early Committed Hematopoietic Cells

a. Fold change of gene expression graphed for GFP+VE-Cadh+CD41- endothelium sorted from Day 6 EBs 48 hours after shRNA/PGK infection. Gene expression was evaluated by qPCR and normalized to GAPDH in each sample before calculating fold change. Blue bars show the fold change of expression comparing shRNA gene expression to PGK control cells. Red bars show fold change of expression comparing shRNA gene expression to uninfected control cells. (Mean +/- Pooled Variance)

b. Fold change of gene expression graphed for GFP+VE-Cadh-CD41+ early committed hematopoietic cells sorted from Day 6 EBs 48 hours after shRNA/PGK infection. (Average of 2 biological replicates (n = 2) with 2 technical replicates each; Mean +/- Pooled Variance)

infection had no effect on gene expression, (ie PGK and uninfected controls behave exactly the same), the blue and red bars should be identical for each gene.

In this graph of fold change, all bars below the x axis indicate reduced expression of that particular gene induced by EfnB2 knock down compared to controls. Bars above the x axis indicate increased gene expression after EfnB2 knock-down. Error bars were calculated as pooled variance, and thus error bars crossing the x axis signify that the detected fold change is not significantly different from control.

In Figure 14a, the fold change of gene expression after EfnB2 knockdown in the Day 6 VE-Cadh+CD41- endothelium is graphed. The first set of bars for EfnB2 expression confirms robust knockdown of EfnB2 expression. In the next bars graphing VE-Cadh fold change, note that there is no significant change in VE-Cadh expression after EfnB2 knock down compared to both PGK and uninfected controls (blue and red bars respectively). This confirms the results of VE-Cadh protein expression showing similar percentages of VE-Cadh+ cells in Day 6 EBs after EfnB2 silencing (Figure 8f). Expression of the arterial-enriched markers Dll4 and Notch1 do not appear significantly affected by EfnB2 knock down in the endothelial population while there is a slight increase in Notch4 expression.

In contrast, there is a consistent decrease in the expression of in all the arterial-associated transcription factors: Hey2, Foxc1, Foxc2, and Sox17. The potent down-regulation of these presumed master regulators of the arterial fate such as Foxc1 and Foxc2 is somewhat surprising, given that EfnB2 is believed to be downstream of these transcription factors. For example, enforced expression of Foxc1 and Foxc2 both induced EfnB2 expression in EC lines.[67] The data here raises the possibility for a reverse feedback loop linking the expression of these transcription factors and EfnB2.

Because these results give only a snapshot of endothelial gene expression profiles after EfnB2 knock-down, the results leave several open questions. First, it is not known if the decreased expression levels of these arterial-enriched transcription factors are due to a failure of gene induction or if EfnB2 is required for the maintenance of expression. It is also not known if the down regulation of these transcription factors will have downstream effects on the expression of the arterial-enriched markers that are not changed at this timepoint. Finally, it is not known if the down regulation of these transcription factors can explain the subsequent hematopoietic defect upon re-plating into OP9 and Methocult3434 culture. The functional results presented here suggest a role for EfnB2 in hematopoiesis from a putative HE, but does not address a potential requirement for other phenotypic markers of arterial endothelium.

Therefore these results cannot address the question if a full arterial gene expression is required for hematopoiesis. Additional epistatic studies need to be performed, including attempts to rescue the hematopoietic defect in EfnB2 silenced putative HE with enforced expression of the arterial-enriched transcriptions factors that have been shown here to be down-regulated after EfnB2 silencing.

Figure 14a also shows down-regulation of both master hematopoietic regulators Scl and Runx1 in the EfnB2 silenced endothelium, suggesting a potential mechanism for the hematopoietic defect caused by EfnB2 knock-down. Scl and Runx1 are potent determinants of the hematopoietic lineage and these results suggest a potential new role for these factors at the step of hematopoietic maturation to the CD45/CD41 single positive non-adherent hematopoietic cells (that are absent in the EfnB2 silenced cultures).

With EfnB2 silencing affecting both arterial-enriched and hematopoietic transcription factors, it remains unclear which factors are critical downstream effectors of EfnB2 in the context of late hematopoietic differentiation from the putative HE. These results do not clarify if EfnB2 acts directly or indirectly through the master regulators Scl and Runx1 to promote hematopoiesis.

The results in Figure 14a show that the definitive hematopoietic surface marker Sca-1 is potently down-regulated in the shRNA cultures compared to PGK controls, while the expression of CD41 was slightly higher in shRNA controls compared to PGK or uninfected controls. This contradictory data on CD41 gene expression is likely attributable to technical reasons since the cells were negatively sorted for CD41 expression; such population should have no detectable CD41 expression that may represent background.

It is noteworthy to mention here that much of the data in this figure recapitulates the qPCR data presented in Figure 8f, which was derived from a separate experiment in which entire sorted GFP+ populations from shRNA and PGK cultures were analyzed. This reinforces the significance and reproducibility of the findings showing that EfnB2 acts upstream of several arterial and hematopoietic master regulators/transcription factors, and that this is a potential mechanism to explain the hematopoietic defect in EfnB2-silenced HE.

Figure 14b also shows the fold change of gene expression after EfnB2 silencing in the Day 6 VE-Cadh-CD41+ early hematopoietic progenitors, sorted at the same time from the cells used in Figure 14a. Again in this figure, the first bars confirm EfnB2 knock-down with a 5.6 fold reduction in EfnB2 by gene expression. VE-Cadh expression appears inconsistent, in that shRNA cells appear to have more VE-Cadh expression than PGK but much less than

uninfected. Since these cells were sorted for VE-Cadh-(CD41+) expression, the results could be from background noise detected by the greater sensitivity of qPCR.

In contrast to the endothelial cells analyzed in Figure 14a, the early hematopoietic VE-Cadh-CD41+ cells knocked down for EfnB2 appear to have increased Dll4, Notch1, Notch4 and Hey2 expression compared to the controls. Given the results in BL-CFC culture in which early committed hematopoietic CD41+ cells potently down-regulate the expression of all "arterial" markers (Figure 7d), these results are not surprising. They suggest that at this timepoint the committed CD41+ cells knocked down for EfnB2 fail to down-regulate the "arterial" markers as drastically as the controls.

Interestingly, in almost direct competition with these notch pathway associated arterial-enriched genes that are up-regulated in the EfnB2 silenced CD41+(VE-Cadh-) early hematopoietic cells, the other non-Notch related arterial-enriched transcription factors Foxc1, Foxc2, and Sox17 are all down-regulated. Indeed, some of these genes, including Foxc2 and Sox17, are down-regulated more profoundly (when compared to uninfected controls) than the expression of EfnB2 upon its own knock-down by shRNA. This interesting contrast suggests that the effect of EfnB2 on the "arterial" gene expression is complex.

In looking at the effect of EfnB2 silencing on the CD41+VE-Cadh- early committed hematopoietic cells on the hematopoietic regulators, the results are very similar to those of the EfnB2-silenced endothelium in Figure 14a. Both Scl and Runx1 are down-regulated, and indeed at levels higher than in the endothelium. This could be because the absolute levels of Scl and Runx1 gene expression are higher in the committed hematopoietic cells of the controls compared to the endothelial HE population, and thus regulation of these genes can be more dynamic. In contrast, the hematopoietic surface markers Sca1 and CD41 are not

significantly changed at the gene expression level in this compartment, which correlates with the previous protein level expression of CD41 not being significantly different in the shRNA infected cells versus the control cells (Figure 9b).

Altogether, the transcriptional profiles of the two different populations in Day 6 EBs (48 hours after infection) elucidate a potential mechanism for the role that EfnB2 has not only on the endothelium (that has become non-hemogenic after EfnB2 silencing based in OP9 and MethoCult3434 re-platings) but also on the decreased hemogenicity of the normally specified early hematopoietic cells. Overall, it appears that EfnB2 knock-down leads to a down-regulation of arterial-associated transcription factors not in the Notch pathway, an up-regulation of those in the Notch pathway, and a consistent down-regulation of the hematopoietic master regulators Scl and Runx1. It still remains to be confirmed whether the role of EfnB2 in hematopoiesis is exerted indirectly through this complex arterial transcription factor network, or directly through these hematopoietic master regulators.

3.3.4 Endogenous EfnB2 Expression Enriches for Endothelial Cells that are Hemogenic in a Tight Time Window

The experiments so far have used the in vitro manipulation of EfnB2 expression to elucidate the role it plays in hematopoiesis. Based on the results suggesting that EfnB2 is required for hematopoietic differentiation, it was interesting to examine if endogenous expression of the protein correlated with levels of commitment to hematopoiesis.

To test this, EfnB2+ and EfnB2- cells were sorted from cultures between Days 4 and 6 of EB differentiation (re-aggregated protocol) into OP9 and MethoCult3434 re-platings. Because VE-Cadh gene expression (Figure 8a) was not detectable until potent up-regulation at Day 5 of EB differentiation, it was not possible to sort for VE-Cadh+ cells (that were EfnB2+ versus

EfnB2-) from Day 4 EBs. Therefore, the data presented in Figure 15 shows results from sorting VE-Cadh- cells based on EfnB2 expression on Day 4 of EB differentiation and sorting VE-Cadh+ cells based on EfnB2 expression on Days 5 and 6 EB when VE-Cadh expression was detectable.

Figure 15a shows a tiled composite of higher magnification images taken at Day 5 of OP9 co-culture for the four different populations sorted by VE-Cadh/EfnB2 expression from Day 5 EBs. First note that both VE-Cadh+ fractions (irregardless of EfnB2 expression; bottom two images) have more hematopoietic colonies than the VE-Cadh- fractions. This supports the claim that the OP9 co-culture supports the differentiation of putative HE.

Within the VE-Cadh- fraction, there does not appear to be a significant difference in the number of hematopoietic colonies from the EfnB2+ versus EfnB2- fractions. However, within the more hematopoietic VE-Cadh+ fractions, it appear that the EfnB2+ re-plated cells formed more colonies. The total number of hematopoietic colonies formed in the four different fractions after OP9 co-culture were counted and graphed in Figure 15b, along with the total colony counts from the populations sorted at the different Days of EB differentiation.

Note from the colony counts in Figure 15b that the Day 4 EBs sorted for just EfnB2 expression (and not VE-Cadh which was not present; data not shown), did not form hematopoietic colonies. This was likely attributable to the fact that the cells sorted from the Day 4 EBs had not matured enough to an endothelial phenotype (no VE-Cadh expression) to re-plate into this definitive hematopoietic assay that supports the maturation of a putative HE.

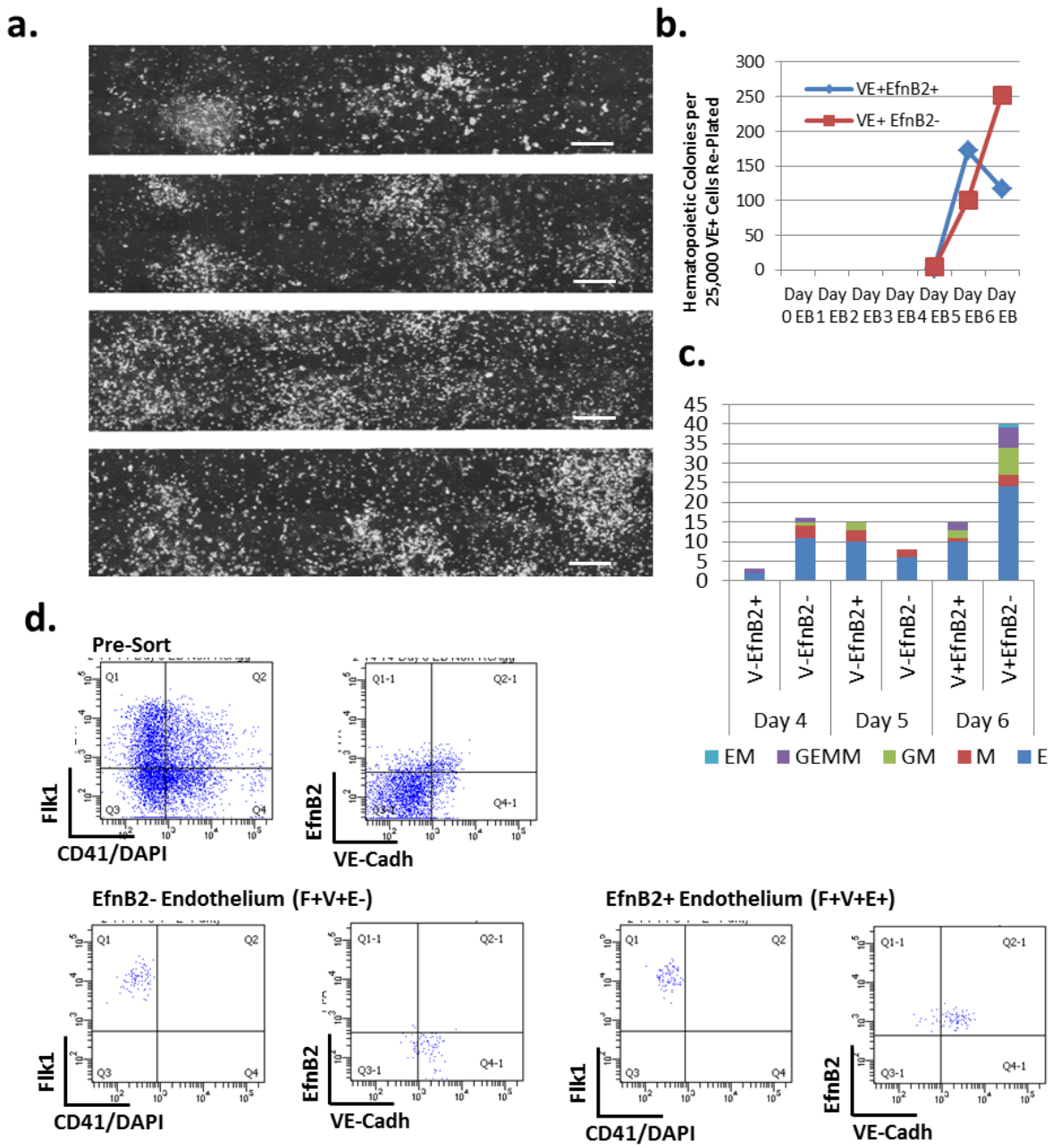

Figure 15. Endogenous EfnB2 Expression Temporarily Enriches for Endothelial Cells that are Hemogenic

a. Tiled brightfield images of Day 5 OP9 co-cultures (of re-plated VE-Cadh/EfnB2 cells sorted Day 5 EBs) show more hematopoietic cells in the EfnB2+ versus EfnB2- population. Scalebar: 500 uM

b. Quantification of the number of hematopoietic colonies in Day 5 OP9 co-culture upon re-plating VE-Cadh/EfnB2 sorted cells from Day 4 – 6 EBs shows temporal enrichment (at Day 5 EB differentiation) of hematopoiesis in the EfnB2+ versus EfnB2- population. (n = 1)

c. Day 10 Methocult3434 colony counts re-plated from Day 4 – 6 EBs sorted by VE-Cadh/EfnB2 expression show similar hematopoietic output trends with OP9 co-culture. (n = 1)

d. Pre-sort and Post-sort purity checks of Flk1/VE-Cadh/EfnB2 expression show efficient separation of EfnB2+ versus EfnB2- endothelium by flow cytometry

In contrast, the hemogenicity of both populations sorted from Day 6 EBs was much higher than at the earlier timepoints, which was expected. Surprisingly, however, was that VE-Cadh+ cells in Day 6 EBs showed more hematopoietic potential in the EfnB2- versus EfnB2+ population, which was the reverse trend of VE-Cadh+ cells from Day 5 EBs.

This surprising reversal of enrichment for hematopoiesis in the EfnB2+ versus EfnB2- populations was re-capitulated in the MethoCult3434 re-plating experiments graphed in Figure 15c. While from VE-Cadh- sorted cells from Day 5 EBs, EfnB2+ cells were more enriched for hematopoiesis than the EfnB2- cells (in this experiment, not enough VE-Cadh+ cells were available, so VE-Cadh- cells were fractionated for Methocult re-plating), the opposite was true when VE-Cadh+ cells from Day 6 EBs were sorted for EfnB2. Note also that in the EfnB2+ population, there was decreased hematopoiesis from the VE-Cadh+ Day 6 EBs compared to the VE-Cadh- Day 5 EBs whereas the opposite was true for the EfnB2- populations sorted from the same timepoints and same VE-Cadh expression.

Figure 15d shows the efficiency and purity of sorting EfnB2+ versus EfnB2- cell populations from the Flk1+VE-Cadh+CD41- endothelial cells from Day 6 EBs. The pre-sort plots in the top row show strong separation of Flk1 and VE-Cadh staining. EfnB2 staining in the pre-sorted population shows poor separation. However, using conservative sort gates, EfnB2+ and EfnB2- populations can be sorted to 95% purity, as shown in this figure.

Therefore, it appears that endogenous EfnB2 expression, in a very tight time window (at Day 5 of EB differentiation), enriches for endothelial cells that are hemogenic. This suggests that EfnB2 expression is needed early to establish or induce the hemogenic potential of the endothelium, but must be down-regulated in order for the proper maturation of the HE.

This view is supported by the results showing the potent down-regulation of EfnB2 upon CD41 induction during BL-CFC differentiation (Figure 6c).

These results raise the possibility that perhaps by Day 6 of EB differentiation, the ECs that express EfnB2 have further committed to a more mature EfnB2+ endothelium that is not hemogenic. It is important to note that in the differentiation of ES cells, there is no temporal or spatial separation as it exists in the embryo, and therefore many developmental processes occur simultaneously and perhaps even asynchronously. Therefore, by Day 6 of EB differentiation, a majority of the cells co-expressing VE-Cadh and EfnB2 may not be a "DA-like" HE, but rather just a more mature arterial endothelium, which upon sorting, dilutes any hemogenic VE-Cadh/EfnB2 double positive cells.

This unexpected result has some technical implications retrospectively for the experiments previously presented, in that perhaps Day 5 EBs would have been a better timepoint to sort endothelium containing the putative HE than from Day 6 EBs, even though the overall hematopoietic potential is much higher in Day 6 VE-Cadh+ cells than in Day 5 VE-Cadh+ cells. This timing issue reflects the challenge of unambiguously correlating in vitro differentiation timepoints with embryonic developmental stages in vivo.

Another significant difference between the experiments analyzing endogenous EfnB2 expression and the genetic experiments manipulating EfnB2 expression was that sorting based on endogenous EfnB2 expression could only be based on one timepoint. Because the expression of EfnB2 was not forced or manipulated, EfnB2- sorted cells did not always remain EfnB2-.

Therefore these experiments differ from the knock down experiments in that it is not known what percentage of the EfnB2- cells sorted had already expressed and subsequently lost, or will express EfnB2 after the sort and re-plating. To answer these questions, other genetic experiments would be necessary such as using a marker gene that would mark any cell that at any point had expressed EfnB2. Such experiments would allow the tracing of all hematopoietic cells to see if they all traced back to an EfnB2+ HE.

Altogether, this experiment suggests that EfnB2 enriches for ECs that are hemogenic within a very tight time window. It also suggests that the expression of EfnB2 must be very tightly regulated endogenously, as EfnB2 time-dependently marks specific populations with distinct hematopoietic potentials.

3.3.5 Administration of EfnB2 Blocking Peptide Re-Capitulates the Effect of EfnB2 Knock-Down

High affinity peptides have been developed through phage display (TNYL-RAW and SNEW) that can interfere with EphB4 and EphrinB2 binding resulting in a block in both forward and reverse signaling.[157]

Given the fact that EfnB2 is a surface molecule that exerts its effects through interactions with its receptor, it is possible to manipulate EfnB2 by administration of peptides that block its binding to its receptor. This tool allows for temporally-controlled and reversible manipulation of EfnB2 signaling to analyze its effects on hematopoiesis. Note, however, that like EfnB2 knock-down, it cannot distinguish between forward and reverse signaling, as it interferes with both reverse and forward signaling in EfnB2-expressing and EphB4-expressing cells (respectively).

As illustrated in Figure 16a, TNYL-RAW blocking peptide was administered at the Day 4 re-aggregation step (at the same time EfnB2 was knocked down with lentivirus previously). Upon re-plating endothelium (VE-Cadh+CD41-) and early hematopoietic cells (CD41+VE-Cadh-) sorted from Day 6 EBs into Methocult3434 culture, the peptide was also added to more closely approximate the knock-down studies which stably knocked down EfnB2 for the duration of the culture.

The results of re-plating the two different populations into Methocult3434 culture (with and without TNYL-RAW blocking peptide) for 9 days is graphed in Figure 16b. First note that in both peptide conditions, the CD41+ fraction gave rise to more hematopoietic colonies than the VE-Cadh+ fraction, similar to the results with the lentiviruses in Figure 12e and Figure 13b. In both CD41+(VE-Cadh-) and VE-Cadh+(CD41-) sorted populations, TNYL-RAW peptide decreased the hematopoietic output of the sorted cells; indeed, the VE-Cadh+(CD41-) population treated with the TNYL-RAW peptide failed to form hematopoietic colonies.

Given that interfering with EfnB2 signaling appears to recapitulate the functional hematopoietic defects of EfnB2 knock-down in Figure 16b, it was interesting to see if blocking peptide treatment affected the transcriptional profiles of the cells, and if these changes were similar to those affected by EfnB2 knock-down. The fold change of gene expression for various arterial-enriched and hematopoietic regulators are graphed in Figure 16. Figure 16c compares TNYL-RAW or SNEW (a different, less potent EfnB2 blocking peptide) treatment with a TNYL-RAW control scrambled peptide while Figure 16d compares TNYL-RAW or SNEW treatment with no peptide control. Overall, it appears that SNEW peptide treatment generally represses gene expression compared to scrambled and no

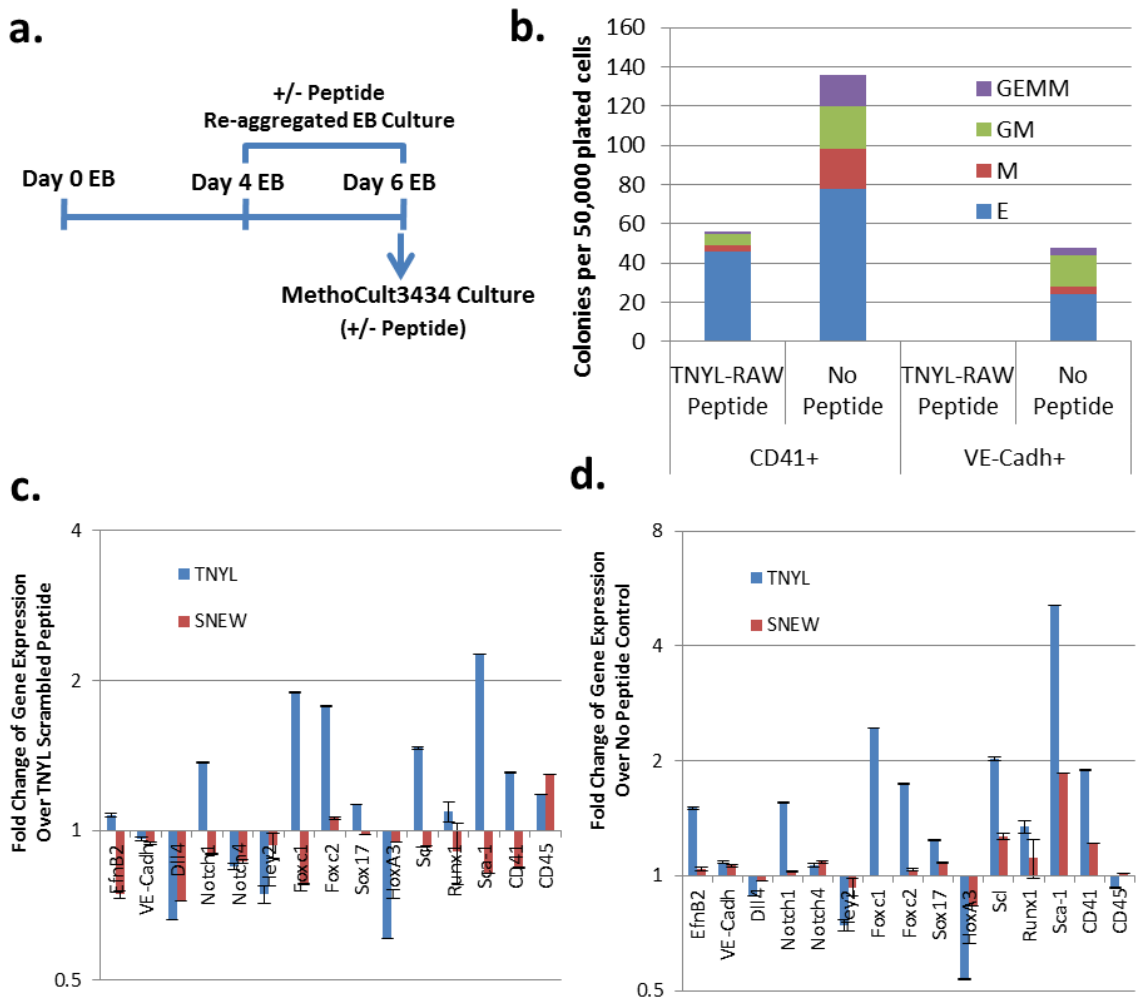

Figure 16. EfnB2 Blocking Peptide Treatment Functionally Recapitulates the Hematopoietic Block but not the Transcriptional Profile Effects of EfnB2 Knock Down

a. Schematic of experiment
b. TNYL-RAW blocking peptide treatment deceases MethoCult3434 colony counts after 9 days of culture. (n = 1)
c. Fold change of gene expression in Day 6 EBs for peptide treated (TNYL-RAW or SNEW; 48 hours) versus TNYL-RAW scrambled peptide-treated cultures. Gene expression is normalized to GAPDH in each sample before calculating fold change. (Mean +/- Pooled Variance)
d. Fold change of gene expression in Day 6 EBs for peptide treated (TNYL-RAW or SNEW; 48 hours) relative to untreated cultures (No Peptide Control). (Mean +/- Pooled Variance; n = 1)

peptide controls, while TNYL-RAW peptide treatment has more dynamic (by absolute value of fold change) effects in both repressive and inductive ways.

Given the many variable effects when looking at the two different blocking peptides against two different controls (scrambled and no peptide), it is easier to comment on trends that are consistent across both peptides compared to both controls. Limited to this analysis, it appears that both TNYL-RAW and SNEW peptides repress expression of Dll4 and Hey2 compared to both scrambled and no peptide controls (more strongly against scrambled than no peptide control). In contrast, Foxc2 expression is induced by both TNYL-RAW and SNEW peptide.

These results differ from the results of EfnB2 knock-down. In those experiments (Figure 14), EfnB2 knock-down led to a down-regulation of arterial-associated transcription factors not in the Notch pathway and an up-regulation of those in the Notch pathway. In the knock-down experiments, there was a consistent repression of the hematopoietic master regulators Scl and Runx1, while in this experiment blocking EfnB2 signaling led to an induction of these two transcription factors. This would suggest a potential Scl/Runx1 independent role of EfnB2 in determining hemogencity of the HE.

Therefore, while abrogating EfnB2 signaling using a blocking peptide (to prevent binding to its receptor) led to functional hematopoietic defects similar to that of knocking-down EfnB2 expression genetically, the transcriptional effects of interfering with EfnB2 signaling were different from those of knock-down. This could be due to temporal differences between manipulating EfnB2 by knock-down versus ligand blocking as well as technical differences between the two techniques. Additional experiments with the peptides would be needed to confirm the results.

3.4 Summary

The results in this chapter provide insight into a potentially important role for EfnB2 during the differentiation of putative HE cells from Day 6 EBs into late "definitive-like" hematopoietic cells. Consistent with EfnB2 expression being tightly controlled throughout development, our results show that endogenous expression of EfnB2 enriches for ECs that are more hemogenic within a very tight time-window of differentiation. Our results also show that the knock-down of EfnB2 impairs the differentiation of putative HE in OP9 co-culture and Methocult3434 colony assays. However, the knock-down of EfnB2 did not impair endothelial commitment and survival, early CD41 expression, endothelial colony formation, and BL-CFC hematopoietic differentiation. Additionally, the knock-down of EfnB2 did not affect the specification to early CD41+ hematopoietic cells, although it decreased the hematopoietic output from these cells. Consistent with these observations, the knockdown of EfnB2 affected the transcriptional profiles of both the endothelium and early hematopoietic cells, linking EfnB2 to several known "arterial" and hematopoietic master transcription factor regulators, suggesting potential mechanisms. The hematopoietic defects were also re-capitulated using blocking peptides that interfered with EfnB2 signaling, suggesting that the role of EfnB2 during hematopoietic differentiation is exerted through its signaling role in the cells.

4 Results Chapter 2: The Role of EfnB2 in the ex vivo Differentiation of the DA Hemogenic Endothelium

4.1 Silencing of EfnB2 ex vivo Recapitulates the in vitro Hematopoietic Defect

To confirm (from the technical standpoint) the hematopoietic defect due to genetic manipulation using shRNA infection during ES differentiation, tissue was dissected from the embryo at the appropriate timepoint during development and re-plated ex vivo with the same knock-down or control shRNA constructs used in vitro. The results of these ex vivo experiments infecting YS and AGM cells from the embryo are shown in Figure 17.

Because EfnB2 expression is detected beginning around E8.25, it was hypothesized that knock-down of EfnB2 would have to occur then, more than two days before the emergence of the HSCs in the DA. Given this relatively early timepoint, it was necessary to add an intermediate step of differentiation/expansion of the AGM explants before re-plating the cells in more stringent hematopoietic differentiation culture.

Figure 17a shows 22 ss (E9.25) YS and AGM dissected and cultured as whole organs in "hanging drop" culture with PGK control lentivirus, imaged 48 hours after dissection and infection. In the low magnification images of the 22 ss YS in hanging drop culture (left column), non-adherent hematopoietic cells can clearly be seen floating around the YS tissue, suggesting that the YS tissue is hematopoietic in this culture. There also appears to be dim GFP expression in the tissue explants at this magnification. However, in the higher magnification zoom of the indicated region (red box; right column), it appears that robust

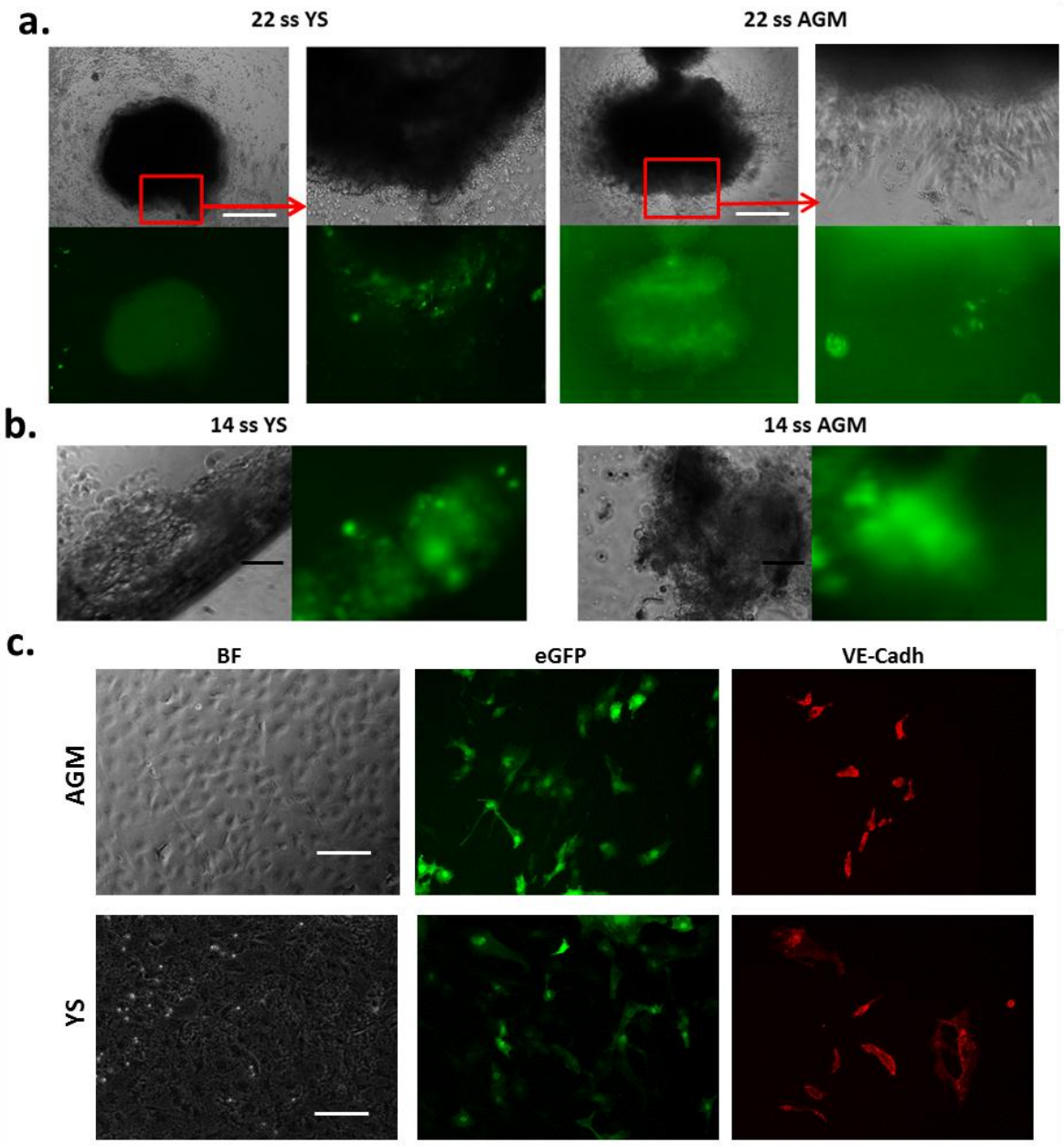

Figure 17. Infection of YS and AGM ex vivo with EfnB2 shRNA Recapitulates the in vitro Hematopoietic Defect

a. YS and AGM from 22 ss embryos cultured as whole organs in hanging drop culture with PGK lentivirus show superficial GFP expression 48 hours post infection. Scalebar: 500 uM

b. YS and AGM from 14 ss embryos dissociated and re-aggregated in hanging drop culture with PGK lentivirus show GFP expression 48 hours post infection. Scalebar: 100 uM

c. YS and AGM from 22 ss embryos re-aggregated with PGK lentivirus were re-plated into OP9 co-culture for 5 days with minimal proliferation. Scalebar: 200 uM

d. YS and AGM from 36 ss embryos split into Methocult3434 cultures with shRNA or PGK lentivirus (0.5 ee for AGM, 0.167 ee for YS) and scored at Day 10 show decreased hematopoietic output with shRNA treatment. (n = 1)

GFP expression is only present in a few cells located at the periphery of the tissue, suggesting that in whole organ culture, there is a lack of tissue penetration for the virus.

The same lack of lentiviral penetration was seen with the AGM organ culture in hanging drop (Figure 17a; right panels). In this culture, endothelial cell sprouting was very apparent from the AGM explant (higher magnification images; right column), however, these cells did not appear to be successfully infected with the PGK virus despite being at the periphery of the organ explant. It is interesting to note that by morphology, the predominant cell type in the YS hanging drop was non-adherent hematopoietic cells while in the AGM hanging drop, it was endothelial cells. This suggests that the earlier waves of hematopoiesis in the YS relative to the later hematopoietic wave (subsequent to endothelial differentiation) in the DA explant were preserved upon ex vivo culture.

Despite the apparent low infection efficiencies in the whole organ cultures with PGK lentivirus, the YS and AGM organs were collected, pooled and trypsinized 48 hours after dissection and infection. The single cell suspensions of infected (GFP unsorted) embryonic tissue were then re-plated with OP9 cells (as was done for the Day 6 putative HE cells in the in vitro experiments). Images from the OP9 co-cultures at Day 5 are included in Figure 17c. In both YS and AGM cultures, adherent GFP+ (control PGK) embryonic cells were seen against the confluent stromal layer of GFP- OP9 cells. These adherent GFP+ YS/AGM cells were also endothelial as they stained for the endothelial marker VE-Cadh (right-most column). A few non-adherent hematopoietic cells could be seen in the images of the YS culture in Figure 17c, and a few sparse hematopoietic cells were also present in other fields of view in this AGM culture (but with no associated with GFP+VE-Cadh+ adherent endothelium; data not shown).

The relatively sparse endothelial and hematopoietic colonies present in these re-plated YS and AGM tissues after infection, however, proved disappointing. The robustness of hematopoiesis from these embryo-derived YS/AGM cells was too low even in the control PGK infected cultures to allow quantitative assessment of differences between embryonic tissues infected with shRNA versus PGK lentivirus.

Figure 17b shows an attempt to get better lentiviral penetration by dissociating the YS and AGM tissue before re-aggregating them in hanging drops with PGK control virus for 2 days. In this experiment, younger 14 somite stage (E8.75) embryos were dissected to try to infect tissues around the time EfnB2 expression is first detected. In both YS and AGM tissues, infection efficiency appeared higher (shown by the percentage of GFP+ cells in the clusters). However, cell yield and viability was much lower in these cultures compared to the organ infected cultures in Figure 17a (data not shown) and there was also concern that the cells were too disrupted from their endogenous in vivo niche to be a good cell source for ex vivo interrogation of hematopoietic potential. Therefore this strategy appeared even more challenging given the disappointing results of the whole organ infected cells in Figure 17b.

To avoid the necessity of having an additional re-aggregation or suspension culture step like in Figure 17a – c, YS and AGM tissue were dissected from older 36 somite stage (E10.5) embryos at the point of HSC emergence, before being re-plated as single cells into MethoCult3434 media with PGK or shRNA virus. Each DA or YS was split into two cultures: one infected with shRNA and the other with control PGK virus. Therefore in this experiment, each shRNA infection condition had a direct PGK control for comparison.

Figure 17d shows that both E10.5 YS and AGM shRNA infected cells formed fewer hematopoietic colonies than PGK control infected cells. This supports the in vitro ES cell

results, in which there was a drastic decrease in the number of hematopoetic cells harvested from the Day 6 ECs re-plated into the same culture. Note that the colonies formed from the explanted YS and AGM tissues were predominantly multipotent GEMM colonies rather than erythroid colonies in re-plated Day 6 EBs (Figure 9g).

It is important to note that in the ex vivo experiment in Figure 17d, the YS and AGM cells were not knocked down for EfnB2 until quite late during differentiation (E10.5). In the ES experiments, EfnB2 was knocked down before endothelial specification (VE-Cadh expression at Day 4 EBs), where it did not affect EC commitment. In this ex vivo experiment, however, the embryonic cells presumably had already expressed EfnB2 since E8.25 (based on previously published LacZ protein staining).[1] Therefore, by infecting the YS and AGM cells at E10.5 when the tissues were dissected, there was a period of over 48 hours in which the putative HE was expressing endogenous EfnB2. The expression of EfnB2 during this critical window without lentiviral manipulation leaves unanswered the question if EfnB2 is required throughout the earliest stages of endothelial/hematopoietic development.

However, the results show a decrease in hematopoiesis from the E10.5 YS and AGM cultures, and in particular the AGM cultures, which is interesting in light of the experiments where EfnB2 was shown to have an effect on the maturation of the VE-Cadh+CD45+ population to the CD45 single positive hematopoietic cells. It can be assumed that this transition is being analyzed here when the E10.5 HE/pre-HSC population is harvested from the AGM and then re-plated into the Methocult3434 assay. If this is the case, the results from this ex vivo experiment further support the notion that EfnB2 plays a role in this final maturation step to the CD45+ putative HSCs.

4.2 EfnB2 Knock-Out Embryos Show Hematopoietic Defects Upon Ex Vivo Re-plating

A more direct strategy to analyze the role of EfnB2 during hematopoietic development in vivo is to analyze the previously characterized EfnB2 knock-out mice. These knock-out mice are growth retarded by E10.0 and die in utero between E10.0 and E11.0, precisely around the time of HSC emergence in the DA around E10.5. Of particular note, the DA in the EfnB2 knockout mice does form, but does not specify to an arterial fate or undergo proper endothelial remodeling.[10, 49] Given these characteristics of the EfnB2 knockout embryos, it would be possible to take the non-arterial specified DA around E10.0 (before the mice die) and to re-plate these potential HE into OP9 co-culture and MethoCult3434 culture to evaluate the hematopoietic potential ex vivo past the developmental stages when the knock-out embryo would be dead.

4.2.1 E10.5 EfnB2 Knock-Out Embryos Show Hematopoietic but not Endothelial Defects

Figure 18a shows the results of re-plating YS and DA dissected from E10.5 embryos of EfnB2 heterozygous parents to evaluate EfnB2-/-, EfnB2+/- ,and EfnB2+/+ littermate embryos. In Figure 18a, images of OP9 co-cultures were taken five days after re-plating one embryo-equivalent DA (dissociated) onto OP9 stroma. Several heterozygous DAs (one shown in the middle panel) showed more robust hematopoiesis in OP9 co-culture than the single re-plated WT DA (top panel). Strikingly, there were no hematopoietic colonies in the single re-plated full knock-out DA (bottom panel).

One potential explanation for the lack of hematopoiesis in the re-plated E10.5 knock-out DA in Figure 18a could be that that this particular knock-out embryo was already dead when

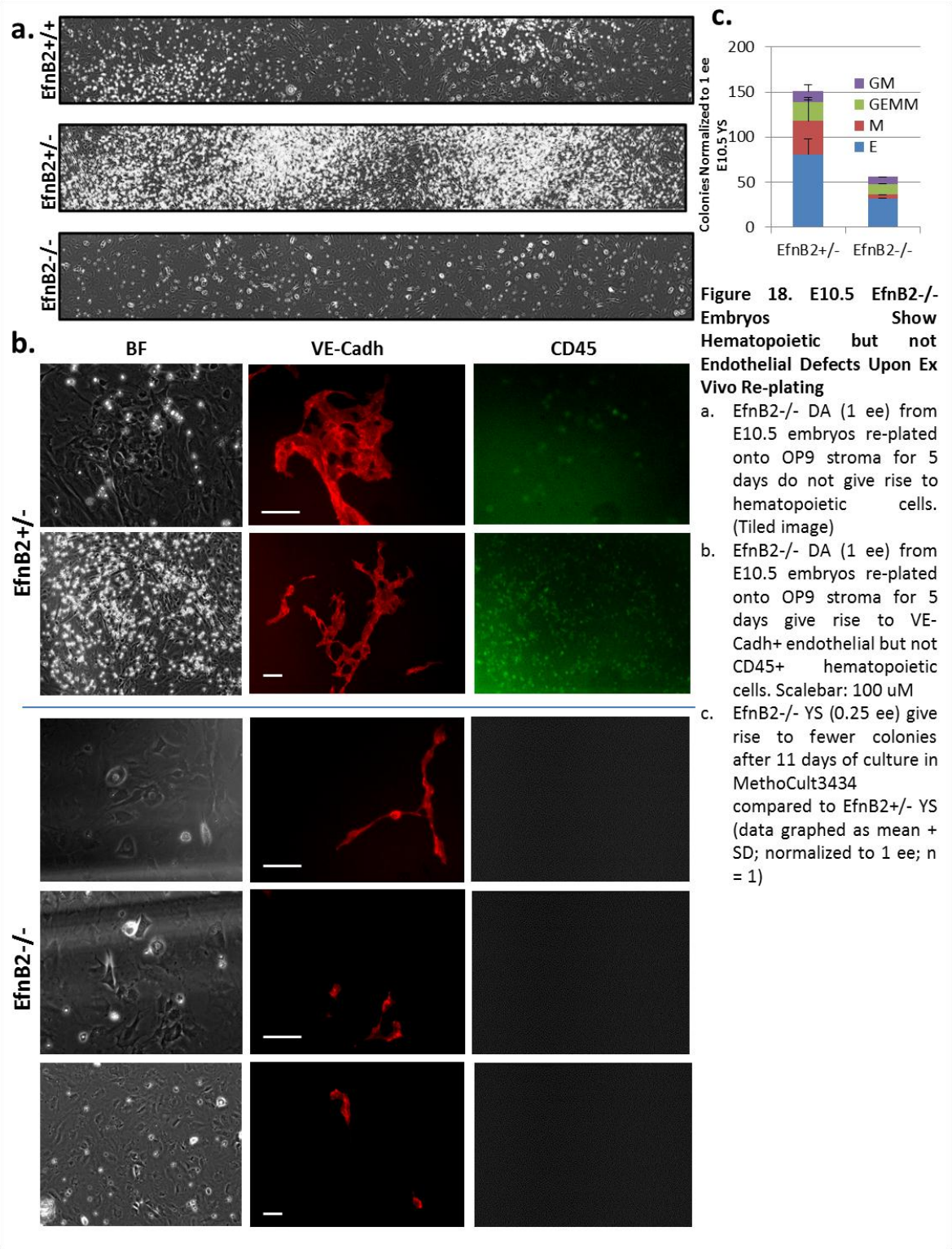

Figure 18. E10.5 EfnB2-/- Embryos Show Hematopoietic but not Endothelial Defects Upon Ex Vivo Re-plating

a. EfnB2-/- DA (1 ee) from E10.5 embryos re-plated onto OP9 stroma for 5 days do not give rise to hematopoietic cells. (Tiled image)

b. EfnB2-/- DA (1 ee) from E10.5 embryos re-plated onto OP9 stroma for 5 days give rise to VE-Cadh+ endothelial but not CD45+ hematopoietic cells. Scalebar: 100 uM

c. EfnB2-/- YS (0.25 ee) give rise to fewer colonies after 11 days of culture in MethoCult3434 compared to EfnB2+/- YS (data graphed as mean + SD; normalized to 1 ee; n = 1)

the DA was dissected out, due to the previously characterized cardiovascular defects found in the full knock-out embryos. Gross defects, including growth retardation, are evident beginning around E10.0, when some knock-out embryos begin to die (embryonic lethality is fully penetrant by E11.0).[10] This particular E10.5 embryo was visibly a knock-out at the time of dissection in that there were gross cardiac defects and bloodless YS and embryonic vessels, a result confirmed by PCR genotyping. Also, the heart was also no longer beating when the embryo was dissected, suggesting that it was dead.

As a result of the gross defects in the full EfnB2 knock-out embryo, the comparison between the full knock-out DA and the developmentally normal heterozygous and WT littermate DA at this timepoint is imperfect. However, VE-Cadh immunostaining showed that the Day 5 OP9 co-cultures (Figure 18a) contain ECs in both the control heterozygous and the full knock-out DA (Figure 18b). The endothelial colonies were much sparser and fewer in the full knock-out DA culture. But the presence of these endothelial colonies five days after re-plating from the dissected embryo suggest that while the knock-out embryo may have been dead upon dissection, the cells re-plated were still alive and capable of re-plating, proliferation, and differentiation. Therefore, the DA endothelium from the EfnB2 full knock-out embryo is non-hemogenic, recapitulating the in vitro results of EfnB2 silencing.

The higher magnification images in Figure 18b further illustrate the virtual absence of CD45 staining in the few non-adherent cells from the full knock-out DA compared to the extensive CD45 staining from the heterozygous DA. Interestingly, there was no CD45 co-staining (in either control or knock-out cultures) with VE-Cadh that was present in the ES differentiation cultures and previously characterized in the literature (Figure 12d).

In this experiment, the YS were also dissected from the embryos and re-plated as single cells after collagenase I digestion (0.25 embryo equivalent) into MethoCult3434 culture for 11 days before scoring. The results (graphed in Figure 18c) show that there was a decrease in hematopoiesis from the full knock-out YS compared to the heterozygous knock-out controls, similar to what was seen in the DA.

This result could be due to the fact that E10.5 reflects a developmental timepoint beyond the peak of YS hematopoietic waves, after circulation is well established in the embryo (Figure 4). Therefore, the results from this experiment may reflect analysis of the already committed hematopoietic progenitors that have traveled (perhaps back) to the YS. If this were the case, the results mirror the results in Figure 13c, where committed CD41+ hematopoietic cells silenced for EfnB2 in Day 6 EBs formed significantly fewer colonies in Methocult3434. These ex vivo results therefore would confirm the continued role of EfnB2 during the maturation and proliferation of committed hematopoietic cells that was characterized in vitro (Results Chapter I).

4.2.2 E9.5 EfnB2 Knock-Out DA are Non-Hemogenic Endothelium

To address the potentially confounding effects of using E10.5 DA from a moribund or already dead full EfnB2 knock-out embryo, DA were dissected from E9.5 embryos for the experiment in Figure 19. At this younger stage of development, it was difficult to visually identify the full knock-out embryo from its littermates. This suggested that it was a good timepoint to dissect out the DA for evaluation of hemogenicity, as there were less confounding factors in the condition of the DA explanted from the different genotypes.

Surprisingly, when the E9.5 DA were re-plated into OP9 co-culture for five days, there were visible hematopoietic colonies in the re-plated EfnB2 full knock-out DA, in contrast to the

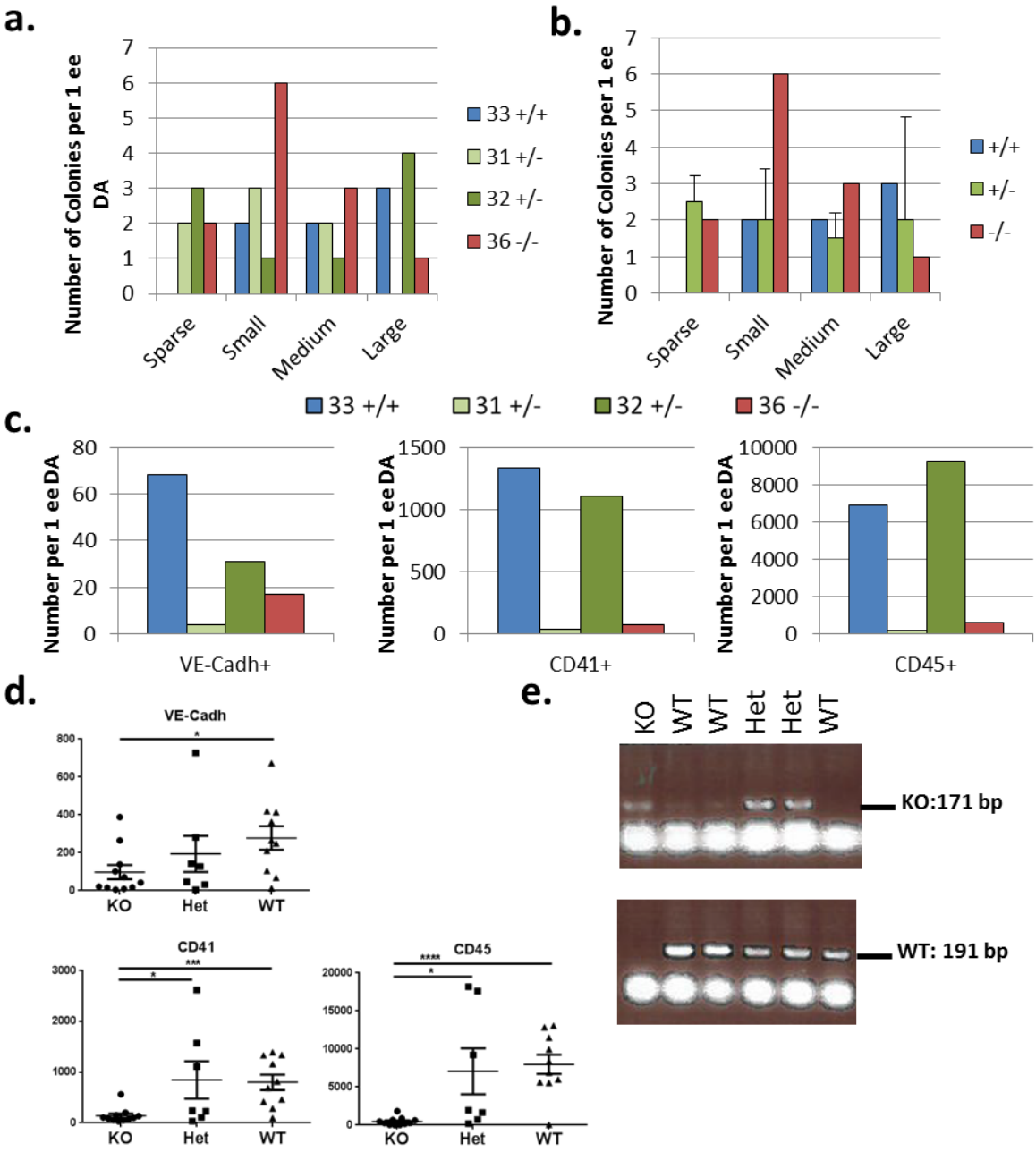

Figure 19. E9.5 EfnB2-/- Embryos Show Hematopoietic but not Endothelial Defects Upon Ex Vivo Re-plating

a. DA from EfnB2-/- E9.5 embryos (1ee) re-plated onto OP9 for 5 days give rise to smaller hematopoietic colonies. Individual embryos (with corresponding genotypes listed) were scored and graphed for the number of hematopoietic colonies present by eye under 10X magnification. Sparse (<20 cells); Small (20-50 cells); Medium (50 – 100 cells); Large (>100 cells).

b. Figure 19a graphed as Mean + SD across genotypes shows variability in the first litter analyzed.

c. DA from EfnB2-/- E9.5 embryos (1ee) re-plated onto OP9 for 5 days gives rise to almost no CD41+/CD45+ hematopoietic cells compared to variable controls in this first litter. The total number of marker+ cells within the live cell gate are graphed.

d. DA from EfnB2-/- E9.5 embryos (1ee) re-plated onto OP9 for 5 days gives rise to significantly lower CD41+/CD45+ hematopoietic cells compared to littermates after analyzing three litters. The total number of marker+ cells per DA are graphed. Each dot is an individual DA analyzed and error bars are SEM. (* $p < 0.05$; **** $p < 0.0001$; Unpaired T test)

e. Sample genotype analysis showing detection of the mutant locus band at 171 bp and WT band at 191 bp with corresponding genotype listed at the top.

E10.5 EfnB2 full knock-out DA re-plated in Figure 18a, which did not give rise to hematopoietic colonies. Nonetheless, all the plates were scored for size and number of hematopoietic colonies and graphed individually in Figure 19a, or as averages across genotypes in Figure 19b. The colonies were scored by approximate number of hematopoietic cells in a particular colony, with sparse colonies defined as those with less than 20 cells, small colonies having between 20-50 hematopoietic cells, medium colonies having between 50-100 cells, and large colonies having more than 100 cells. The cells were scored by eye under a brightfield microscope lens.

Note first in Figure 19a that the WT DA formed almost equal numbers of small, medium, and large hematopoietic colonies (embryo 33; blue bars). In the two heterozygous knock-out DA, there was variability in that one embryo (embryo 32; dark green) formed all sizes of hematopoietic colonies, but particularly large hematopoietic colonies (at a level equivalent to WT) while the other heterozygous knock-out DA (embryo 31; light green) formed only sparse to medium colonies and no large colonies. Given this variability between the two heterozygous knock-out DA, the averaged data graphed in Figure 19b show large error bars. The single full knock-out embryo (embryo 36; red bars) formed predominantly small to medium-sized colonies, with only one large colony scored.

To further quantify the level of hematopoietic differentiation in the different cultures, plates were carefully harvested and stained for VE-Cadh, CD41, and CD45. The entire samples were then FACS counted for the total number of VE-Cadh+, CD41+, and CD45+ cells in the live cell gate (by propidium iodide staining). The results are graphed in Figure 19c with each embryo separately graphed.

The WT DA had robust numbers of endothelial (VE-Cadh+) and hematopoietic (CD41+ or CD45+) cells compared to the other embryos (blue bars). Note that for all the embryos (including this WT DA), there were relatively few VE-Cadh+ endothelial cells and a lot of CD45+ hematopoietic cells (more than CD41+). The low numbers of VE-Cadh+ endothelial cells was surprisingly low compared to the percentage in Day 6 EBs re-plated into OP9 co-culture (Figure 12d). Also, there was not a distinct VE-Cadh/CD45 double positive population that was identified in the EB re-platings (data not shown).

The variability noted between the two different heterozygous knock-out DA scored in Figure 19a is also evident in the counts of VE-Cadh+ endothelial cells and CD41+/CD45+ hematopoietic cells in Figure 19b. Similar to the results in Figure 19a where embryo 32 formed almost the same number of large hematopoietic colonies as the WT DA, embryo 32 (dark green bars) had almost the same total number of CD41+ and CD45+ cells as the WT embryo. Interestingly, there was a 50% decrease in the number of VE-Cadh+ ECs in the heterozygous DA compared to WT. However, the other heterozygous knock-out DA (embryo 31; light green bars) had almost no endothelial or hematopoietic cells (4 VE-Cadh+; 35 CD41+; 197 CD45+).

The results of the CD41+/CD45+ counts for the full knock-out DA (embryo 36; red bars) show that there were only 69 CD41+ and 624 CD45+ hematopoietic cells in the live cell gate. If this is compared to the WT and heterozygous knock-out DA (embryo 32), it represents an over 90% decrease in the number of hematopoietic cells derived from re-plated E9.5 DA. However, one heterozygous knock-out DA that had even lower levels of endothelial and hematopoietic differentiation (from embryo 31) than the full knock-out DA

All together, the results of scoring different sized hematopoietic colonies before harvesting the plates for immunophenotypic counting by flow cytometry seems to be highly predictive method of measuring hematopoietic output. In particular, the number of large hematopoietic colonies scored by eye seems to be most directly related to the hemogenicity of the culture as measured by the total number of live CD41+/CD45+ cells after OP9 co-culture and FACS analysis. Therefore, while the E9.5 DA from the full EfnB2 knock-out embryo had many (small to medium) sized hematopoietic colonies in the culture (in comparison to the E10.5 DA and the silenced EBs), the hemogenicity was significantly lower when quantified immunophenotypically.

There could be several possible explanations for the difference between colony counts (especially the small to medium sized colony counts) and final immunophenotypic cells counts. First, when the colonies were scored by eye, it was not possible to know for certain if the non-adherent cells were (1) alive and (2) hematopoietic. The FACS results gated out dead cells, which could have been counted when scoring the colonies. The other explanation could be that the non-adherent "hematopoietic" cells in the knock-out cultures did not express the traditional hematopoietic markers CD41/CD45.

The results of this ex vivo experiment analyzing DA from an earlier time in development to prevent confounding effects of pan-EfnB2 knock-out are very encouraging. The results suggest that the non-arterial specified DA in the full knock-out embryos is not hemogenic compared to the DA from littermate controls. This represents a confirmation that EfnB2 plays a critical role in regulating hematopoiesis from the DA. Interestingly, it has been documented that EfnB2 heterozygous embryos are born at half the predicted Mendelian ratios (and this appeared to be the case in breedings here as well), suggesting that that

there is a dosage effect of EfnB2 knock-down on embryonic viability.[49] The variability seen in the ex vivo re-platings, therefore, may be a direct reflection of the varied penetrance and phenotype in the heterozygous EfnB2 knock-out. This is particularly interesting given that haploinsufficiency of Dll4 has been thoroughly characterized.

These results have been confirmed with three additional litters. The pooled results from all experiments are graphed in Figure 19d and show that E9.5 EfnB2-/- DA display a significant hematopoietic defect. Each dot in the plots represents the number of VE-Cadh+, CD41+, or CD45+ cells from an individually re-plated DA after 5 days of OP9 co-culture. Note that a significantly lower number of VE-Cadh+ cells is found in the EfnB2-/- versus EfnB2+/+ littermates. No such difference is observed when EfnB2-/- and EfnB2+/- littermates are compared. Importantly, the EfnB2-/- DA produced significantly fewer CD41+ and CD45+ cells compared to both EfnB2+/- and EfnB2+/+ littermates. Therefore, the DA from E9.5 EfnB2-/- mice are less hemogenic than their heterozygous and wild-type littermates upon ex vivo re-plating.

Figure 19e is an example of genotyping the embryo remnants after dissecting out the DA to determine the appropriate genotype of each re-plated DA. Two separate genotyping reactions are performed on the DNA; one for the endogenous EfnB2 locus, and one for the targeted locus (with LacZ primers). As illustrated in this figure, embryos with only the KO band but not the WT band are identified as EfnB2-/- embryos (Lane 1). Embryos with only the WT band but no KO band are identified as EfnB2+/+ mice (Lanes 2, 3, and 6), while embryos with both WT and KO bands are identified as EfnB2+/- embryos (Lanes 4 and 5).

YS from the E9.5 embryos were also dissected and re-plated into Methocult3434 culture. At Day 9 of culture, it was grossly evident that there was a defect in colony formation in the

knock-out YS (Embryo 36) because there were no red colonies observable by the naked eye compared to all the other wells (Figure 20a; Image taken by iPhone, so low resolution). Under the microscope, the predominant colony type in the knock-out embryo is pictured in Figure 20c. These colonies were scored as "Erythroid" but note that there is no red pigment in any of the colonies. The images here were taken by a monochrome camera attached to the microscope, so the red color cannot be detected under brightfield, but in the +/+ and +/- images, the dark centers were red by eye. The colony counts are graphed in Figure 20b and show some variability within the different genotypes (+/+ and +/-). Note however, the big decrease in the number of multipotent GEMM colonies that were present in the knock-out embryo. The non-red erythroid colonies are graphed in the light pink bar.

Note that by total colony counts, there does not appear to be a difference in hemogenicty of the knock-out YS. Further analysis will need to be done to confirm the identity of these cells, including staining for hemoglobin, such as with benzidine. It is interesting to note that one of the gross defects observed in the E10.5 knock-out embryos (24 hours after this dissection) is a pale YS. The ex vivo re-plating of this E9.5 YS seems to support this erythro-deficiency.

4.3 EfnB2 Conditional Knock-Out in Tie2+ Endothelium Shows Inconsistent Hematopoiesis

To further evaluate the endothelial-specific, cell autonomous requirement for EfnB2 during hematopoietic development that was suggested by the in vitro studies (Results Chapter I), conditional knock-out mice in which EfnB2 deletion was targeted to the endothelium using either tamoxifen induced VE-Cadh–Cre or constitutive Tie2-Cre was performed. At the time of writing this thesis, only $EfnB2^{fl/+}$;Tie2-Cre males were bred with $EfnB2^{fl/fl}$ females.

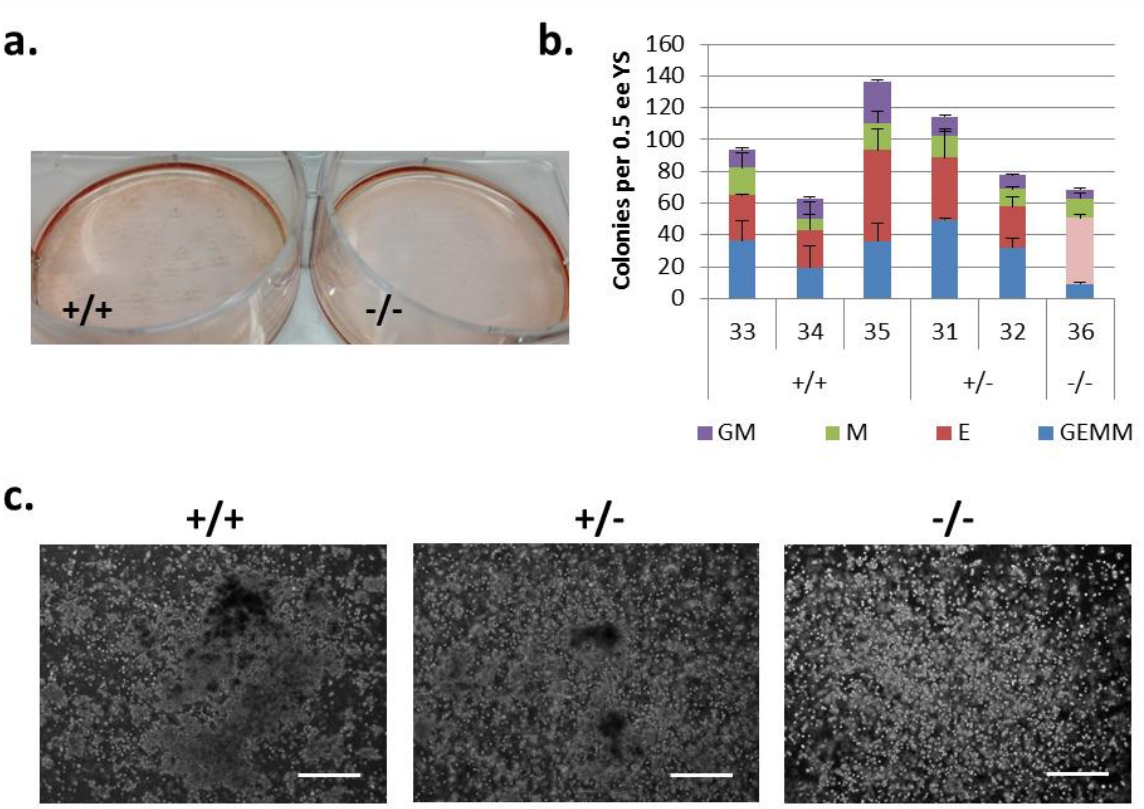

Figure 20. E9.5 EfnB2-/- YS Shows Abnormal Erythroid Re-plating ex vivo

a. E9.5 EfnB2-/- YS plated for 9 days in MethoCult3434 do not show macroscopic red erythroid colonies.

b. E9.5 EfnB2-/- YS (0.5 ee) re-plated into MethoCult3434 and scored at Day 9 show comparable number of total colonies. The light pink bar in the EfnB2-/- YS (embryo 36) represents scored erythroid colonies that are not red (n = 1 litter)

c. Brightfield images (monochrome) taken of Day 9 MethoCult3434 cultures showing the lack of hemoglobin in EfnB2-/- YS erythroid colonies. Scalebar: 200 uM

The ex vivo re-plating of YS and AGM cells from the Tie2 targeted knock-out of EfnB2 mice are shown in Figure 21. In this experiment, the embryos were dissected at E10.0. At the time of dissection, one embryo (embryo 42) was visually a conditional knock-out mouse, a result confirmed by genotyping. This embryo was growth retarded compared to its littermates and had severe cardiac defects and very little blood in either the YS or embryonic vessels. The other conditional knock-out embryo (embryo 41) appeared developmentally normal compared to its littermates and was not identified as a conditional knock-out until genotyping was performed. This difference between the two conditional knock-out littermates suggests that there is variability in the penetrance of this EfnB2 conditional knock-out, perhaps with more variability than in the full EfnB2 knock-out embryos (Figure 19).

In this experiment, the DA were dissected, dissociated, and re-plated (1 ee) onto OP9 stroma for four days before being scored by size and number for hematopoietic colonies, as was done previously (Figure 19). The results are graphed for each individual embryo in Figure 21a as averages across the different genotypes in Figure 21b.

It is evident from Figure 21a that there is variability in the level of hematopoiesis within the different mice genotypes when scored by eye. In this figure, embryos of the same genotype are graphed as the same color to allow easier grouping when looking at the data. Within each group of colored bars (ex. red EfnB2$^{fl/+}$;Tie2-Cre+ mice) there is often more than 50% variability in the number of colonies scored in the same colony size category. This is particularly noticeable in Figure 21b when the averages of each colony type are graphed within each genotype category with error bars.

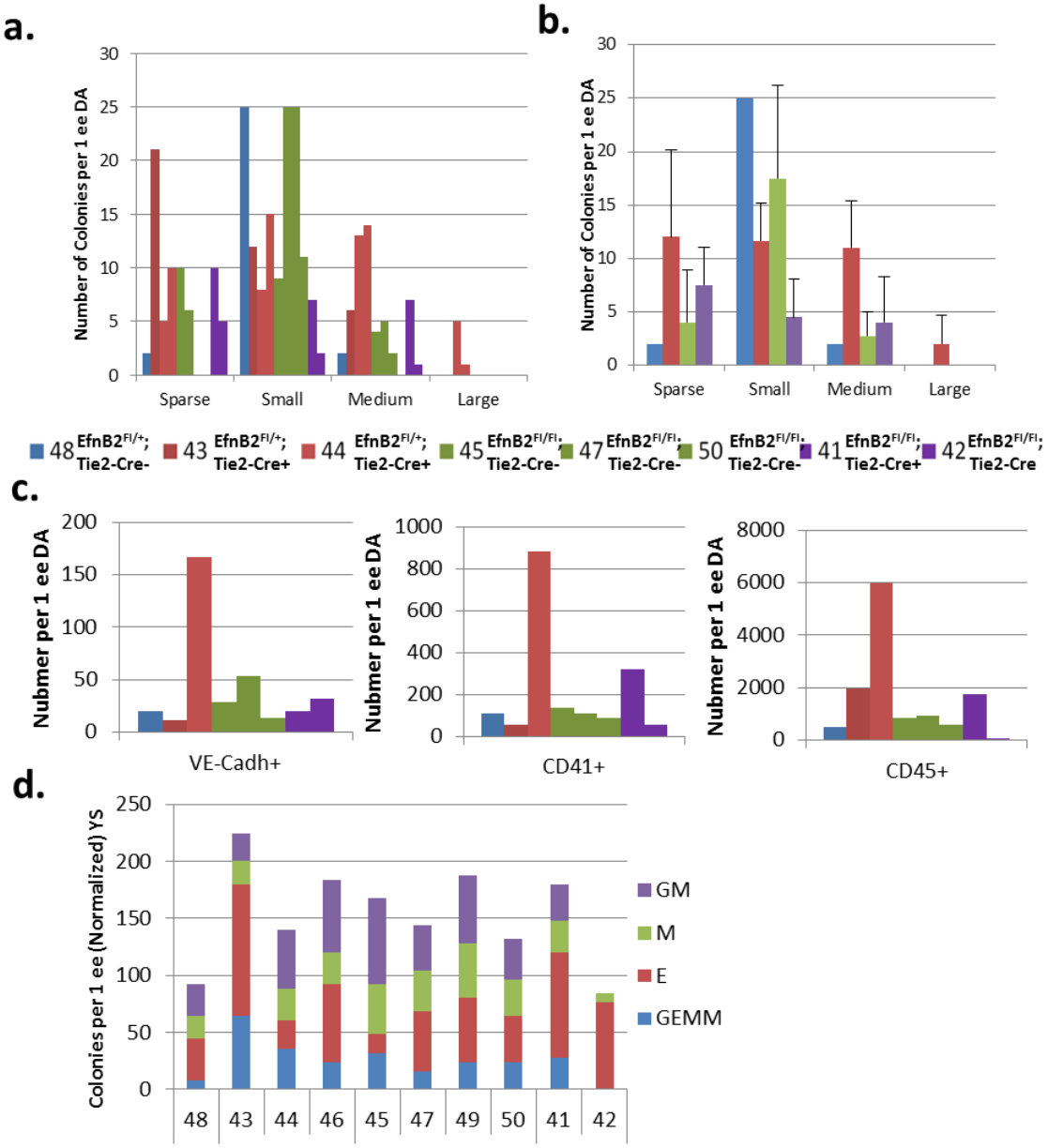

Figure 21. E10.0 EfnB2 Tie2 Conditional Knock-Out Embryos and Controls do not Show Consistent Hematopoietic Output

a. DA from E10.0 embryos (1ee) re-plated onto OP9 for 5 days show varied colony formation within different genotypes. Individual embryos (with corresponding genotypes listed) were scored and graphed for the number of hematopoietic colonies present by eye under 10X magnification. Sparse (<20 cells); Small (20-50 cells); Medium (50 – 100 cells); Large (>100 cells).

b. Figure 21a graphed as Mean + SD summarizes variability within each genotype.

c. Counts of the total number of VE-Cadh+/CD41+/CD45+ cells from re-plating 1 ee E10.0 DA in 5 days of co-culture on OP9 stroma show variable hematopoiesis.

d. E10.0 YS (0.25 ee) re-plated into MethoCult3434 and scored at Day 8 show comparable levels of hematopoiesis between all genotypes (Data normalized to 1 ee; n = 1)

The only standout trends in Figure 21a and b include the observation that the one EfnB2$^{fl/+}$;Tie2-Cre- embryo did not have very much hematopoietic activity, with a lot of small hematopoietic colonies, but only one medium or large colony (reminiscent of the EfnB2 full knock-out embryo in Figure 19a). This is surprisingly in that the EfnB2$^{fl/+}$;Tie2-Cre- embryo would have been predicted to be the least developmentally disrupted embryo as it only has one floxed EfnB2 allele and no Cre.

In comparison, the EfnB2$^{fl/+}$;Tie2-Cre+ and EfnB2$^{fl/fl}$;Tie2-Cre- mice overall had much higher levels of hematopoiesis compared to EfnB2$^{fl/+}$;Tie2-Cre- or the conditional knock-out EfnB2$^{fl/fl}$;Tie2-Cre+ mouse. Indeed in all these mice, there were many more sparse, medium and in some cases large colonies in the EfnB2$^{fl/+}$;Tie2-Cre+ and EfnB2$^{fl/fl}$;Tie2-Cre- mice than the EfnB2$^{fl/+}$;Tie2-Cre- or EfnB2$^{fl/fl}$;Tie2-Cre+ mice. However, the actual counts of each of the colonies varied substantially within the EfnB2$^{fl/+}$;Tie2-Cre+ and EfnB2$^{fl/fl}$;Tie2-Cre- genotype categories. Nonetheless, the high levels of hematopoiesis observed in the EfnB2$^{fl/+}$;Tie2-Cre+ and EfnB2$^{fl/fl}$;Tie2-Cre- embryos are encouraging in that they confirm individually that the presence of Tie2 Cre with only one floxed allele and the presence of two floxed alleles do not seem to affect hematopoiesis. This would suggest that a conditional knock-out phenotype re-capitulating a full EfnB2 knock-out would require both alleles of to be floxed in conjunction with a Tie2 transgene.

To more quantitatively assess the hematopoietic output of each culture, the plates were harvested for FACS analysis to count the total number of live (propidium iodide negative) VE-Cadh+/CD41+/CD45+ cells in each culture. The results are graphed for each individual embryo in Figure 21c and as averages across the different genotypes in Figure 21d. The

variability in hemogenicity of the different embryos within each genotype category is again very evident from these graphs.

The data in Figure 21c further confirm the results in Figure 21a; for instance the EfnB2$^{fl/+}$;Tie2-Cre- embryo (embryo 38) which had few scored colonies also showed relatively low cell counts in Figure 21c (20 VE-Cadh+, 112 CD41+ and 486 CD45+). If this EfnB2$^{fl/+}$;Tie2-Cre- embryo is used as a baseline with which to compare all the other embryos, the variability becomes strikingly obvious. With only three exceptions (the number of CD41+ cells in EfnB2$^{fl/fl}$;Tie2-Cre- embryos and the number of CD45+ cells in EfnB2$^{fl/+}$;Tie2-Cre+ and EfnB2$^{fl/fl}$;Tie2-Cre- embryos—which were all consistently higher than the EfnB2$^{fl/+}$;Tie2-Cre- cell counts) there was always at least one embryo in each genotype that had less and one embryo that had more of that particular cell compared to the EfnB2$^{fl/+}$;Tie2-Cre- embryo.

It is not known why there was so much variability in this experiment, though it was the only experiment with the Tie2 conditional knock-out mice done before writing these results. The variability is most likely related to technical issues during the dissection and dissociation of the DA. It is important to note that with collagenase I digestion, it was not possible to achieve completely homogeneous single cell suspensions from the tissue, such that some tissue clumps were re-plated. It can be assumed that even with incomplete tissue digestion, there were probably single cells that were over-digested and therefore did not successfully re-plate. Every care was taken during the digestions, and all the plates were visualized after plating the dissociated tissues onto the OP9 stroma to confirm that the cells were relatively healthy. These experiments will have to be optimized and repeated to decrease the

variability in the control embryos before any conclusions can be drawn regarding the endothelial cell autonomous role for EfnB2 during ex vivo DA hematopoiesis.

YS from the E9.5 embryos were also dissected and re-plated into Methocult3434 culture and scored in Figure 21d. The variability in the DA re-platings was also reflected in the YS colony counts, though with somewhat better consistency. The two biggest outliers were the EfnB2$^{fl/+}$;Tie2-Cre- YS (Embryo 48) and one of the EfnB2$^{fl/fl}$;Tie2-Cre+ conditional knock-out YS (Embryo 42). However, while the EfnB2$^{fl/+}$;Tie2-Cre- YS had some multipotent GEMM colonies, the EfnB2$^{fl/fl}$;Tie2-Cre+ embryo did not, nor did it have GM colonies. Indeed, the colony counts of the EfnB2$^{fl/fl}$;Tie2-Cre+ (Embryo 42) were almost exclusively erythroid. However, this result was not confirmed in the other EfnB2$^{fl/fl}$;Tie2-Cre+ conditional knock out embryo (Embryo 41), which appeared to have comparable hematopoietic output in line with the controls.

Embryo 42 was identified at the time of dissection as the targeted knock out due to its growth retardation and lack of blood. It is interesting that upon re-plating, this YS gave almost exclusively erythroid colonies (and absolute numbers higher than most of the other controls), suggesting that environmental/niche factors may play a role in the lack of erythroid differentiation in the conditional knock-out embryo. This result also in contrasts with the explanted YS from the full EfnB2 knock-out embryo (Figure 20c), which showed aberrant hemoglobin-negative erythroid colony formation.

Overall, the experiments with the targeted knock out mice remain to be optimized to minimize the variability between the different embryos before any definitive conclusions can be drawn from the experiments. These results can potentially shed light on whether or not the requirement for EfnB2 during developmental hematopoiesis is EC autonomous.

While it is hoped that optimization will increase consistency in the controls, it is also understood that conditional deletion of proteins is never as clean as in full knock out embryos due to variability in penetrance and timing of deletion.

4.4 EfnB2 May Play a Potential Role in the Hematopoietic Stromal Niche

The key difference between the experiments using the full EfnB2 knock-out embryos with the Tie2+ endothelial targeted EfnB2 knock-out embryos can be useful in dissecting out whether the role of EfnB2 in hematopoiesis is cell autonomous or not. The hypothesis that EfnB2 is required cell autonomously in the HE comes from the finding that the ECs of the DA are EfnB2+. From the previously published LacZ staining of $EfnB2^{LacZ/+}$ embryos, there did not appear to be EfnB2 expression in the subaortic mesenchyme or in the basement membranes surrounding the YS vessels, though this needs to be further confirmed.[1]

However, there could be a non-cell autonomous role for EfnB2, in which the surrounding niche of the HE could be inducing the Ephrin signaling necessary for hematopoiesis. Given the variable results of re-plating the Tie2 conditional knock-out of EfnB2 DA onto the OP9 stroma in Figure 21, it seemed important to address the possibility of a non-cell autonomous role for EfnB2 in the developmental hematopoietic niche.

This seems less likely given the fact that EfnB2 expression or signaling was never manipulated in the OP9 cells that served as the supportive niche environment in the in vitro experiments (Results Chapter I), as the shRNA/PGK control virus was never in the media when the Day 6 EBs were re-plated. However, the OP9 cells could potentially provide the critical Eph/Ephrin signals that the knocked-down ES cells could not respond to.

The OP9 cells used in the co-culture re-platings were derived from adult mouse bone marrow as a stromal cell line supportive of definitive hematopoiesis and hematopoietic stem/progenitor differentiation in the bone marrow.[82, 158] In an attempt to better characterize if there were Eph/Ephrin proteins that could play an instructive role for the HE or hematopoietic progenitors, including CD34+ human HSCs, the OP9 cells were analyzed by flow cytometry for EfnB2 and EphB4 expression.

Figure 22a shows that by flow cytometry, 47.5% of the OP9 cells express the receptor EphB4. This could be interesting in that the putative HE cells sorted from the EB differentiation express EfnB2, and therefore there could be reciprocal interactions and signaling between the ES cells (as well as the re-plated DA) and the stromal layer. It would be interesting to extend this analysis to the embryo and see if EphB4 were expressed in the subaortic mesenchyme or tissue surrounding the YS vessels.

In contrast, EfnB2 protein was not detectable by flow cytometry on the surface of OP9 cells, whereas phosphorylated EfnB (in the intracellular domain) was detected in 83.2% of the fixed and permeabilized OP9 cells. Although it is possible that the EfnB2 is all internalized in the OP9 cells, it is more likey that these results are attributable to the antibodies used. The phospho EfnB antibody is not specific to EfnB2 (recognizing the phosphorylated EfnB1/B2/B3 proteins) and has also not been documented in the literature for use in intracellular flow cytometry.

To functionally test if EfnB2 was expressed and required in the OP9 stromal cells, the EfnB2 shRNA (that was previously used on the ES cells) was used to knockdown EfnB2 expression. The results of knockdown four days after infection are imaged in Figure 22b and show that the knockdown of EfnB2 caused severe morphological and proliferation defects in the OP9

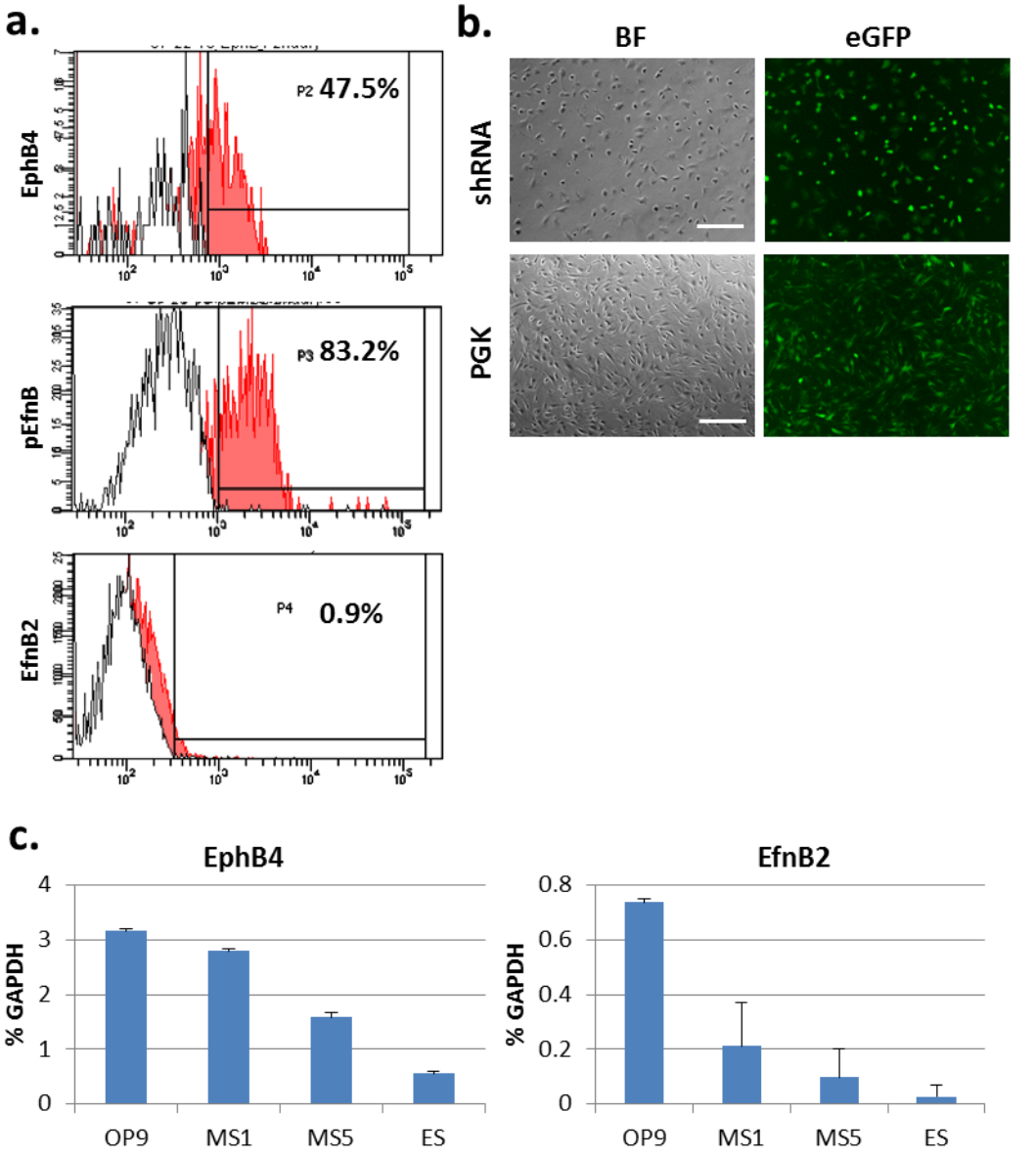

Figure 22. EfnB2 and EphB4 are Expressed in the Hematopoietic Stromal Support OP9 Cells

a. OP9 cells show EphB4 and phospho-EfnB but not EfnB2 surface protein by FACS (red histogram against secondary only control in black; Representative of 2 experiments)

b. OP9 stromal cells infected with shRNA lentivirus show abnormal morphology and decreased proliferation compared to PGK controls. (n = 1) Scalebar: 200 uM.

c. qPCR confirms relatively high EphB4 and EfnB2 gene expression in OP9 stromal cells compared to other cell lines (MS1: EC cell line, MS5: stromal cell line, ES: ES cell line; Graphed as Mean + SD; normalized to GAPDH; n = 1)

stromal cells. The shRNA infected cells lost their spindle-like morphology and began to round up and lose all contacts with surrounding cells. The silenced cells also stopped proliferating while the control PGK-infected cells continued to grow until confluency.

The results of this knockdown experiment in OP9 cells suggest that EfnB2 is expressed by the OP9 cells, as knockdown of the protein had profound morphological and proliferative effects on the cells. By flow cytometry, its receptor EphB4 appeared to also have been expressed.

This finding was confirmed by qPCR by comparing the gene expression levels of EphB4 and EfnB2 in various cell lines (Figure 22c). In this experiment, OP9 stromal cells in maintenance media express high levels of EphB4 and especially EfnB2 when compared to MS1, a pancreatic endothelial cell line, and MS5, a bone marrow stromal cell line. The expression levels of both genes are also much higher than the expression levels of EphB4 and EfnB2 in ES cells during maintenance on LIF.

These results raise an interesting situation where EfnB2 and EphB4 appear to be expressed in both the OP9 stromal cells and the re-plated endothelium (by qPCR analysis; Figure 8a). It raises the possibility that reciprocal interactions between EfnB2 and EphB4 on both cell populations could play a role in the hematopoietic differentiation of the putative HE.

It was not tested whether EfnB2 knockdown in OP9 cells would reduce their supportive function for HE cells co-cultured on them. However, the profound morphological changes in EfnB2-silenced OP9 cells raises the possibility that EfnB2 expression is required. If so, the question would be whether or not a similar requirement applies to the situation in vivo. It is

not known how equivalent the OP9 stromal cell line is to the subaortic mesenchyme during development.

The characterization of the supportive OP9 stromal cell line in these experiments raises the distinct possibility that EfnB2 may be required non cell-autonomously by the HE. Both EfnB2 and EphB4 appeared to be expressed by the stromal cell line, and EfnB2 expression was required for the maintenance of the OP9 cells. Given the profound gross morphological changes that occurred in the stromal cells, it is hypothesized that re-plating of HE cells from either Day 6 EBs or the embryo would probably not have led to hematopoiesis in vitro. This result could be a limited demonstration of a niche-factor role for EfnB2, though it remains to be tested and further explored. Finally, the results showing EfnB2 expression and its requirement for OP9 cell survival potentially extend the role of EfnB2 to adult life-long hematopoietic maintenance and differentiation.

4.5 Summary

In this chapter, several in vivo models and techniques were used to verify and extend the in vitro findings presented in Results Chapter I, which suggested a role for EfnB2 in the putative DA-like HE. To confirm technically the genetic manipulation of EfnB2 using shRNA infection, embryonic cells at the appropriate time of development were dissected and re-plated ex vivo with the same shRNA or control PGK constructs used in the ES experiments. Though cell viability was poor in these experiments, cells from YS and AGM tissue from older 36 somite stage (E10.5) embryos infected with shRNA showed less hematopoiesis than control PGK infected cells, supporting the results using the constructs in the in vitro differentiation of ES cell-derived endothelium into hematopoietic cells.

Critical experiments were presented in this chapter using full EfnB2 knock-out and Tie2 endothelial targeted EfnB2 knock-out embryos. Results in the full EfnB2 knock-out embryos suggest that there is a hematopoietic defect (as measured by the total number of live CD41+/CD45+ cells after ex vivo re-plating on OP9 stromal cells), which critically extend the in vitro results with ES cells to in vivo embryonic development.

Characterization of the OP9 stromal cell line used in the in vitro (and ex vivo) re-platings of the HE also suggested a potential non cell-autonomous role for EfnB2 during hematopoietic development, at least in vitro on the stromal cell layer. These findings potentially extend the current findings into adult hematopoiesis as OP9 stromal cells were derived from adult mouse bone marrow.

5 Results Chapter 3: The Role of Constitutive Expression of WT and Mutant EfnB2 in the Differentiation of Hemogenic Endothelium in vitro

5.1 EfnB2 Over-Expression Increases Hematopoietic Commitment in BL-CFC Differentiation

In the original experiments in which EfnB2 was knocked down in ES cells after mesoderm specification, there was no hematopoietic defect in BL-CFC differentiation. To further assess whether EfnB2 plays a role in this system, it was constitutively over-expressed in the ES cells before re-plating into BL-CFC culture. This experiment is schematized in Figure 23a and b. ES cells were infected with a lentivirus encoding full length EfnB2 during ES cell maintenance and sorted for GFP expression (from both EfnB2-WT and PGK cultures) before differentiation into EBs. At Day 3.5 of EB differentiation, GFP+Flk1+ cells were sorted into arterial monolayer culture (see below) and GFP+Flk1- cells were sorted into BL-CFC differentiation (Figure 23b).

The arterial monolayer culture is a different re-plating assay for Flk1+ mesoderm where instead of being re-plated into a methocellulose-based culture with hematopoietic cytokines (like BL-CFC and MethoCult3434), the cells are re-plated as single cells onto a Collagen IV-coated plate in the presence of a high concentration of the EC growth factor VEGF (50ng/mL) to promote the formation of an arterial endothelial colony (much like those produced on the OP9 stromal cells) from which non-adherent hematopoietic cells bud beginning around Day 2 of Arterial Monolayer re-plating. An image of an adherent arterial endothelial colony (on Collagen IV) with budding hematopoietic cells in suspension is included in Figure 23a.

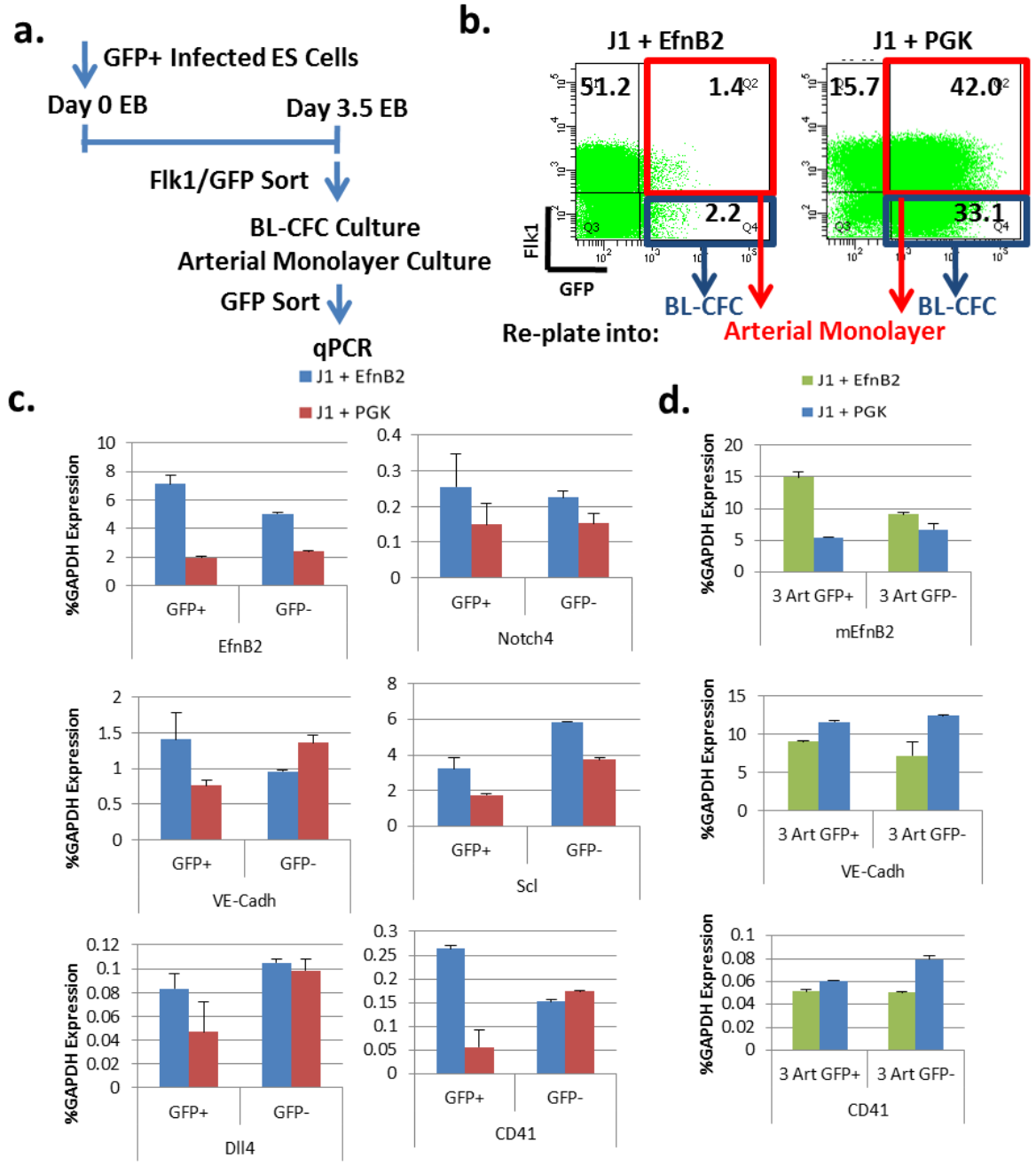

Figure 23. EfnB2 Over-Expression Increases Hematopoietic Commitment in BL-CFC Differentiation

a. Schematic of experiment.

b. Flk1 staining of Day 3.5 EBs infected with EfnB2-eGFP or PGK control virus do not show effect of infection on mesoderm commitment. GFP+Flk1+ cells (red box) were re-plated into arterial monolayer cultures while GFP+Flk1- cells (blue box) were re-plated into BL-CFC culture for Figure 23c and d.

c. Day 4 BL-CFC (from re-plated GFP+Flk1- Day 3.5 EBs) from EfnB2-over-expression vector infected cells show increased expression of various endothelial and hematopoietic markers compared to control PGK. (n = 1; Mean + SD)

d. Day 3 arterial monolayer cells (from re-plated GFP+Flk1+ Day 3.5 EBs) from EfnB2-over-expression vector infected cells do not show different expression of various endothelial and hematopoietic markers compared to control PGK. (n = 1; Mean + SD)

Note in Figure 23b that GFP expression is very low in the EfnB2 over-expressing cells because the EfnB2 molecule is expressed as a fusion protein with GFP. In subsequent data (Figure 26b), it will be shown that GFP expression vastly underestimates the number of cells expressing the EfnB2-eGFP fusion (surface staining of the cells with an EfnB2 antibody detects an eight-fold increase in the number of cells compared to control infected cultures).

In this experiment, however, because successful infection was determined by GFP expression, very few GFP+Flk1+ cells could be sorted for re-plating. Therefore, only GFP+Flk1+ cells (red box) were re-plated into arterial monolayer culture (as previous experiments showed that only Flk1+ cells could be used in this assay since Flk1- cells formed adherent secondary EBs that prevented the growth of endothelial colonies). Flk1-GFP+ cells were also re-plated into BL-CFC culture (blue box) as this culture was less stringent and blast colonies did differentiate from Flk1- cells.

The results of the BL-CFC re-plating of GFP+Flk1- (EfnB2 over-expressing) cells are shown in Figure 23c. For the qPCR analysis, the BL-CFC cultures were harvested at Day 4 and sorted again for GFP expression before RNA extraction and cDNA synthesis. The qPCR results confirm over-expression of EfnB2 in both the GFP+ and GFP- fractions (a 3.5 fold increase in the GFP+ and an over 2 fold increase in the GFP- fraction), confirming that over-expression was not limited to the GFP+ fraction (though it did appear more potent in the GFP+ fraction).

VE-Cadh expression was somewhat up-regulated upon constitutive EfnB2 expression in the GFP+ fraction but not in the GFP- fraction, while the arterial-enriched surface markers Notch1 and Dll4 were slightly up-regulated in both the GFP+ and GFP- fractions. Together

these results suggest that constitutive EfnB2 expression promotes expression of endothelial markers in BL-CFC culture.

Interestingly, both Scl and CD41 (archetypical markers of hematopoiesis) were also increased in the GFP+ EfnB2 over-expression cultures. This suggests that both endothelial and hematopoietic commitment are increased by enforced EfnB2 over-expression. The results need to be confirmed, however, with re-plating Flk1+ mesoderm (instead of the Flk1- cells re-plated in this experiment), to verify that the increased differentiation was not attributable to potential differences in the Flk1- population (for instance delayed hemangioblast commitment).

The increased endothelial and hematopoietic commitment in BL-CFC differentiation were not confirmed, however, by the GFP+Flk1+ EfnB2 over-expressing cells re-plated into the arterial monolayer cultures (Figure 23d). Similar to the BL-CFC qPCR experiment, the arterial monolayer cells were sorted for GFP expression after three days of re-plating. EfnB2 over-expression was confirmed by the qPCR in the GFP+ fraction where EfnB2-WT cells expressed three-fold more EfnB2 than PGK controls. Over-expression was much less potent in the GFP- fraction in this culture (in contrast to the BL-CFC GFP- cells in Figure 23c), and showed only about 20% more EfnB2 expression. Note that all cells (control or over-infected) expressed more EfnB2 in the arterial monolayer culture compared to the BL-CFC culture. This is not surprising as the arterial monolayer culture more stringently selects for arterial endothelial differentiation.

Despite the persistent EfnB2 over-expression in the arterial monolayer cultures at this timepoint, both endothelial differentiation and hematopoietic differentiation (analyzed by

VE-Cadh and CD41 RNA transcript levels respectively) were decreased in both GFP+ and GFP- fractions of EfnB2 infected populations when compared to control infection samples.

The results from these EfnB2 over-expression experiments need to be further refined. The analysis of endothelial and hematopoietic commitment was only done at one specific timepoint in each culture, and therefore could not account for subsequent changes/compensation of differentiation kinetics. Also, differentiation should be confirmed at a protein level by flow cytometry, which can assess levels of hematopoietic markers at a single cell level, rather than the population averages of RNA expression by these qPCR results. Finally, the most striking results of EfnB2 knockdown were in the later re-platings of endothelium into definitive colony assays of OP9 co-culture and MethoCult3434, and not of mesoderm replatings into BL-CFC (which showed no effect of EfnB2 knock-down). Therefore, it will be necessary to over-express EfnB2 and analyze its effect in these later re-plating cultures.

If EfnB2 over-expression can promote both endothelial and potentially hematopoietic differentiation (CD41 and Scl expression) as it does in BL-CFC culture, this could be a potentially applicable strategy for expansion of hematopoietic progenitor/stem populations in vitro. Because EfnB2 is a signaling ligand expressed at the cell surface, there are molecules that can be used to temporally and reversibly activate EfnB2 in vitro, without the required genetic mutations needed for transcription factors and other nuclear-restricted regulators. This could be a potentially promising strategy to increase hematopoiesis in vitro.

5.2 "Arterial" Gene Expression is Intact and Persistent in Scl-/- ES Cells During BL-CFC Differentiation

The transcription factor Scl has been shown to be critical for all waves of hematopoiesis. To characterize the role of Scl in the differentiation of mesdoerm in BL-CFC culture, qPCR was performed over the timecourse of EB and BL-CFC differentiation for both WT (J1) and Scl-/- ES cells.

In this experiment, EBs were differentiated until Day 3.5, whereupon Flk1+ cells were sorted from both control J1 and Scl-/- samples and re-plated into BL-CFC culture. The qPCR results are graphed in Figure 24a after harvesting whole cultures at the indicated timepoints (except for the Day 3.5 EB sample, which contains only Flk1+ sorted cells). In these graphs, gene expression is corrected for GAPDH expression and so the scales for the J1 and Scl-/- samples are the same, allowing quantitative comparison between the two different cell lines.

The knockout of the Scl alleles can be confirmed by the timecourse (top right of Figure 24a) showing that Scl expression was never detected across differentiation compared to the WT J1 ES cells in which Scl expression was induced upon replating into BL-CFC culture. The master regulator of definitive hematopoiesis, Runx1, was also expressed at drastically lower levels in the Scl-/- cells through BL-CFC differentiation compared to J1 WT, confirming the block in hematopoiesis of the Scl-/- ES cells. The observation that Runx1 expression appeared induced at the correct time in the J1 control and Scl-/- ES cells between Day 0 and Day 1 of BL-CFC differentiation (though at reduced levels in the Scl-/- ES cells) suggest that Scl is not absolutely necessary for the induction of Runx1 expression, but rather for full

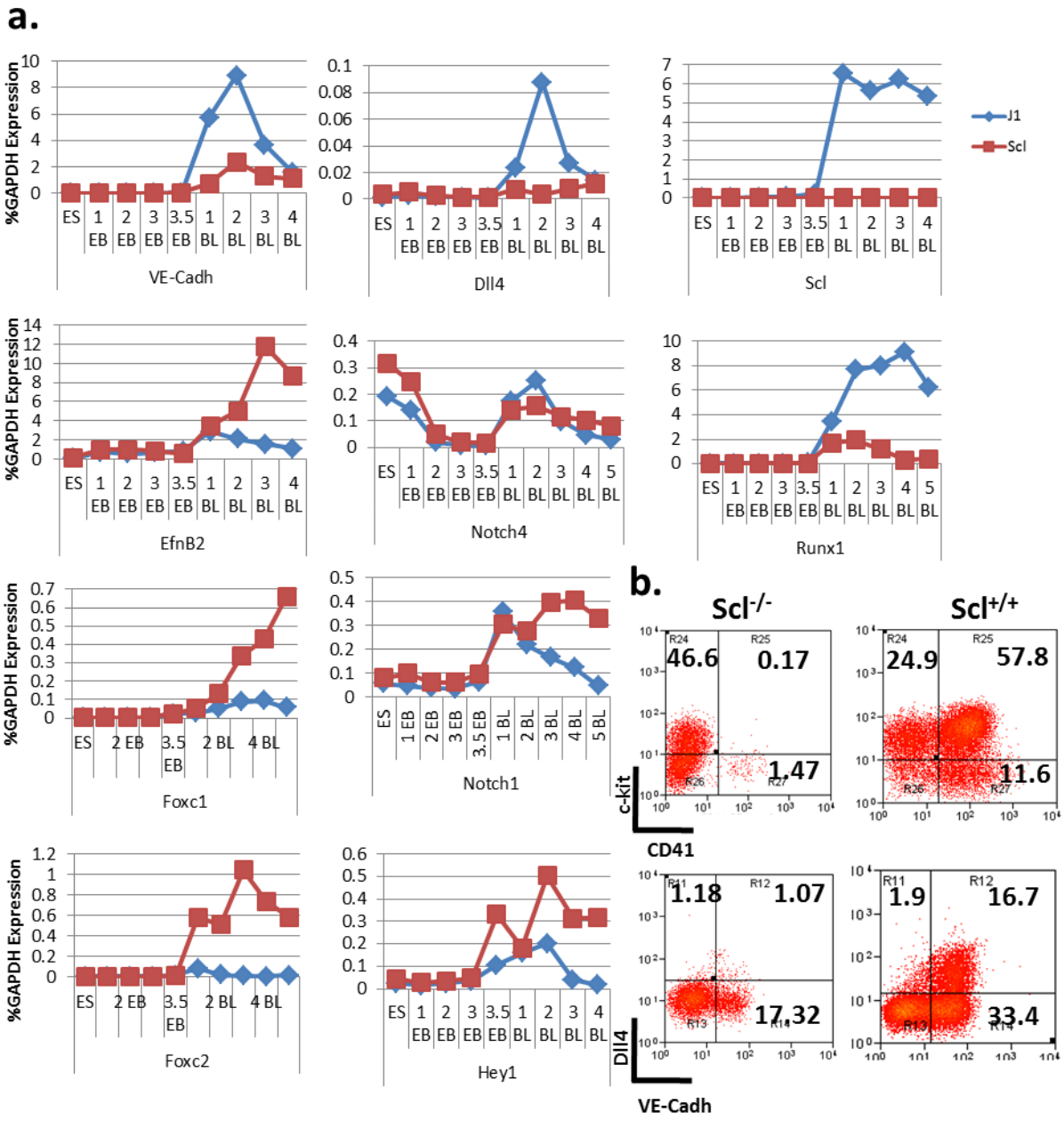

Figure 24. "Arterial" Gene Expression is Intact and Persistent in Scl$^{-/-}$ ES Cells During BL-CFC Differentiation with the Exception of VE-Cadh and Dll4

a. Gene expression across EB and BL-CFC differentiation shows persistent expression of many arterial-associated genes. BL-CFC samples were from re-plated Flk1+ Day 3.5 EBs. (Data normalized to GAPDH; n = 1)

b. Day 1.75 BL-CFC stained for c-kit/CD41 and VE-Cadh/Dll4 show decreased VE-Cadh, Dll4, and CD41 protein expression in Scl-/- versus Scl+/+ cultures. (Representative of 3 experiments)

induction or subsequent maintenance of expression throughout hematopoietic differentiation in BL-CFC culture.

As shown previously, the endothelial marker VE-Cadh was also upregulated upon WT J1 BL-CFC re-plating; it peaked at Day 2 of BL-CFC differentiation before decreasing (when the hematopoietic marker CD41 increases; Figure 5b). The kinetics of VE-Cadh expression were similar in the Scl-/- ES cells, but the expression levels of VE-Cadh were markedly lower compared to WT J1 differentiation, suggesting that endothelial commitment was reduced, though not delayed, in BL-CFC differentiation.

The arterial-enriched marker, Dll4, was strikingly decreased in the Scl-/- cells differentiated in BL-CFC culture. While the expression of Dll4 in WT J1 differentiation is almost completely in the EC VE-Cadh+ population, there was very little Dll4 expression detected in the Scl-/- ES cells throughout BL-CFC differentiation (Figure 24a).

This result is in direct contrast to the gene expression profiles of the other arterial-associated regulators and markers Foxc1, Foxc2, Notch1, and Hey1. All of these genes were properly induced in the Scl-/- ES cells upon re-plating into BL-CFC culture, but showed a pattern of persistent expression after the peak of expression at Day 2 BL-CFC culture in the WT J1 ES cells.

A possible explanation for this pattern of expression is that the Scl-/- cells failed to differentiate into hematopoietic cells (marked by CD41, Scl and Runx1 expression), and were stuck in an endothelial phenotype that continued to express those arterial-associated markers. It is interesting, however, that the endothelial marker VE-Cadh was still much

lower than WT J1 controls, despite a failure to continue on to hematopoietic differentiation and subsequent down-regulation of endothelial-associated genes.

The only arterial-enriched marker that exhibited normal expression kinetics in the Scl-/- cultures compared to the J1 WT cultures was Notch4, which was interestingly also the only marker that was more highly expressed before differentiation (in ES cells compared to EBs and BL-CFC cells) and which showed a pattern of down-regulation during early EB differentiation before being upregulated again upon BL-CFC re-plating. Notch4 (and Notch1) are the receptors for Dll4, which was not induced in the Scl-/- cultures.

The block in hematopoiesis and Dll4 expression in the Scl-/- cultures are shown at the protein level by the flow cytometry results of Day 1.75 BL-CFC in Figure 24b. In these plots, the WT J1 ES cells had over 69% of the cells expressing the hematopoietic marker CD41, whereas only 1.6% expressed CD41 in the Scl-/- BL-CFC cultures. Dll4 was also undetectable in the Scl-/- cultures, whereas a distinct population (16.7%) co-expressed VE-Cadh and Dll4 in the WT J1 cultures. The decrease in VE-Cadh expression shown in the qPCR results (Figure 24a) were also confirmed by the flow cytometry results, where the percentage of VE-Cadh+ cells was lower in Scl-/- compared to the J1 WT BL-CFC cultures (18.4% versus 50.1%).

These results confirm a clear block in hematopoiesis by Scl-/- ES cells but present a mixed picture of “arterial” gene expression in the knockout cells. Dll4 expression is very specifically affected by the knockout of Scl, but the other arterial-enriched markers were induced at the correct time during BL-CFC culture, but instead persisted at much higher levels than seen in WT J1 differentiation.

5.3 Enforced EfnB2 Expression is not Sufficient to Rescue Scl-/- Hematopoietic Defects

Given that the transcription factor Scl plays a critical role in modulating arterial-enriched marker expression and promoting hematopoiesis in WT J1 cells, coupled with the finding EfnB2 overexpression induced a slight increase in hematopoiesis in these cells, it was hypothesized that EfnB2 overexpression in the Scl-/- ES cells could potentially rescue hematopoiesis in these cells. The schematic for this experiment is included in Figure 25a, in which a lentivirus encoding full length EfnB2 (WT) was infected into Scl-/- ES cells during maintenance and sorted for GFP expression before differentiation in EB culture. At Day 3.5 of EB differentiation, GFP+Flk1+ cells were sorted into BL-CFC or arterial monolayer culture to evaluate the occurrence of hematopoietic rescue.

The FACS plots for Day 3.5 EB differentiation are included in Figure 25b, where GFP expression in the EfnB2 overexpression cultures was low due to the fact that GFP was fused to the EfnB2 protein. The overexpression of EfnB2 did not affect mesoderm commitment in the Scl-/- ES cells over-expressing EfnB2, as the percentages of Flk1+ cells was similar to that found in the PGK control infected J1 WT ES cells (51.2% and 57.7% respectively).

The results of re-plating the GFP+Flk1+ cells (outlined by the red box in Figure 25b) from the over-expression (Scl-/-) and control infected (J1 WT) EB cultures into BL-CFC culture are shown in Figure 25c. The J1 ES cells with control PGK infection served as a positive control for hematopoiesis, without an Scl-/- non-infected/PGK infected negative control.

Despite the missing Scl-/- negative control sample, the FACS analysis of Day 4 BL-CFC culture in Figure 25c shows that there was no rescue of hematopoiesis based on cell surface staining for c-kit, CD41, CD45, and CD150. All four hematopoietic markers were not

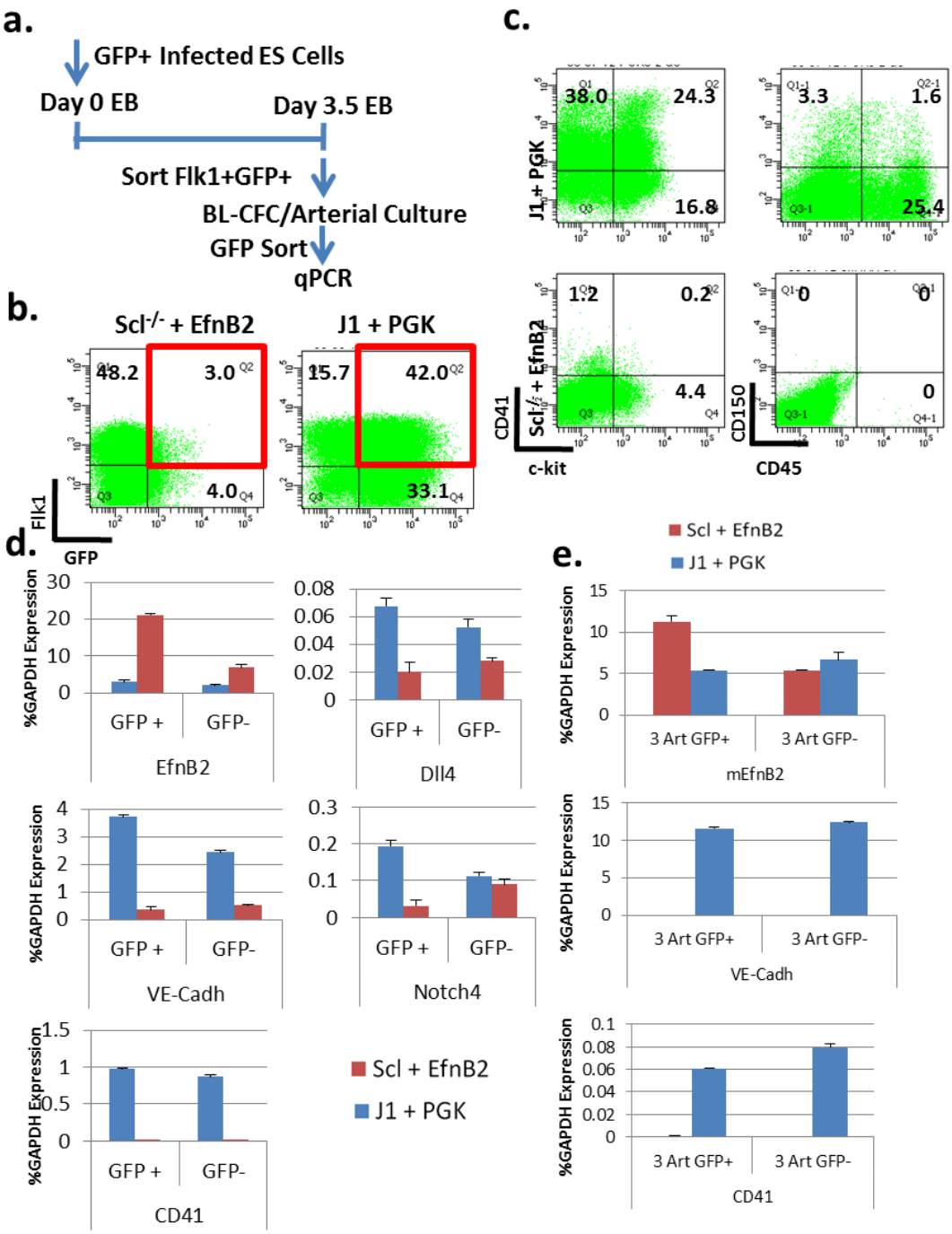

Figure 25. Enforced EfnB2 Expression is not Sufficient to Rescue Scl$^{-/-}$ Hematopoietic Defects

a. Schematic of experiment

b. Flk1/GFP plots of Day 3.5 EBs shows the efficiency of infection and lack of Flk1 mesoderm perturbation due to EfnB2 over-expression. Flk1+GFP+ cells (red box) were sorted and re-plated into BL-CFC and arterial monolayer culture.

c. Scl-/- Day 4 BL-CFC cultures infected with EfnB2-WT do not show rescue of hematopoietic CD41/CD45/CD150 expression compared to J1 (WT) + PGK positive control cultures. (Mean + SD; n = 1)

d. Scl-/- Day 4 BL-CFC cultures infected with EfnB2-WT sorted for GFP+ versus GFP- cells do not show rescue of hematopoietic genes compared to J1 (WT) + PGK positive control cultures. (Mean + SD; n = 1)

e. Scl-/- Day 3 arterial cultures infected with EfnB2-WT sorted for GFP+ versus GFP- cells do not show rescue of hematopoietic genes compared to J1 (WT) + PGK positive control cultures. (Mean +SD; n = 1)

detected at any appreciable level in the Scl-/- cultures overexpressing EfnB2 compared to the WT J1 cells that served as positive controls.

To further confirm this lack of hematopoiesis in the EfnB2 over-expression Scl-/- cultures, the plates were harvested at Day 4 BL-CFC culture and sorted again for GFP expression before qPCR analysis. The results in Figure 25d of the qPCR results confirm the over-expression of EfnB2 in both the GFP+ and GFP- fractions when comparing Scl-/- cells overexpressing EfnB2 with control infected J1 WT ES cells (albeit not a fair comparison). The induction of EfnB2 was very high in this experiment, and almost 15 fold comparing the Scl-/- to J1 controls.

Despite such a profound increase in EfnB2 over-expression in the Scl-/- infected cells, the arterial-enriched markers Dll4, VE-Cadh, and Notch4 were not rescued to the levels found in J1 control infected cells. Most notably, however, was that CD41 was not detectable by qPCR, which was consistent with the results from all previous Scl-/- differentiations. Therefore, based on these results, over-expression of EfnB2 did not rescue endothelial or hematopoietic differentiation in the Scl-/- BL-CFC cultures.

These results of re-plating Scl-/- cells over-expressing EfnB2 into BL-CFC culture were re-capitulated in the arterial monolayer cultures analyzed in Figure 25e. Although these samples also lacked the proper Scl-/- infected/uninfected controls, EfnB2 over-expression was confirmed by comparing Scl-/- EfnB2-WT samples with WT J1 PGK controls. The degree of overexpression was not nearly as profound as in the BL-CFC cultures presented in Figure 23d (here there was only two fold induction). In this arterial monolayer culture, which is more stringent than BL-CFC culture, the Scl-/- cells over-expressing EfnB2 did not replate well, similar to all previous experiments with the uninfected parental Scl-/- ES cells. The

EfnB2 over-expressing Scl-/- cells formed monolayers of cells that looked more stromal than endothelial, and correspondingly, there was no detectable expression of VE-Cadh or CD41 by qPCR in the results graphed in Figure 25e.

Therefore, despite the lack of proper controls for the Scl-/- cells (PGK control infected and uninfected), it is clear that endothelial differentiation (marked by VE-Cadh) and hematopoietic differentiation (marked by CD41 expression) were not rescued to J1 WT levels by enforced EfnB2 expression. This is not surprisingly given the data presented in Figure 24, where it was shown that Scl-/- ES cells express several arterial-enriched markers (with the exception of Dll4), but have an abnormal persistence of these markers. This effect of Scl knock-out was not appreciated, however, until after the rescue experiments were performed and failed, as described here.

5.4 EfnB2 5Y Signaling Mutant Decreases Hematopoietic Output

The striking effects on hematopoietic development of EfnB2 knock down in vitro described in Results Chapter 1 can be attributed to either through disrupted forward signaling of the EfnB2 ligand to its receptor EphB4, or reverse signaling through the ligand itself. The knock-out of EphB4 in ES cell differentiation has been previously published, and interestingly, there was an observed decrease in the number of blast colonies in the EphB4-/- ES cultures (approximately 1/3 less), whereas there was no difference in these experiments knocking down EfnB2 (Figure 7). However, the results of the EphB4-/- ES cells showed much fewer Flk1+ cells at Day 4 when they were re-plated (13% versus 52%) and the EBs were not sorted for Flk1 expression before plating into BL-CFC culture. Therefore the published difference in blast colony counts could have been due to fewer Flk1+ “hemangioblasts” re-plated into BL-CFC culture. The results also do not preclude the possibility that the effect of EphB4 knock

out was exerted through EfnB2. Their results show down-regulation of EfnB2 as a result of EphB4 knock-out by RT-PCR.[109]

EfnB2 signaling is complex, in that it may be induced by phosphorylation of tyrosine resides, by PDZ domain protein-protein interactions or other mechanisms.[45] To test whether tyrosine-phophorylation-dependent signaling could explain the characterized hematopoietic defect, a mutant EfnB2 protein where the intracellular tyrosines have been mutated so that it cannot be phosphorylated (EfnB2-5Y) was over-expressed in the putative HE. In this mutant EfnB2-5Y protein, the extracellular domain remains intact, and therefore maintains its ability to bind and activate the receptor EphB4 for normal forward signaling.

The schematic of the experiment using this EfnB2-5Y mutant construct is included in Figure 26a. Figure 26b shows the successful overexpression of EfnB2 at the protein level using the EfnB2-WT and EfnB2-5Y constructs, as detected by EfnB2 antibody staining of Day 6 EBs, 48 hours after infection. Infection with both constructs expectedly increased the number of EfnB2 positive cells by over eight fold (39% versus 4.6%) when detected by EfnB2 antibody staining, an expression level that was much higher than that determined by GFP expression alone (as the GFP signal was very low due to it being fused with the EfnB2 protein).

Co-staining with VE-Cadh and CD41 antibodies in Figure 26c shows that both early hematopoiesis (CD41 single positive cells) and endothelial commitment (VE-Cadh single positive cells) were both slightly increased in the EfnB2-5Y and EfnB2-WT cultures compared to PGK controls (6.5% versus 4.6% and 6-7% versus 3.3% respectively).

However, upon re-plating into the definitive colony MethoCult3434 assay, the EfnB2-5Y cells had decreased hematopoietic output as measured by the number of scored colonies at Day

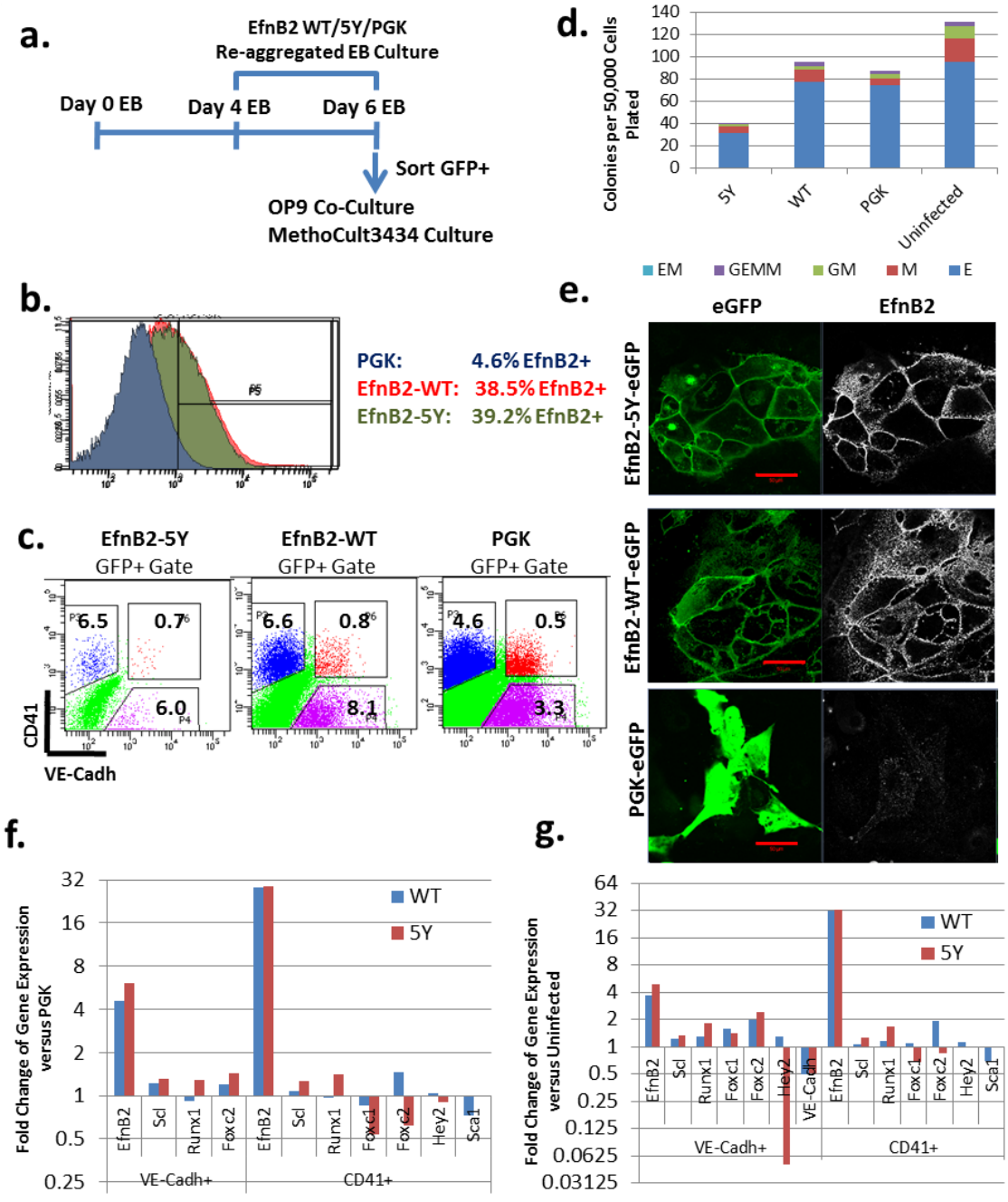

Figure 26. EfnB2 5Y Reverse-Signaling Mutant Over-expression Decreases Hematopoietic Output

a. Schematic of experiment
b. Over-expression of mutant (5Y) and WT EfnB2 in Day 6 EBs (48 hours after infection) is confirmed by FACS staining with EfnB2 antibody.
c. Staining with VE-Cadh/CD41 in Day 6 EBs shows comparable hematopoietic specification in EfnB2-over-expressing cultures compared to PGK control. (n = 1)
d. EfnB2 reverse-signaling mutant (5Y) infected cultures give rise to fewer colonies after 9 days of culture in MethoCult3434 compared to PGK/uninfected controls and EfnB2-WT over-expressing cultures. (50,000 GFP+ Day 6 EBs re-plated; n = 1)
e. Confocal images of Day 5 OP9 cultures show GFP and EfnB2 staining confirming over-expression. Scalebar: 50 uM
f. Fold change of gene expression for GFP+VE-Cadh+(CD41-) and GFP+CD41+(VE-Cadh-) Day 6 EBs (48 hours after infection with either EfnB2-WT or EfnB2-5Y) relative to PGK control infected cells
g. Fold change of gene expression for GFP+VE-Cadh+(CD41-) and GFP+CD41+(VE-Cadh-) Day 6 EBs (48 hours after infection of either EfnB2-WT or EfnB2-5Y) relative to uninfected control cells

9 (by about 50%). This result suggests the possibility that the effect of EfnB2 knock-down presented earlier might be attributable to defective reverse signaling in the EfnB2-ligand expressing cell (as the EfnB2-5Y mutants have defective intracellular tyrosine-dependent signaling but normal extracellular domains that can forward signal to EphB4 expressing cells).

There are two technical issues that need to be noted in this experiment concerning the EfnB2-5Y construct. First, it is not known if the EfnB2-5Y mutant is a dominant negative in ES cells as it is in primary ECs. As is evidenced by the staining of control PGK-infected EBs in Figure 26b, there are cells (4.6%) that express endogenous EfnB2, which may properly reverse signal through this pathway. If so, there would be a properly functional EfnB2 in the putative HE cells even after EfnB2-5Y infection (albeit a minority).

Also because infection with EfnB2-5Y leads to a severe over-expression of EfnB2 protein (notably extracellular domain), it is not possible to de-couple the effects of EfnB2 over-expression with the effect of having blocked reverse signaling due to the mutated tyrosines. However, the results in Figure 26d suggest that EfnB2 over-expression by itself (EfnB2-WT) does not significantly affect hematopoietic output in the MethoCult3434 re-plating assay. If so, these results provide initial evidence that the effect of EfnB2-5Y infection on hematopoiesis is due to defective tyrosine-dependent reverse signaling. A more definitive approach would be to knock-in the 5Y mutant into the EfnB2 endogenous locus of an ES cell line.

The Day 6 EBs infected with EfnB2-5Y/EfnB2-WT/PGK virus were also re-plated into OP9 co-culture. After 5 days, the cultures were stained and imaged by confocal microscopy (Figure

26e). Day 5 cultures show persistent over-expression of EfnB2 by the two (mutant and WT) over-expression constructs.

To see if there were any transcriptional changes due to the over-expression of the mutant or WT EfnB2 protein, EfnB2+ endothelium (VE-Cadh+CD41-) and early committed hematopoietic cells (CD41+VE-Cadh-) were sorted and analyzed by qPCR in Figure 26f and g. In Figure 26f, the data of the WT and 5Y samples were normalized to PGK control sorted cells, while in Figure 26g, they were normalized to uninfected controls. Overall, with the exception of EfnB2 expression which was massively induced in both VE-Cadh+ and CD41+ subsets, most of the other arterial-enriched and hematopoietic regulators/markers were minimally affected. The overall trend appears only to be slight induction of the arterial-enriched and hematopoietic transcription factors, especially in the VE-Cadh+ compartment at this early timepoint (48 hours after infection).

The results presented here, notably the decrease in hematopoietic colonies after MethoCult3434 re-plating of the EfnB2-5Y infected endothelium (compared to EfnB2-WT, EfnB2-PGK and uninfected controls; Figure 26d) suggest that the effects seen with EfnB2 knock-down may be due to a requirement for tyrosine-dependent reverse-signaling.

However, both EfnB2-5Y mutant knock-in mice and EfnB2-ΔPDZ mutant mice (with a deleted PDZ domain) survive to term, suggesting that any hematopoietic defects they may have are not embryonic lethal.[48] The results of the EfnB2-5Y infection presented here, however, do suggest that at least temporarily in vitro, there is a hematopoietic defect when reverse signaling of EfnB2 is inhibited by over-expression of an EfnB2-5Y mutant.

5.5 Summary

In this chapter, the role of EfnB2 during developmental hematopoiesis was further investigated by using techniques to over-express the protein, not only in the WT context but also in an Scl mutant ES cell line that was not hematopoietic. Constitutive EfnB2 expression in the WT ES cells promoted endothelial differentiation (marked by increased VE-Cadh, Notch1 and Dll4 expression) and hematopoiesis (marked by increased Scl and CD41 expression) in BL-CFC culture, but did not rescue the Scl-/- hematopoietic defect. Upon closer investigation of the differentiation of Scl-/- ES cells, it was noted that many of the arterial-associated regulators, including EfnB2 and others in the Notch pathway were improperly persistent during culture.

In the final experiments, a mutant EfnB2 protein with mutated tyrosines that cannot be phosphorylated, but which has an intact extracellular domain that can properly bind and forward signal to its receptor EphB4, was overexpressed in WT ES differentiation to try to de-couple the reverse and forward signaling roles of EfnB2 in the HE in vitro. Preliminary results suggested that there was at least a contribution of reverse signaling to the role of EfnB2 during developmental hematopoiesis, as over-expression of the EfnB2-5Y mutant (but not EfnB2-WT) led to decreased hematopoietic output in MethoCult3434 re-plating assays. Altogether, the data presented in this chapter furthers the knowledge garnered through EfnB2 knock-down and knock-out experiments presented in the first two chapters to form a more complete picture of the role of EfnB2 during developmental hematopoiesis.

6 Discussion and Future Plans

The results presented in this thesis provide insight into a potentially important role for EfnB2 during the differentiation of late "definitive-like" hematopoietic cells from an endothelial intermediate. EfnB2 expression was tightly controlled throughout development, and knock-down of EfnB2 in vitro had very specific effects on the latest stages of hematopoietic differentiation with minimal effects on putative HE/endothelial commitment and survival, early CD41 expression, endothelial colony formation, and BL-CFC hematopoietic differentiation. The requirement for EfnB2 was only seen upon re-plating EfnB2-silenced endothelium into OP9 co-culture and Methocult3434 colony assays, where knock-down prevented the maturation of CD45 single-positive non-adherent hematopoietic cells. These results were tentatively re-capitulated using EfnB2/EphB4 blocking peptides, suggesting that the effects of EfnB2 were exerted through its signaling role.

To confirm the results of genetically manipulating EfnB2 using shRNA infection, YS and DA cells were dissected from WT 36 somite stage (E10.5) embryos and infected with shRNA/PGK lentivirus. The EfnB2-silenced YS and DA showed less hematopoiesis ex vivo, confirming the ES cell results. The EfnB2 knock-out and Tie2 endothelial-targeted EfnB2 knock-out embryos were also analyzed for hematopoietic potential from the YS and DA HE. Preliminary results in the EfnB2 knock-out embryos suggest a DA HE hematopoietic defect (as measured by the total number of live CD41+/CD45+ cells after ex vivo re-plating of the DA on OP9 stromal cells) and a massive skewing of myeloid differentiation of the YS HE (MethoCult3434 culture). Combining many of the most striking results presented in this thesis, several broad and distinct conclusions can be drawn, which are discussed below.

6.1 EfnB2 Gene Expression Precedes Hematopoietic Commitment During Development and in a Tight Time Window Enriches for Hematopoiesis

An EfnB2+ HE was characterized immunophenotypically in BL-CFC culture, within which five distinct populations were identified based on c-kit/CD41 expression that represented populations at different levels of hematopoietic maturation (Figure 5c). A lineage relationship between the five populations was elucidated (schematized in Figure 27) in which a c-kit/CD41 double negative mesoderm population (Population 1) precedes a c-kit single positive hematopoietic competent/HE population (Population 2), followed by CD41+ hematopoietic committed cells (Population 3) that then lose c-kit expression (Population 4) before up-regulating CD41 expression (Population 5).

Identification of this framework allowed for a detailed examination of various arterial-enriched markers in the distinct stages of endothelial/HE/hematopoietic development demarcated by the c-kit/CD41 staining. "Arterial" gene expression in the putative HE (VE-Cadh+c-kit+CD41-) was confirmed by almost homogeneous staining with EfnB2 and Notch1 and heterogeneous staining with Dll4 and Notch4. These arterial-enriched markers were highly correlated with each other.

A dynamic pattern of these markers was identified when analyzing their expression in relation to the distinct c-kit/CD41 compartments. All the markers were up-regulated upon hematopoietic competence (Population 1 to Population 2; acquisition of c-kit) and then massively down-regulated upon hematopoietic commitment (Population 2 to Population 3; CD41 acquisition). This finding, schematized in Figure 27, strongly suggests that "arterial" gene expression precedes hematopoietic commitment during ES differentiation, which is well documented in zebrafish and xenopus.[29, 159]

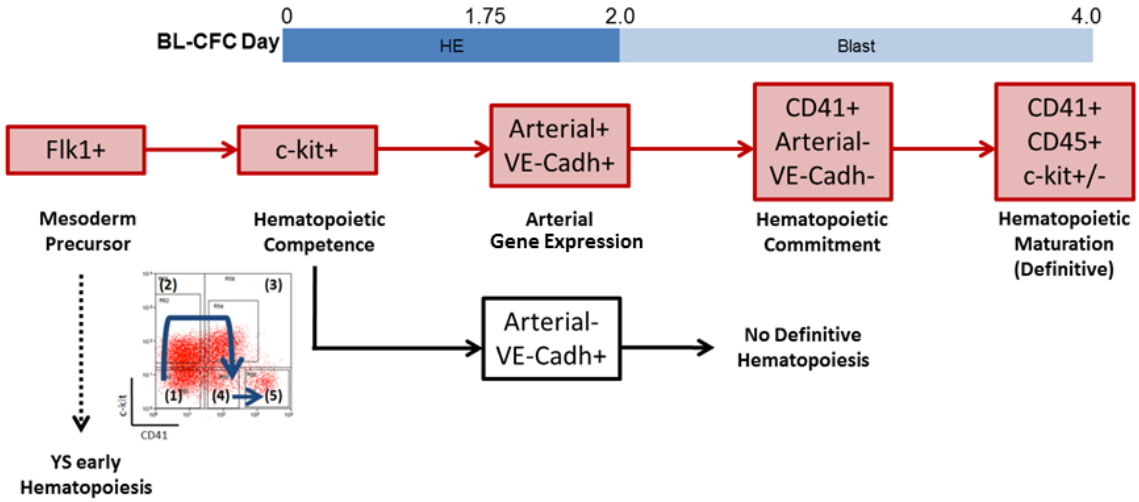

Figure 27. Model of "Arterial" Gene Expression Preceding Hematopoiesis in BL-CFC Differentiation
The transitions of the five c-kit/CD41 populations (Populations 1 – 5) are schematized linearly here across time (through BL-CFC differentiation) to illustrate the idea that arterial markers are up-regulated after hematopoietic competence in the HE compartment (c-kit expression; HE Population; Population 2) before hematopoietic commitment (CD41 expression; Populations 3 -5). The hypothesis derived from this model is that the arterial HE is more enriched for definitive hematopoiesis (DA-like hematopoiesis; CD45 expression) than the non-arterial putative HE.

The identification of an EfnB2+ HE during ES differentiation in vitro prompted us to ask whether this EfnB2+ HE could represent a more DA-like HE with enriched hematopoietic potential. Sorting endothelial cells (VE-Cadh+CD41-) based on EfnB2 expression showed that EfnB2 does enrich for hematopoiesis within a tight time window upon re-plating into OP9 and MethoCult3434 hematopoietic cultures, and thus supports the notion that this EfnB2+ endothelium is more hemogenic than the EfnB2- endothelium.

Further studies should be done to characterize what, if any, molecular differences distinguish between the EfnB2+ versus EfnB2- endothelium in the ES system that have these different hematopoietic potentials. This could identify EfnB2 targets or links between the arteriogenic and hematopoietic programs. It would also be interesting to identify if and when the hematopoietic regulators (Scl, Runx1, etc.) are first induced in either the EfnB2+ or EfnB2- endothelium.

6.2 EfnB2 De-Couples Endothelial and Hematopoietic Commitment

Many studies have unequivocally established the generation of HSCs from the DA endothelium. Due to the critical HE intermediate, it is not surprising that many mutant mice with vasculogenic defects during development show severe subsequent hematopoietic defects (ex. Flk1-/-, VE-Cadh-/-, Dll4+/- and Dll4-/- mice, and Scl-/- ES cells).[24, 41, 106, 114] While the EfnB2-/- mice have been previously characterized to have only arteriogeneic and angiogenic defects during development, it was necessary to first confirm that endothelial differentiation and vasculogenesis was not impaired in EfnB2-silenced ES cells and in the EfnB2-/- DA.[10, 49] This was especially important because EfnB2 expression appeared early during EB differentiation, before VE-Cadh and CD41 was detectable by qPCR (Figure 5b).

By knocking-down EfnB2 in ES cells after mesoderm specification at Day 4 of EB differentiation when its expression was at its maximum level, VE-Cadh and CD31 expression were unaffected compared to controls (Figure 8d - f). Therefore, the profound hematopoietic defects characterized upon re-plating of the EfnB2-silenced ECs into the hematopoietic assays was not attributable to an endothelial defect that propagated through development. Indeed, the EfnB2-silenced endothelium did not appear to have a defect in endothelial re-plating or cell survival. The VE-Cadh+CD45+ population was also formed at a normal frequency upon re-plating of this "non-hemogenic" EfnB2-silenced endothelium (Figure 12d).

Altogether, these data suggest that EfnB2 can decouple endothelial and hematopoietic commitment and that knockdown of EfnB2 results in a non-hemogenic endothelium. This adds EfnB2-silenced endothelium to the growing list of non-hemogenic endothelium in the literature including from Notch1-/-, Jagged1-/-, Sox17-/-, and Runx1-/- mice, VEGF medium and long form morphant zebrafish, and Runx1-/- ES cells (which have normal Tie2 expression during BL-CFC differentiation) which all showed normal levels of VE-Cadh and DA formation.[29, 63, 99, 102, 119, 132, 160]

While all of these different endothelial populations were non-hemogenic, the preservation of "arterial" gene expression in the different mutant non-hemogenic endothelium was not consistent. The E9.5 P-Sp of Notch1-/- mice (and Sox17-/- ES cells) had decreased EfnB2 expression (by RT-PCR), but the E10.5 Jagged1-/- mouse (by immunofluroescence) and VEGF medium and long isoform morphant zebrafish (by in situ hybridization) both had normal EfnB2 expression in the DA, with no results published on the Runx1-/- DA.[29, 102, 160] The results here with the EfnB2-/- mice suggest that EfnB2 expression in the DA is absolutely

required for hematopoiesis from the DA (though probably not sufficient given the VEGF morphant).[29, 102, 160]

The non-hemogenic endothelial populations in these mutant mice have not been systematically characterized, making it difficult to draw firm conclusions relative to whether arteriogenesis is required for hematopoiesis. Another difficulty preventing definitive conclusions is that there is no generally accepted definition for what an arterial endothelium is outside of an anatomically preserved vascular system. EfnB2 has been considered a classic "arterial" marker based on its expression and functional requirement in vivo. We therefore consider "arterial" gene expression to signify expression of EfnB2 in addition to broad expression of the panel of different arterial-enriched markers and transcriptions factors including Notch, Fox, and Sox pathway genes.

This goal was complicated by the fact that while EfnB2 knockdown in vitro did not affect vasculogenesis, it did affect "arterial" gene expression in the VE-Cadh+ endothelial compartment including Foxc1, Foxc2, Sox17 and Hey2 (Figure 14a). This arteriogeneic defect needs to be confirmed in the dissected DA from the EfnB2 knock-out embryos.

Note that these results give only a snapshot gene expression profile of the EfnB2-silenced non-hemogenic endothelium, leaving several open questions. First, it is not known if the decreased levels of these arterial-associated transcription factors are due to a failure of gene induction or if EfnB2 is required for the maintenance of these genes. It is also not known if or how the down-regulation of these arterial-associated transcription factors explains the subsequent hematopoietic defect upon re-plating into OP9 and Methocult3434 culture. For instance, it is not known if EfnB2 exerts its effects directly on the hematopoietic regulators Runx1 and Scl (shown to be down-regulated in the EfnB2-silenced non-

hemogenic endothelium), or if it does so indirectly potentially through one of the arterial-associated regulators. More epistatic studies would need to be performed, such as trying to rescue the hematopoietic defect in these EfnB2-silenced endothelium with enforced expression of the transcriptions factors down-regulated by EfnB2 to see if it is an indirect effect.

It is surprising that EfnB2 knock-down in vitro led to a decrease in the FoxC family and Notch pathway genes given the classical arterial genetic cascade with Hedgehog, VEGF, Notch, and FoxC all upstream of EfnB2. This result needs to be confirmed in the EfnB2-/- DA, but it suggests that there is a feedback loop with EfnB2 and these upstream arterial-associated regulators. Unfortunately, this result also complicates any conclusions as to whether EfnB2, as an "arterial" marker, can directly determine hemogenicity in an endothelium.

Additionally, EfnB2 was shown to not be sufficient to rescue the Scl-/- hematopoietic defect, though it was discovered that induction of "arterial" gene expression was intact in these cells (Figure 24). It would be interesting to see if enforced expression of EfnB2 is sufficient to rescue other non-hemogenic endothelial populations.

6.3 Requirement for EfnB2 Potentially Identifies a YS-like versus DA-like HE Population in vitro

During embryonic development in vivo, there is both temporal and spatial separation of the different hematopoietic waves (summarized in Table 2 and Figures 3 and 4). The spatial separation is almost completely lost in vitro since the ES cells differentiate as one heterogeneous clump of cells. Therefore, while it is hypothesized that all hematopoietic waves of development can and potentially are being modeled in the ES/EB system, it is hard to dissect them out in vitro.

In this thesis, the expression and requirement for EfnB2 was used to further dissect the different hematopoietic waves and intermediates. As a result of our investigation, it is proposed that there are three different waves and hematopoietic intermediates being modeled in the ES/EB system: a YS mesoderm/hemangioblast that gives rise to CD41+ cells in Day 6 EBs, a YS-like HE that gives rise to CD41+ cells in BL-CFC culture, and a DA-like HE from VE-Cadh+EfnB2+ Day 6 EBs that gives rise to CD41+/CD45+ cells in OP9 co-culture or MethoCult3434 medium. This model is schematized in Figure 28. The rationale for defining these distinctions is covered in the Introduction in Section 1.8.4, which lays out the definitions for the different in vitro populations relative to the previously characterized populations in the literature.

EfnB2 was hypothesized to be able to distinguish between the different YS and embryonic waves of hematopoiesis based on its expression patterns during development. In the DA, EfnB2 expression is first detected by LacZ staining at E8.25, over 48 hours before HSC emergence. EfnB2 expression in the YS vasculature was observed beginning at E8.5, which is not only after expression is detected in the DA, but it is also after both primitive (primitive erythrocyte) and pro-definitive (erythro/myeloid) hematopoiesis occurs in the YS. This suggested the existence of some waves of YS hematopoiesis that do not require EfnB2.

The first population is the YS mesoderm that can directly give rise to hematopoietic cells without an endothelial intermediate. This wave is hypothesized to give rise to the CD41+ cells that appear between Day 4 – 6 of EB culture. During this period, VE-Cadh and CD41 mRNA expression appear almost synchronously at Day 4 of EB differentiation (Figure 8a) when there is no detectable VE-Cadh protein by flow cytometry. The simultaneous induction of these markers suggests that it is unlikely that the CD41 expression is derived

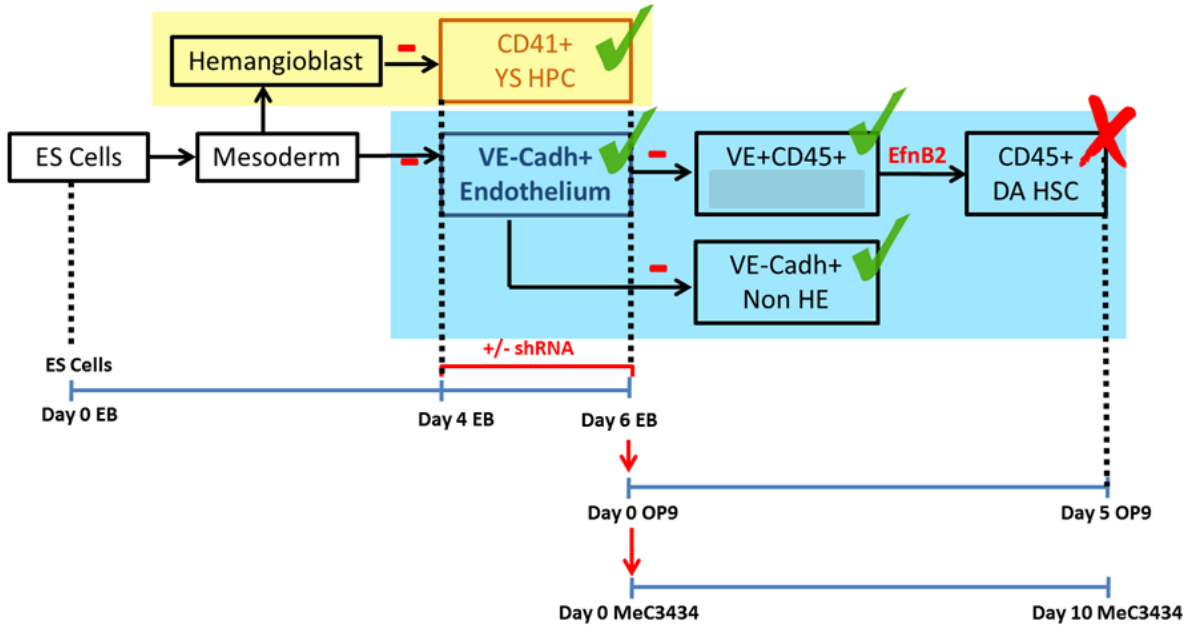

Figure 28. Model of EfnB2 De-Coupling Early YS-like and Late DA-like Waves of Hematopoiesis in vitro
The process of generating early YS-like hematopoieic cells through a hemangioblast intermediate is schematized in the yellow shaded box. Later DA-like hematopoiesis through a HE is schematized in the shaded blue box. Red bars represent transitions that do not require EfnB2. Green check marks identify populations that specify normally despite EfnB2 KD in vitro while the red cross marks the population that is affected by EfnB2 KD. The dotted black lines correlate the schematized populations with the corresponding experimental timepoints.

from a VE-Cadh+ endothelium. The emergence of these CD41+ hematopoietic cells appears to be unaffected by EfnB2 knock-down both before and after mesoderm specification (Figure 9 and Figure 11), supporting the hypothesis that YS waves of hematopoiesis would not require EfnB2 and thus be insensitive to knock-down.

While these YS-like CD41+ cells in Day 6 EBs specify normally without EfnB2, they appear to require EfnB2 for further expansion or maturation, as the CD41+ cells knocked-down for EfnB2 show decreased hematopoietic output in both OP9 co-culture and MethoCult3434 re-platings (Figure 13).

The YS-like HE is hypothesized to be modeled in the BL-CFC re-plating of Day 3.5 Flk1+ mesoderm. The HE was originally identified and published using this culture and the gene expression kinetics in this thesis supports it, as VE-Cadh expression peaks before CD41 expression begins to pick up in BL-CFC culture (Figure 5). However, because EfnB2 knock-down in this endothelium does not affect its hemogenicity, it is believed to not represent the DA-like HE that requires EfnB2. It is interesting to note that while EfnB2 does not appear to be necessary, it is actually highly expressed in this YS-like HE (96% in the c-kit+CD41- compartment; Population 2; Figure 6c). EfnB2 is expressed in the YS beginning around E8.5, suggesting that this YS-like HE could be the intermediate for the third YS wave: YS Meta-Definitive.

The Day 6 VE-Cadh+CD41-EfnB2+ population is hypothesized to model the more DA-like endothelium. The first distinctive feature of this wave is that, much like DA hematopoiesis in the embryo, it occurs temporally after the YS-like mesodermal precursors and YS-like HE. This wave in EB differentiation occurs two days later, beginning at Day 6 EB, and it is interesting to note that DA hematopoiesis also occurs about 48 hours after YS

hematopoiesis. It is of particular interest that the slightly less mature Day 4 EBs (that temporally align with the Day 4 – Day 6 EB and Day 0 – Day 4 BL-CFC culture cells described previously) cannot re-plate into OP9 co-culture (Figure 15b). This suggests that Day 4 EB cells contain the mesodermal precursor while Day 6 EBs contain the bona fide HE. It also suggests that OP9 co-culture and MethoCult3434 culture, which were both designed to support definitive hematopoiesis from adult hematopoietic stem/progenitor populations select for more definitive-like hematopoiesis in ES/EB culture and cannot support hematopoiesis from an earlier developmental progenitor.

The functional evidence that this Day 6 EB population models the DA-like HE is its requirement for EfnB2. The results in Figure 12 show that the VE-Cadh+CD41- endothelium containing the putative HE in Day 6 EBs becomes non-hemogenic upon EfnB2 silencing.

These results are reflected with the ex vivo re-platings of DA and YS from litters of EfnB2-/- and EfnB2+/+ and +/- embryos. At both E9.5 and E10.5 timepoints, the clonogenicity of re-plated YS into MethoCult3434 are less affected in the EfnB2-/- YS compared to littermates (though there appears to be a skewing towards myeloid and no erythroid differentiation). However, at the corresponding timepoints, the DA does not give rise to any significant numbers of hematopoietic cells upon co-culture with OP9 stroma (Figure 18 and Figure 19).

To fully characterize the hematopoietic potential of the YS, it will be necessary to also re-plate YS from younger embryos (Primitive and Pro-Definitive waves) and before circulation is established between the YS and embryo to confirm the dispensability of EfnB2 in all the YS waves (despite the eventual presence of EfnB2 expression in the YS arteries). There is one additional confounding factor (in addition to circulation between the YS and DA) which is the finding that the hypothesized CD41+ YS-mesoderm-derived cells in ES cells do show

decreased expansion/proliferation upon re-plating into MethoCult3434 despite the population being specified normally after EfnB2 silencing. This could explain the decrease (but not block) of ex vivo colony formation in these experiments. From the embryo, it is difficult to de-couple specification from expansion/proliferation.

The approach of using different culture conditions to identify more or less definitive hematopoietic intermediates is not unique to the results here. In fact the results here correlate very well with those previously published. Choi et al first proposed the idea that in human ES culture, BL-CFC differentiation represents a YS-like HE while co-culture on OP9 stroma represents a DA-like HE. The functional studies of EfnB2 appear to extend this idea in mouse ES differentiation.[101]

Irion et al. identified two Flk1+ waves, with the first at Day 3.25 of EB differentiation and the second at Day 5.25.[132] Using Sox17 expression and hematopoietic re-plating assays, they identified the first wave as more YS-like and the second wave as more P-Sp-like. This coincides with the Day 3.5 YS mesoderm and Day 5.25 DA HE characterized here. Their work brings to light another interesting idea which is that in addition to two distinct YS versus DA-like HE, there are probably two distinct YS versus DA-like mesodermal populations. This is because in their cultures to generate the Flk1 waves, the first comes from the Flk1+ cells at Day 3.25 of EB differentiation, while the second one comes from re-aggregation of the Flk1- cells at Day 3.25. It can be assumed in the cultures presented here, that the Day 6 DA-like HE also came from the Flk1- cells at Day 3.5 EB that were not sorted into the BL-CFC experiments.

The ability of one arterial-enriched marker to potentially discriminate against YS versus DA hematopoiesis is not limited to EfnB2, as it has previously been shown that Notch1-/- E9.5

P-Sp had an ex vivo hematopoietic defect but not the E7.0 YS from these mutant mice. This is, however, potentially the first time it has been used in ES differentiation to distinguish the two YS versus DA-like HE populations.

6.4 EfnB2 Potentially Exerts its Effects Through Cell-Autonomous Signalling in the Endothelium

The striking functional requirement for EfnB2 in hematopoietic differentiation can be explained by the transcriptional effects that its knock-down had in the endothelium. It was shown that EfnB2 silencing decreased the expression of arterial-enriched transcription factors Hey2, Foxc1, Foxc2, and Sox17 and the hematopoietic master regulators Scl and Runx1 (Figure 14a). In contrast, the early hematopoietic VE-Cadh-CD41+ cells silenced for EfnB2 had increased Dll4, Notch1, Notch4 and Hey2 expression suggesting that these cells had failed to down-regulate arterial-enriched markers, as it was normally observed upon CD41 acquisition, potentially explaining their decreased hematopoietic output upon MethoCult3434 re-plating.

To confirm that these changes were attributable to EfnB2 signaling functions and not just to its expression, peptides blocking EfnB2/EphB4 binding were used to block both binding and forward and reverse signaling in the EfnB2- and EphB4- expressing cells. Peptide treatment functionally recapitulated the EfnB2 silencing results, suggesting that the effects of EfnB2 knock-down were due to its signaling role.

Despite the same functional effects between EfnB2 knock-down and blocking peptide treatment, however, both TNYL-RAW and SNEW peptide treatment led to transcriptional effects in exact opposition with the effects of EfnB2 genetic knock-down. Instead of a down-regulation of arterial-associated transcription factors not in the Notch pathway and

an up-regulation of those in the Notch pathway, peptide treatment increased Foxc2 and Sox17 while decreasing Dll4 and Notch4. Instead of the repression of the hematopoietic master regulators Scl and Runx1, blocking EfnB2 signaling led to an induction of these two transcription factors (Figure 16). These results need to be repeated and could provide insight into what is absolutely essential downstream of EfnB2 for the endothelium to be hemogenic.

To further confirm that EfnB2 was required in the EfnB2-ligand expressing cell (presumably as it is in the DA where the endothelium and not mesenchyme is EfnB2+) for reverse signaling, genetic experiments were done over-expressing an EfnB2 reverse signaling mutant that could properly activate forward signaling in EphB4+ cells but not reverse-signaling through tyrosine phosphorlyation into itself. These mutant-infected cells also recapitulated the knock-down and blocking peptide-treated endothelium. Interestingly, the gene profiles of the mutant-infected endothelium recapitulated the blocking peptide-treated cells more than the EfnB2-silenced cells. The transcription factor Foxc2 and the hematopoietic master regulators Scl and Runx1 were all increased in the endothelium compared to PGK infected controls (Figure 26f and g).

Studies of EphB4-/- ES cells suggest that the receptor is required for hematopoiesis. However, the results may not be conclusive in that they were based on blast colony counts after re-plating whole EBs. Results in the paper show a 75% decrease in Flk1+ cells in the EBs, and the graph of blast colony counts shows about a 75% decrease in colony counts as well.[109] Therefore, the BL-CFC defect could potentially be attributable to a hemangioblast specification defect. Also, at the time of HSC emergence, EphB4 should no longer be expressed in the DA, and thus not be required cell-autonomously in the HE.[10, 49]

Altogether, these results suggest that EfnB2 is required cell-autonomously in the DA for reverse signaling to induce definitive hematopoiesis. The endothelial-specific requirement for EfnB2 needs to be further confirmed with the targeted deletion of EfnB2 in mice using inducible VE-Cadh-Cre and Tie2-Cre mice crossed with EfnB2 floxed mice. Preliminary results presented here show too much variability to draw definitive conclusions.

The requirement for EfnB2 during hematopoietic differentiation from the HE may parallel the requirement for EfnB2 in vessels engaged in angiogenic sprouting. In the absence of EfnB2, lymphatic endothelial cells do not respond to VEGF-C stimulation due to defective VEGFR3 receptor internalization and signaling.[54] In addition, mature lung endothelial cells lacking EfnB2 or expressing a mutant EfnB2 with defective PDZ-dependent signaling do not respond to VEGF-A stimulation due to defective VEGFR2 internalization and signaling.[53]

Therefore, it is possible that the requirement for EfnB2 during hematopoietic differentiation of the HE rests on the essential contribution of EfnB2 to VEGF/VEGFR signaling. This is consistent with the requirement for VEGF during ES culture and with VEGFR2 (Flk1) receptor expression on mesodermal cells. Further work will be required to test if this is the case. However, given the more severe phenotype of VEGFR knock-out mice (Flk1(VEGFR2)$^{-/-}$ mice die at around E8.5-9.5 whereas EfnB2$^{-/-}$ mice die at E11.0), some level of VEGFR signaling may be preserved despite loss of EfnB2, at least in the earliest stages.[10, 23] Consistent with this, it was reported that some VEGFR internalization is preserved in EfnB2-deficient angiogenic vessels studied (measured by percent perinuclear accumulation of VEGFR2/3).[53, 54] However, a significant decrease in VEGFR internalization could potentially explain the decreased endothelial and hematopoietic output observed in vitro and ex vivo in the current study.

The establishment of a requirement for EfnB2 in the DA would be interesting in light of the fact that other signaling pathways, most notably Notch and VEGF, have been shown in lower vertebrates to be required non-cell-autonomously from the somite.[29, 103]

6.5 EfnB2 May Play an Unidentified Role in the Hematopoietic Niche

The original aim of this project was to focus on the HE both during the in vitro differentiation of ES cells and in vivo in the knock-out mice. In selecting EfnB2, a surface signaling molecule, an interesting aspect presented itself in regards to the interaction of this EfnB2+ HE with its surrounding niche. The characterization of the OP9 stromal cell line used in the in vitro (and ex vivo) re-platings of the HE not only suggested that EphB4 and activated intracellular phosphorylated EfnB was present in the OP9 stroma (Figure 22), but that silencing EfnB2 functionally affected the morphology and proliferation of the stromal cell line, probably to a degree that it could not support hematopoiesis from the ES and embryo-derived HE populations, though this remains to be tested.

To further understand the potential role of EfnB2/EphB4 in the stem cell-niche interaction during developmental hematopoiesis, it will first be necessary to return to a careful descriptive analysis in vitro, and especially in vivo (of embryo sections), to define expression of EfnB2 versus EphB4. Particular attention will need to be taken towards the expression of molecules in the sub-aortic mesenchyme before and during the time of HSC emergence (E10.5) to identify potential niche signals in inducing the hematopoietic program. Characterization of the endothelial-targeted EfnB2 knock-out mice (by inducible VE-Cadh and Tie2) will also aide in dissecting out endothelial cell-autonomous versus non-cell-autonomous requirements for EfnB2 in the HE population.

Another interesting hematopoietic niche for investigation in the future would be the adult bone marrow, from which the OP9 stromal cell line was derived. Given the in vitro analysis here, there may also be a role for EfnB2 and EphB4 during adult hematopoietic maintenance and differentiation in the bone marrow.

6.6 Concluding Remarks

It was hypothesized at the beginning of this work that studying EfnB2 would allow for the potential identification of a more "DA-like" HE in ES/EB culture and potentially elucidate the arterial and hematopoietic characterics of the DA HE. Through immunophentyping, genetic and molecular manipulations of EfnB2, and characterization of the knock-out mice presented in this thesis, the first of these goals looks achievable with a few more experiments. EfnB2 expression and function appears to be recapitulated in a specific endothelial population, identified here in Day 6 EBs, that closely relates to the DA HE. This work may lay the groundwork for further studies to bring the identification of a true LT-HSC in ES differentiation possible.

The studies in this thesis, however, have also revealed far deeper complexities regarding EfnB2 and its role in both arteriogenesis and hematopoiesis. The data provide many clues to the role that EfnB2 plays in the functional context. However, the molecular and genetic mechanisms underlying its role in specifying "arterial" gene expression and hematopoietic potentials remain elusive. Further work will be done to continue pursuing these questions, with the hope that the role of EfnB2 in determining hemogenicity in an arterial endothelium can be fully understood.

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