The Specification Of Hemogenic Endothelium During Embryogenesis And Beyond

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Abstract
The primary goal of regenerative medicine is the in vitro derivation of cells that are functional and safe for transplantation into patients. Although progress has been made towards this goal there are no clinical applications that utilize cells derived in vitro from pluripotent stem cells or reprogrammed primary cells. The foremost reason for this is an incomplete understanding of the development processes that regulate cell identity. In order to gain a better understanding of the specification of hematopoietic stem cells (HSC), I studied their de novo generation during embryogenesis.

Using confocal microscopy, I mapped out hematopoiesis during midgestation and discovered that it is less spatially restricted than previously thought. I identified two new sites of blood formation, the dorsal longitudinal anastomotic vessels and the intersomatic vessels. I also identified three waves of hematopoiesis in the heart. During the final wave of cardiac hematopoiesis, spherical protrusions of endocardium encapsulating hematopoietic cells called cardiac blood islands (CBIs) form on the ventricles. CBIs pinch off from the ventricle and contribute to the coronary vasculature in a novel mechanism of vasculogenesis. Interestingly, loss of the RAS GTPase activating protein Nf1 significantly increases the total number of CBIs. Furthermore, I found that the mechanism of vasculogenesis employed by the heart also occurs from the umbilical and vitelline arteries. During midgestation clusters of hematopoietic cells detach from the artery forming extravascular islands. The extravascular islands than sprout and contribute to nearby vessels. My studies revealed new sites of hematopoiesis and shed light on a novel mechanism of vasculogenesis that is coupled with embryonic hematopoiesis.

In the final section of this dissertation, I discuss the precursors of HSCs called hemogenic endothelium (HE). HE undergoes an endothelial to hematopoietic transition (EHT), which is dependent on the transcription factor, Runx1. Runx1 activates hematopoietic genes and inhibits endothelial genes resulting in the endothelial to hematopoietic transition. By ectopically expressing Runx1 in endothelium, I found that embryonic and fetal populations are competent to respecification into multilineage hematopoietic cells but adult endothelium is not. Understanding the specification of HE will be imperative for the successful derivation of HSCs in vitro.

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THE SPECIFICATION OF HEMOGENIC ENDOTHELium DURING EMBRYOGENESIS AND BEYOND

Amanda D. Yzaguirre

A DISSERTATION

in

CELL AND MOLECULAR BIOLOGY

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ABSTRACT

THE SPECIFICATION OF HEMOGENIC ENDOTHELium DURING EMBRYOGENESIS AND BEYOND

Amanda D. Yzaguirre
Nancy A. Speck

The primary goal of regenerative medicine is the *in vitro* derivation of cells that are functional and safe for transplantation into patients. Although progress has been made towards this goal there are no clinical applications that utilize cells derived *in vitro* from pluripotent stem cells or reprogrammed primary cells. The foremost reason for this is an incomplete understanding of the development processes that regulate cell identity. In order to gain a better understanding of the specification of hematopoietic stem cells (HSC), I studied their *de novo* generation during embryogenesis.

Using confocal microscopy, I mapped out hematopoiesis during midgestation and discovered that it is less spatially restricted than previously thought. I identified two new sites of blood formation, the dorsal longitudinal anastomotic vessels and the intersomitic vessels. I also identified three waves of hematopoiesis in the heart. During the final wave of cardiac hematopoiesis, spherical protrusions of endocardium encapsulating hematopoietic cells called cardiac blood islands (CBIs) form on the ventricles. CBIs pinch off from the ventricle and contribute to the coronary vasculature in a novel mechanism of vasculogenesis. Interestingly, loss of the RAS GTPase activating protein Nf1 significantly increases the total number of CBIs. Furthermore, I found that the mechanism of vasculogenesis employed by the heart also occurs from the umbilical and vitelline arteries. During midgestation clusters of hematopoietic cells detach from the artery forming extravascular islands. The extravascular islands then sprout and
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In the final section of this dissertation, I discuss the precursors of HSCs called hemogenic endothelium (HE). HE undergoes an endothelial to hematopoietic transition (EHT), which is dependent on the transcription factor, Runx1. Runx1 activates hematopoietic genes and inhibits endothelial genes resulting in the endothelial to hematopoietic transition. By ectopically expressing Runx1 in endothelium, I found that embryonic and fetal populations are competent to respecification into multilineage hematopoietic cells but adult endothelium is not. Understanding the specification of HE will be imperative for the successful derivation of HSCs in vitro.
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CHAPTER 1: Introduction

The role of Runx1 in embryonic blood cell formation
Introduction

The *de novo* generation of hematopoietic stem and progenitor cells (HSPC) occurs solely during embryogenesis from a population of epithelial cells called hemogenic endothelium (HE). During midgestation HE cells in multiple intra- and extraembryonic vascular beds leave the vessel wall as they transition into HSPCs in a process termed the endothelial to hematopoietic transition (EHT). Runx1 expression in HE cells orchestrates the transcriptional switch necessary for the transdifferentiation of endothelial cells to functional HSPCs. Runx1 is widely considered the master regulator of developmental hematopoiesis because it plays an essential function during specification of the hematopoietic lineage during embryogenesis. Here I review the role of Runx1 in embryonic HSPC formation, with a particular focus on its role in hemogenic endothelium.

Almost all blood cells in the adult mammal differentiate from hematopoietic stem cells (HSCs) in the bone marrow. However HSCs do not originate in the bone marrow, and instead differentiate in the embryo before bone or bone marrow forms (Müller et al., 1994). The majority of HSCs, defined as cells that can engraft adult transplant recipients, differentiate from immature HSC precursors called pre-HSCs (Rybtsov et al., 2011, Taoudi et al., 2008). Pre-HSCs, in turn, differentiate from a small population of hemogenic endothelial cells (Zovein et al., 2008, Chen et al., 2009). The maturation of pre-HSCs into HSCs predominantly takes place in the fetal liver, which is colonized by pre-HSCs via the circulation (Rybtsov et al., 2016, Kieusseian et al., 2012). Following birth, HSCs leave the fetal liver and settle in the bone marrow, where they remain for the rest of adult life.
HSCs develop at midgestation in the mouse embryo, and at 1 month of gestation in the human embryo (Ivanovs et al., 2011, Müller et al., 1994). However, before HSCs are present, several other primitive types of blood cells emerge that are essential for embryonic viability. Hematopoietic progenitors (cells that can produce differentiated blood cells, but do not possess long-term multilineage reconstitution potential) and HSCs form in three waves, as described below. Runx1 is important for the differentiation of all embryonic blood cell lineages, and is particularly essential for the differentiation of blood cells in the second two waves from hemogenic endothelium.

**Primitive hematopoiesis—the first wave**

Primitive hematopoietic cells are one of the earliest functional cell populations to appear during embryogenesis. They emerge in the extraembryonic yolk sac shortly after gastrulation, and prior to the onset of circulation, a functional vascular system, or the development of HSCs (Palis et al., 1999, Ferkowicz and Yoder, 2005). Primitive hematopoietic cells in this first wave include unipotent primitive erythrocyte progenitors, bi-potent erythrocyte/megakaryocyte progenitors, and primitive macrophages (Xu M et al., 2001, Tober et al., 2007, Palis et al., 1999, Moore and Metcalf, 1970, Haar and Ackerman, 1971, Tracey et al., 1998). These primitive blood cells have distinct morphological and functional features compared to their “definitive” counterparts that form during the second and third waves of hematopoiesis. For example, primitive erythrocytes are larger than definitive erythrocytes, they express embryonic and adult globins, and they retain their nucleus when entering the circulation (Palis et al., 1999,
Palis, 2014, Kingsley et al., 2004). Primitive megakaryocytes have a lower ploidy class than definitive megakaryocytes, and more rapidly produce platelets that prevent hemorrhaging in the primitive vascular plexus as it develops into a functional cardiovascular system (Xu M et al., 2001, Potts et al., 2014).

The mesodermal cells that give rise to primitive hematopoietic cells originate from a population of proximal epiblasts that migrate through the primitive streak and into the extraembryonic yolk sac early during gastrulation (Lawson et al., 1991). The mesoderm accumulates to form thickened regions called mesodermal masses that then differentiate into blood islands consisting of primitive erythroblasts, and into angioblasts that will form the vascular plexus of the yolk sac (Ferkowicz and Yoder, 2005, Haar and Ackerman, 1971). Due to their parallel development and close physical association, it was initially hypothesized that blood and endothelial cells in the yolk sac shared a common progenitor called the hemangioblast (Murray, 1932, Sabin, 1920). This idea was supported by the demonstration that hemangioblast-like progenitors that gave rise to both blood and endothelial cells could be isolated from embryonic stem (ES) cell cultures (Choi et al., 1998, Zambidis et al., 2005). It later became apparent that the putative bi-potent hemangioblast was actually a tri-lineage progenitor that could also give rise to smooth muscle cells (Ema et al., 2003). However, in vivo clonal analyses provided evidence against the existence of a bi-potential hemangioblast in the yolk sac, and instead suggested that yolk sac endothelium and hematopoietic cells are derived from adjacent but independent regions of the epiblast, and are thus specified prior to entering the primitive streak (Padron-Barthe et al., 2014, Ueno and Weissman, 2006).
Runx1 is expressed in the mesodermal mass in the yolk sac, and in the progenitors of primitive hematopoietic cells in the mouse embryo with the exception of primitive erythrocytes that initially express Runx1 but rapidly downregulate its expression shortly after emergence (North et al., 1999, Zeigler et al., 2006, Lacaud et al., 2002). Two of the three primitive hematopoietic lineages, primitive erythrocytes and megakaryocytes can form in the absence of Runx1, however their normal development is affected by Runx1 loss. Runx1-deficient embryos produce numbers of primitive erythroid colonies comparable to littermate controls and do not appear anemic (Yokomizo et al., 2008, Lacaud et al., 2002). However, more detailed analysis revealed reduced expression of cell surface Ter119 and the hematopoietic transcription factors KLF1 and GATA1, and defective maturation of Runx1-deficient erythrocytes (Yokomizo et al., 2008, Castilla et al., 1996). Furthermore, about 30% of primitive erythrocytes derived from Runx1-/- embryos displayed a deformed shape characterized by a rough punctate surface (Yokomizo et al., 2008). Despite these abnormalities Runx1-/- primitive erythrocytes are functional, as indicated by normal levels of benzidine staining (hemoglobinization) and the fact that Runx1-/- embryos survive until E12.5, which is longer than GATA1-deficient embryos, which die by E10.5 with severe anemia due to the lack of functional primitive erythrocytes (Yokomizo et al., 2008, Fujiwara et al., 1996, Okuda et al., 1996, Wang et al., 1996a). Runx1 is not required for the formation of primitive diploid megakaryocytes, although their numbers were lower in Runx1 deficient yolk sacs (Potts et al., 2014). Primitive macrophages, on the other hand, absolutely require Runx1, as they are lacking in Runx1-/- embryonic stem cell differentiation cultures (Lacaud et al., 2002) and embryos (Li et al., 2006). In summary, in the absence of Runx1, primitive macrophages are absent, diploid megakaryocytes are reduced in
number, and primitive erythropoiesis is abnormal. Although it is often stated that Runx1 is required for definitive, but not primitive hematopoiesis, this is inaccurate as Runx1 is strictly required for the development of one primitive blood cell lineage, and important for the normal development of two others.

Runx1 has also been shown to play a role during primitive hematopoiesis in zebrafish and Xenopus embryos. In Xenopus embryos, Runx1 is expressed in the ventral blood island (VBI), which is analogous to mouse yolk sac blood islands (Tracey et al., 1998). Inhibiting Runx1 function via the injection of a dominant negative form of Runx1 mRNA prior to the VBI stage drastically reduced the number of Benzidine$^+$ primitive erythrocytes (Tracey et al., 1998). Similarly, in zebrafish embryos, morpholino knockdown of Runx1 expression at the one to eight cell stage resulted in fewer primitive erythrocytes (Kalev-Zylinska et al., 2002). The primitive macrophage and megakaryocyte populations were not examined in either species. The decrease in primitive erythrocytes in both zebrafish and Xenopus embryos is contrary to what is observed in the mouse and suggests that Runx1 plays a more essential role in primitive erythropoiesis during zebrafish and Xenopus development.

**Definitive hematopoiesis-the second and third waves**

The term “definitive” in the context of developmental hematopoiesis has several meanings, but was originally used to describe adult erythrocytes, which unlike primitive erythrocytes are small and concave, lose their nuclei before entering the circulation, and do not express embryonic globin (Palis et al., 1999, Palis, 2014, Kingsley et al., 2004).
Defined this way, definitive hematopoiesis encompasses two overlapping waves of blood development. Wave 2 is characterized by the generation of erythro-myeloid progenitors (EMPs) and lymphoid progenitors in the yolk sac and embryo proper (Yoder, 2014). EMPs can be found as early as E8.25 in the murine yolk sac (Palis et al., 1999, McGrath et al., 2015) and heart (Nakano et al., 2013). The next wave 2 progenitor to appear are lymphoid progenitors, which are found at E9.5 in the yolk sac and the paired dorsal aorta, and by E10.5 in the umbilical artery (UA) and vitelline artery (VA) (Yoshimoto et al., 2011, Yoshimoto et al., 2012). Adult repopulating HSCs (wave 3) do not appear until E10.5; they are generated initially in the dorsal aorta (DA), UA, and VA, and can subsequently be found in the yolk sac, head and placental vasculature (Li et al., 2012, Li et al., 2016, Rhodes et al., 2008, Gordon-Keylock et al., 2013, de Bruijn et al., 2000, Gekas et al., 2005). They are thought to arrive via circulation in these latter sites, instead of being generated in situ (Dieterlen-Lievre, 1975, Cumano et al., 2001, Medvinsky and Dzierzak, 1996).

Definitive hematopoietic cells are derived from a population of epithelial cells called hemogenic endothelium (HE) that are part of the interior lining of specific blood vessels in the embryo (Swiers et al., 2013b). HE is a transient population that gives rise to hematopoietic progenitors and stem cells in a process termed the endothelial to hematopoietic transition (EHT) (Kissa and Herbomel, 2010). Live-imaging studies of HE cells in vitro and in vivo have captured this dynamic process (Kissa and Herbomel, 2010, Boisset et al., 2010, Bertrand et al., 2010, Lancrin et al., 2009). Initially, HE cells appear flat in images generated by confocal microscopy, and integrated in the endothelial monolayer. Scanning electron microscopy of mouse HE cells revealed them to be more
oblong, with rounded cell bodies and filopodia-like protrusions of the membrane as compared to non-hemogenic endothelial cells (Bos et al., 2015). As the EHT progresses, the HE cell bends away from the vessel wall until it rounds up and detaches from the endothelial layer becoming a mobile hematopoietic cell (Kissa and Herbomel, 2010, Boisset et al., 2010, Bertrand et al., 2010, Lancrin et al., 2009, Eilken et al., 2009).

In mouse embryos, HE is localized in the yolk sac, the large arteries of the embryo proper, the heart, and the chorionic plexus (Rhodes et al., 2008, Li et al., 2012, Nakano et al., 2013, Yzaguirre and Speck, 2016a). HE cells are identified based on Runx1 expression (North et al., 1999) (Fig. 1.1). Runx1 is a critical regulator of the EHT and as such, suppresses an endothelial transcriptional program and initiates a hematopoietic program in HE allowing the EHT to occur (Lancrin et al., 2012a, Chen et al., 2009, North et al., 1999, Yokomizo et al., 2001). Transcriptional and functional analyses demonstrated that HE cells derived from E8.5 mouse embryos preferentially form endothelial tubules in culture conditions that support both endothelial and hematopoietic cells (Swiers et al., 2013a). In contrast, E10.5 HE preferentially forms hematopoietic cells in vitro. The functional change that occurs between E8.5 and E10.5 was accompanied by a transcriptional shift characterized by the upregulation of hematopoietic factors such as Runx1, Meis1, Gata2, Gata3 and Myb suggesting that initially HE cells are functional endothelial cells, but as the hematopoietic program ramps up during midgestation HE loses endothelial function and gains hematopoietic potential (Swiers et al., 2013a).

In mammalian embryos, after the EHT occurs, newborn hematopoietic cells adhere to the vessel wall within the lumen forming clusters of hematopoietic cells. The
peak of EHT in the mouse embryo (E10.5) is marked by the formation of hundreds of Kit$^+$ hematopoietic clusters within the lumens of the DA, VA and UA and dozens residing within the vascular plexus of the yolk sac (Yokomizo and Dzierzak, 2010, Frame et al., 2015, Yzaguirre and Speck, 2016a). Analysis of the Kit$^+$ hematopoietic clusters within the embryo proper between E10.5 and E11.5 has revealed that they consist of lymphoid progenitors, a small number of myeloid progenitors, and pre-HSCs that can mature into HSCs capable of long-term multilineage reconstitution (Boisset et al., 2015, Li et al., 2014, Taoudi et al., 2008, Rybtsov et al., 2011). By E12.5 most hematopoietic cluster cells have entered the circulation and made their way to the fetal liver where they undergo maturation and proliferation, expanding the pool of HSCs and hematopoietic progenitors (Ema and Nakauchi, 2000, Kieusseian et al., 2012, Rybtsov et al., 2016). Beginning at E17.5 HSCs migrate to the bone marrow where they will reside throughout the lifetime of the animal (Christensen et al., 2004).

In zebrafish embryos the EHT occurs away from the lumen of the dorsal aorta, and the newly formed hematopoietic cells must traverse through the subaortic space and enter circulation via the axial vein (Kissa et al., 2008a). Once in circulation hematopoietic cells migrate to the caudal hematopoietic tissue that is akin to mammalian fetal liver where they differentiate and expand before traveling to definitive hematopoietic organs (Murayama et al., 2006).

**Runx1 is required during definitive hematopoiesis**
Runx1 is expressed in all sites of blood formation. During gastrulation Runx1 is expressed in the extraembryonic mesoderm that gives rise to primitive hematopoietic cells (Swiers et al., 2013a, Lacaud et al., 2002, Zeigler et al., 2006). During definitive hematopoiesis Runx1 is the most reliable marker of hemogenic endothelium and is expressed by all hematopoietic cells with the exception of erythrocytes (North et al., 2004, North et al., 1999, North et al., 2002, Lorsbach et al., 2004). In addition to hematopoietic tissues, Runx1 is expressed in the olfactory epithelium, spinal ganglia, maxillary processes and the mesenchyme that flanks the ventral length of the dorsal aorta (North et al., 1999, Levanon et al., 2001a). Germline deletion of Runx1 results in the elimination of all definitive hematopoietic cells and embryonic lethality by E12.5 (Wang et al., 1996a, Okuda et al., 1996). Embryonic lethality of Runx1 deficient embryos is due to hemorrhaging within the ventricle of the central nervous system, the pericardial space, and the peritoneal cavity (Okuda et al., 1996, Wang et al., 1996a). The hemorrhaging is likely secondary to the lack of definitive hematopoietic cells because hematopoietic cells are involved in vascular remodeling during embryogenesis. For example, hematopoietic cells express angiopoietin-1 (Ang-1), a chemoattractant that promotes blood vessel sprouting (Witzenbichler et al., 1998). Analysis of the vasculature of Runx1 deficient embryos revealed decreased branching in the head, pericardium and vitelline artery in the yolk sac (Takakura et al., 2000). When Runx1 deficient explants were supplemented with hematopoietic cells or Ang-1 the vascular defects were rescued, suggesting that the vascular defects that cause hemorrhaging in Runx1 deficient embryos are due to the loss of Ang-1 expressing definitive hematopoietic cells (Takakura et al., 2000).
Runx1 can bind DNA as a monomer *in vitro*, but when Runx1 heterodimerizes with its non-DNA binding subunit CBFβ, flexible DNA-recognition loops in Runx1 are stabilized and its binding affinity for DNA increases (see Bushweller and Tahirov, this volume). Embryos deficient for CBFβ died by E12.5 with hemorrhaging akin to Runx1 deficient embryos and had significantly fewer definitive hematopoietic progenitors in their fetal livers when compared to littermate controls (Sasaki et al., 1996, Bresciani et al., 2014, Wang et al., 1996b, Niki et al., 1997). Similar results were obtained in CBFβ deficient zebrafish, confirming that CBFβ is required for Runx1 to function during definitive hematopoiesis (Sasaki et al., 1996, Bresciani et al., 2014). Interestingly, definitive hematopoiesis is not completely blocked in CBFβ deficient embryos to the same extent as in Runx1 deficient embryos. For example, the hematopoietic-specific transcription factor c-Myb is not expressed at sites of definitive hematopoiesis in Runx1 deficient zebrafish embryos, but it is expressed in the dorsal aorta of CBFβ deficient zebrafish embryos (Bresciani et al., 2014). Furthermore, definitive erythroid and myeloid progenitors are never found within the fetal livers of Runx1 deficient embryos but small numbers (approximately 40-fold less than wildtype controls) are present in CBFβ deficient fetal livers (Wang et al., 1996b). These studies suggest that the low-affinity binding of Runx1 to DNA in the absence of CBFβ is enough to initiate definitive hematopoiesis but is not sufficient to supply enough definitive hematopoietic cells to prevent embryonic lethality.

Unlike Runx1 and CBFβ deficient embryos, Runx1 heterozygous embryos survive well into adulthood and have relatively minor defects in hematopoietic development. There are fewer erythroid/myeloid progenitors in the yolk sacs, fetal livers
and aorta/gonad/mesonephros regions of Runx1\textsuperscript{+/-} embryos compared to wild type littermate controls (Wang et al., 1996b, Wang et al., 1996a, Mukouyama et al., 2000). Unexpectedly, the development of HSCs in Runx1\textsuperscript{+/-} embryos is accelerated and spatially shifted (Cai et al., 2000a). Specifically, HSCs were readily detected in the E10.5 AGM, and could also be detected in the yolk sacs of Runx1\textsuperscript{+/-} embryos at E10.5 (Cai et al., 2000a). This is in contrast to wild type embryos in which very few HSCs are present in the E10.5 dorsal aorta and are found in the yolk sac approximately 24 hours later (Müller et al., 1994). Therefore, reduced Runx1 dosage suppresses definitive hematopoiesis (wave 2) and changes the spatial and temporal development of HSCs (wave 3). The mechanism behind the temporal and spatial shift in HSC development associated with Runx1 heterozygosity is not known, but a subsequent study on the differentiation of Runx1 heterozygous embryonic stem (ES) cells provided a clue. The commitment of Runx1\textsuperscript{+/-} ES cells to hemangioblasts, and subsequently to hematopoietic lineages was found to be accelerated by approximately 12 hours compared to that of wildtype ES cells (Lacaud et al., 2004). Therefore the acceleration in HSC formation may originate at a very early stage in hematopoietic development, in the formation of the tri-lineage hemangioblast, in which Runx1 is expressed (Lacaud et al., 2002).

**Runx1 is required in hemogenic endothelium for the development of definitive hematopoietic cells**

The studies of Runx1 and CBF\(\beta\) knockout mice demonstrated that Runx1 and CBF\(\beta\) are essential for definitive hematopoiesis but did not pinpoint when and in which
cell population Runx1 is necessary. The observation that Runx1 is expressed in endothelial cells at all sites of hematopoietic cluster formation led to the hypothesis that Runx1 is required for the transition from endothelial to definitive hematopoietic cells. To test this hypothesis Runx1 was ablated in endothelial cells via endothelial specific Cre-recombinase mediated excision, which led to the complete abrogation of definitive hematopoiesis and embryonic lethality by E13.5 (Li et al., 2006, Chen et al., 2009). Also, endothelium sorted from the yolk sac and embryo proper of E10.5 Runx1−/− mice could not generate hematopoietic cells when plated on an OP9 stromal cell layer in conditions that support EHT (Yokomizo et al., 2001). These findings suggest that Runx1 expression is required in endothelial cells for the de novo generation of definitive hematopoietic cells. This point was further supported by a study that took the reverse approach by restoring endogenous Runx1 expression in Tek-expressing endothelial cells in Runx1 reversible knockout mouse embryos (Liakhovitskaia et al., 2009). Restoration of Runx1 expression in endothelial cells was sufficient to rescue lymphoid lineages, myeloid lineages and HSCs, and prolonged the life of the embryos up until birth (Liakhovitskaia et al., 2009). Postnatal lethality of these mice likely resulted from the loss of Runx1 expression in non-hematopoietic tissues. In fact, Runx1 null mice in which Runx1 expression is restored in only endothelial/hematopoietic cells have defects in neuronal differentiation and mineralization of the skull and sternum, demonstrating additional roles of Runx1 during development (Kobayashi et al., 2012, Liakhovitskaia et al., 2010).

After HE cells transition into hematopoietic cells they continue to express Runx1, which led to the hypothesis that Runx1 remains essential even after the EHT. However, conditional deletion of Runx1 in hematopoietic cells via Vav1-Cre, did not result in the
ablation of EMPs or HSCs, nor did it affect embryonic or adult viability, indicating that Runx1 is not required in Vav1 expressing hematopoietic cells (Chen et al., 2009). However, Runx1 deletion in hematopoietic cells does cause defects that include thrombocytopenia and defective lymphopoiesis in adult mice (Chen et al., 2009, Ichikawa et al., 2004, Grownney et al., 2005, Putz et al., 2006). Therefore, although Runx1 expression in hematopoietic cells is not essential for the generation and survival of definitive hematopoietic cells, it is required for lineage-specific differentiation and homeostasis. One caveat of this study is that Vav1-Cre is active in circulating and fetal liver hematopoietic cells but not in hematopoietic cluster cells within the dorsal aorta, leaving open the possibility that Runx1 is required for a short period after the EHT and before fetal liver colonization.

To more precisely determine the temporal requirement of Runx1 expression in hemogenic endothelium, Tober et al. conditionally deleted Runx1 during 24-hour intervals between E7.5 and E11.5 using a tamoxifen-inducible endothelial-specific Cre driven from vascular endothelial cadherin (Cdh5) regulatory sequences (Cdh5-CreERT) then assessed hematopoiesis (Tober et al., 2013). They found that when Runx1 was deleted between E8.25 –E9.25 that EMP numbers were dramatically reduced, indicating that Runx1 is critical in that time frame for the formation of EMPs from hemogenic endothelium. On the other hand deletion between E9.0 - E10.0 had no effect on EMP numbers, indicating that by E10.0 the requirement for Runx1 in hemogenic endothelium for the majority of EMP formation has ended. In contrast, the de novo development of HSCs was dependent on Runx1 expression in the endothelium up until E11.5. Thus, the requirement for Runx1 expression in HE for the development of EMPs and HSCs is
temporally uncoupled, which is consistent with the sequential development of EMPs and HSCs during embryogenesis. This study however, did not determine if Runx1 was required in hematopoietic cluster cells because although vascular endothelial cadherin protein (CD144) is expressed on the surface of hematopoietic cluster cells, Cdh5 mRNA is downregulated 6-fold in hematopoietic cluster cells (Tober et al., 2013, North et al., 2002, Jaffredo et al., 2005, Fraser et al., 2003). It was unclear whether Cre$^{ERT}$ protein levels correlated with Cdh5 mRNA or vascular endothelial cadherin protein levels, and was present and active in hematopoietic cluster cells.

The molecular basis for the transient requirement for Runx1 was explored in a mouse embryonic stem (ES) cell model (Hoogenkamp et al., 2009, Lichtinger et al., 2012). Using a Runx1$^{-/}$ mouse ES cell line expressing inducible Runx1, Hoogenkamp et al. demonstrated that Runx1 bound to an upstream regulatory element (URE) of the Spi1, encoding the hematopoietic transcription factor Pu.1. Spi1 is a downstream Runx1 target that is required for myelopoiesis. Runx1 initiated chromatin unfolding in the Spi1 URE at the onset of hematopoietic development (Hoogenkamp et al., 2009). Furthermore, using ChIP and in vivo footprinting they found that weak and transient binding of Runx1 to the URE was sufficient to establish stable transcription factor complexes at cis-regulatory elements that could sustain Spi1 expression even after removal of Runx1 (Hoogenkamp et al., 2009).

Genome-wide analysis by the same group using the same ES cell differentiation model compared ES-derived HE cells before and after the induction of Runx1 (Lichtinger et al., 2012). They found that after Runx1 induction in HE, Runx1 bound to sites that contained little or no H3K9Ac and subsequently strongly increased H3K9Ac levels,
illustrating that Runx1 does not require high levels of active chromatin marks to bind to its target sites, but once bound can induce chromatin activation. Furthermore, Runx1 was shown to recruit hematopoietic regulators, SCL/TAL1 and FLI1 to target sites in HE cells to activate a hematopoietic transcriptional program (Lichtinger et al., 2012). This study illustrates Runx1’s ability to orchestrate a hematopoietic-specific program in HE by changing the binding profiles of hematopoietic regulators and insuring proper progression through the EHT.

As hemogenic endothelial cells begin to transition into hematopoietic cells, one of the earliest hematopoietic markers to be expressed is the αIIb integrin subunit CD41 (Mikkola et al., 2003). A subset of endothelial cells in the dorsal aorta of Runx1 deficient embryos express CD41, suggesting that in the absence of Runx1, hemogenic endothelium is at least partially specified and can switch on hematopoietic gene expression (Liakhovitskaia et al., 2014). To determine if Runx1 expression close to the onset of EHT is sufficient for generating definitive hematopoietic cells, Liakhovitskaia et al., restored Runx1 expression in CD41⁺ cells in Runx1 deficient embryos via CD41 (Itga2b)-Cre (Liakhovitskaia et al., 2014). Restoring Runx1 expression in CD41⁺ cells rescued the generation of HSCs, and the embryos survived until birth, suggesting that Runx1 is required and sufficient for the progression of CD41⁺ cells into HSCs (Liakhovitskaia et al., 2014). CD41⁺ cells isolated from wild type mouse embryos or embryonic stem cell cultures can give rise to hematopoietic cells but cannot generate endothelial progenitors, indicating that CD41⁺ cells are committed to the hematopoietic lineage (Hashimoto et al., 2007, Li et al., 2005). The finding that restoring Runx1 expression in CD41⁺ (Itga2b-Cre expressing) cells can rescue HSCs suggests that
Runx1 is not required until the endothelial to hematopoietic transition is initiated and hematopoietic fate has been cemented. However, transcriptional analysis of hemogenic and non-hemogenic endothelial cells isolated from E8.5 embryos revealed that while CD41 protein at the surface of either cell population is low to non-existent at E8.5, both hemogenic and non-hemogenic endothelial cells express Itga2b mRNA (Swiers et al., 2013a). Therefore the Itga2b-Cre used by Liakhovitskaia et al. (Liakhovitskaia et al., 2014) may have restored Runx1 expression in all endothelium at E8.5 rather than specifically in HE cells initiating the EHT. Thus, it is formally possible that Runx1 expression in Itga2b-expressing endothelial cells earlier in development, prior to the onset of EHT, is necessary for the de novo generation of definitive hematopoietic cells.

Regulation of Runx1 expression during the specification of hemogenic endothelium

Although Runx1 is required for the successful transition of HE cells into hematopoietic cells it is not required for the specification of hemogenic endothelium. This was perhaps best illustrated in live-imaging studies of EHT in Runx1 deficient zebrafish embryos. In Runx1 morphant zebrafish embryos, HE cells bend away from the endothelial monolayer, initiating the EHT, but fragment before forming a hematopoietic cell (Kissa and Herbomel, 2010, Zhen et al., 2013), a phenomenon that was also observed in Runx1−/− mouse ES-derived HE cells (Lancrin et al., 2009, Eilken et al., 2009). Furthermore, as mentioned above CD41 is expressed by HE cells in the DA of
E10.5 Runx1^{−/−} mouse embryos, indicating that the hematopoietic program is at least partially initiated in the absence of Runx1 expression (Liakhovitskaia et al., 2014).

Although Runx1 is not required for the specification of HE it was proposed to play a role in determining cell fate in mesoderm-derived progenitors. Etv2^{+} Flk1^{+} mesodermal cells give rise to both endothelial cells and blood (Kataoka et al., 2011, Wareing et al., 2012). Whether the Etv2^{+} Flk1^{+} mesodermal progenitor gives rise to a non-hemogenic endothelial cell or a HE cell was recently reported to be controlled, at least in part, by Runx1 (Eliades et al., 2016). At E7.5, Runx1^{+} Etv2^{+} Flk1^{+} cells reside within the extraembryonic yolk sac and co-express mesodermal and endothelial specific markers. At E8.5, a subset of Etv2^{+} cells migrate from the area at the boundary of the yolk sac and embryo proper into the embryo proper and downregulate mesoderm-specific genes (Eliades et al., 2016). A similar observation was made by Tanaka et al., who reported that between E7.5 and E8.5 Runx1^{+} Gata1^{−} cells located at the boundary between the extraembryonic yolk sac and the embryo proper, migrate to the embryo proper where they contribute to the intraembryonic vasculature and blood (Tanaka et al., 2014). Interestingly, the Etv2^{+} population at E7.5 expresses Runx1 and has hemogenic potential, likely representing at least in part the yolk sac blood island cells. At E8.5, in contrast most Etv2^{+} cells do not express Runx1, and lack hematopoietic potential, from which it was suggested that Runx1 is silenced in the majority of Etv2^{+} cells between E7.5 and E8.5 (Eliades et al., 2016). The mechanism of silencing involves Bmi1, a member of the Polycomb Repressive Complex 1 (PRC1) (Eliades et al., 2016), which physically interacts with Runx1 (Yu et al., 2012). Ectopic expression of Runx1, or inhibition of PRC1 conferred hemogenic potential to the E8.5 Etv2^{+} population,
suggesting that the hemogenic potential of the E8.5 Etv2+ population is restricted through Runx1 silencing (Eliades et al., 2016). These results demonstrate that the default program in Etv2+ Flk1+ progenitors may be the hematopoietic program, initiated by Runx1. Bmi1 then represses Runx1 expression at E8.5 to promote a vascular fate.

Silencing of Runx1 expression in endothelium is also mediated through the homeobox protein, HoxA3. During hematopoietic development the expression of Runx1 and HoxA3 in the endothelium is mutually exclusive, in part because HoxA3 directly interacts with and represses Runx1 expression (Iacovino et al., 2011). Ectopic expression of HoxA3 during ES cell differentiation and in cells derived from E10.5 mouse embryos resulted in the downregulation of hematopoietic markers and inhibited hematopoietic specification, and increased the expression of endothelial-specific genes, suggesting that HoxA3 reinforces an endothelial fate while suppressing the hematopoietic potential of endothelial progenitors (Iacovino et al., 2011). Interestingly, when Runx1 is ectopically expressed in HoxA3-induced ES-derived endothelial progenitor cells the expression of hematopoietic genes is rescued, indicating that high levels of Runx1 can override HoxA3 activity (Iacovino et al., 2011).

**Does Runx1 function as a master regulator of hematopoiesis?**

The term “master regulator” is often used to describe a gene that sits at the very top of a regulatory hierarchy. However a stringent test of a master regulator is whether it can reprogram one cell type into another (Chan and Kyba, 2013). Logically, the most likely cells that would respond to direct reprogramming by Runx1 are endothelial cells.
However direct reprogramming studies have shown that Runx1 alone is not sufficient to reprogram either human umbilical vein endothelial cells (HUVECs) or human adult dermal endothelial cells (hDMECs) into hematopoietic progenitor cells (Sandler et al., 2014). Only when Runx1 was combined with Spi1, Fosb and Gfi1 could relatively efficient reprogramming of endothelial cells be achieved (Sandler et al., 2014). Interestingly, both Spi1 (Pu.1) and Gfi1 are direct downstream targets of Runx1 (Lancrin et al., 2012a, Huang et al., 2008, Hoogenkamp et al., 2009), but when they were individually removed from a transduction cocktail containing all four transcription factors the efficiency of reprogramming significantly decreased, suggesting that ectopic Runx1 alone was unable to efficiently drive their expression (Sandler et al., 2014). Therefore, by this strict definition Runx1 is not a master regulator, as it is not by itself sufficient to reprogram HUVECs or hDMECs into blood cells. The reason for this is unclear, but may be because Runx1 cannot access various downstream targets in specific endothelial subtypes. Endothelial cells of different tissues and developmental stages are diverse in function, phenotype, transcription and chromatin state (Nolan et al., 2013, Aird, 2012, Chi et al., 2003, Casanello et al., 2014), therefore it would be interesting to determine if other endothelial subtypes are more permissive to respecification by Runx1. Runx1 can induce a hematopoietic program in E8.5 Etv2⁺ endothelial cells therefore the ability of endothelial cells to respond to Runx1 activity may be lost as development proceeds (Eliades et al., 2016).

Downstream targets of Runx1 that regulate the EHT
In order to transition morphologically and functionally into hematopoietic cells, HE cells must extinguish their endothelial-specific transcriptional program and upregulate a hematopoietic program; a transcriptional switch that is largely orchestrated by Runx1. Two direct targets of Runx1, Gfi1 and Gfi1b, encode nuclear zinc finger transcriptional repressors that inhibit expression of endothelial genes in HE during the EHT (Lancrin et al., 2012a). In Gfi1/Gfi1b deficient mouse embryos, HE cells in the yolk sac fail to transition morphologically into hematopoietic cells and remain locked in the endothelial layer (Lancrin et al., 2012a). However, dissociation of Gfi1/Gfi1b deficient yolk sac frees the hematopoietic cells, which can then form hematopoietic colonies in clonogenic assays, suggesting that Gfi1/Gfi1b deficient HE cells can form functional hematopoietic progenitors but are unable to physically transition into a morphological hematopoietic cell (Lancrin et al., 2012a). Conversely, when Gfi1 and Gfi1b expression was induced in Runx1-/- embryonic stem cell derived-HE, the HE cells could undergo the morphological transition into rounded cells but the round cells could not form colonies in hematopoietic clonogenic assays, thus illustrating that during the EHT Gfi1 and Gfi1b repress an endothelial fate allowing for the morphological transition of flat HE cells into rounded hematopoietic cells (Lancrin et al., 2012a). Interestingly, a subsequent study found that hematopoietic clusters did not form in the arteries of Gfi1/Gfi1b deficient embryos, and Gfi1 expressing cells remained within the endothelial layer. However, unlike in the yolk sac, dissociated cells from the arteries could not differentiate into hematopoietic colonies, indicating that Gfi1 and Gfi1b have functions in blood cell formation in the major arteries in addition to their requirement for the EHT (Thambyrajah et al., 2016).
Identifying the transcriptional program regulated by Runx1 in hemogenic endothelium is challenging because HE is a rare population that exists only transiently during midgestation. To overcome these challenges Lie-A-Ling et al. (Lie-A-Ling et al., 2014) used an alternative technique to chromatin immunoprecipitation called DNA adenine methyltransferase identification (DamID). DamID relies on the fusion of a transcription factor (such as Runx1) to the *Escherichia coli* DNA adenine methyltransferase (Dam). When the transcription factor binds DNA the fused Dam protein adds stable methylation tags to adenines within nearby GATC sequences allowing for identification of transcription factor binding sites without the need for antibodies (Lie-A-Ling et al., 2014). To identify Runx1 targets in HE, Lie-A-Ling et al. established Runx1<sup>−/−</sup> ES cell lines containing doxycycline inducible Runx1-Dam, and then differentiated the ES cells into HE. Fortuitously, the inducible system was leaky, allowing for low levels of Runx1 expression in the absence of doxycycline that were not sufficient for EHT, but were sufficient for the detection of Runx1 occupancy by DamID (Lie-A-Ling et al., 2014). Comparison of the Runx1-DamID methylation and RNA-Seq datasets led to the identification of 235 genes that were both bound by Runx1 and differential expressed in HE cells generated from wild type and Runx1<sup>−/−</sup> ES cells (Lie-A-Ling et al., 2014). The expression of 80 of the genes was negatively correlated with Runx1 occupancy and 155 genes were positively correlated (Lie-A-Ling et al., 2014), consistent with Runx1’s ability to function as a transcriptional repressor or activator in the same cell type (Canon and Banerjee, 2003). The target genes that were positively correlated with Runx1 expression were associated with cell adhesion, integrin signaling, cellular movement and interaction with the extracellular matrix (Lie-A-Ling et al., 2014). Interestingly, very few hematopoietic genes were identified as Runx1 targets, suggesting that the HE was in an
early stage of differentiation and had not yet initiated a hematopoietic-specific program. Thus an early function of Runx1 in HE is to regulate the expression of genes involved in the activation of migration and adhesion of HE cells prior to the EHT.

Transcriptional and translation regulation of Runx1 expression during embryonic hematopoiesis

The spatio-temporal specific expression pattern of Runx1 during embryonic hematopoiesis is controlled, in part, through transcriptional regulation. In vertebrates, Runx1 is transcribed from two alternative promoters, the distal (P1) promoter and the proximal (P2) promoter (Ghozi et al., 1996, Rennert et al., 2003, Levanon et al., 2001b, Bee et al., 2009b, Telfer and Rothenberg, 2001). The P2 promoter differs from the P1 promoter in that it is associated with a large CpG island that may influence differential regulation of P1 versus P2 transcription (Levanon et al., 2001b, Bee et al., 2009b). Furthermore, the conserved binding sites associated with each promoter are different; P1 contains a cAMP-responsive element, a CCAAT box, GATA, SMAD and RUNX motifs whereas P2 contains CCATT boxes, initiator sequences, a GC-box, OCT and ETS motifs (Ghozi et al., 1996, Bee et al., 2009a, Bee et al., 2010, Martinez et al., 2016). Differential promoter usage in addition to RNA splicing leads to a vast array of Runx1 isoforms. The full-length isoforms generated from the P1 and P2 promoters are referred to as Runx1c and Runx1b, respectively. Runx1c (465aa) is larger than Runx1b (451aa) due to a difference of 19aa at their N-termini, but there are no data that suggest these
differences confer distinct properties to the Runx1c and Runx1b proteins (Fujita et al., 2001, Challen and Goodell, 2010).

Differential promoter usage during hematopoiesis does, however, control the timing and level of Runx1 expression. Analysis of Runx1 promoter activity in mouse embryos and ES cell differentiation models revealed that the P2 promoter is dominant early during primitive hematopoiesis and at the onset of definitive hematopoiesis, whereas P1 activity ramps up later in development during fetal liver and bone marrow hematopoiesis (Bee et al., 2009b, Bee et al., 2010, Fujita et al., 2001, Pozner et al., 2007, Sroczynska et al., 2009). In mice, abrogation of the P2 promoter via insertion of a neomycin resistance gene resulted in fewer hematopoietic clusters in the large arteries of the embryo proper, significantly fewer hematopoietic progenitors in the fetal liver and yolk sac, reduced thymopoiesis, and perinatal lethality (Pozner et al., 2007, Bee et al., 2010). The prolonged survival of P2-attenuated mice compared to Runx1 null mice (E12.5 lethality), is likely due to the overlap of P1 and P2 activity in hemogenic endothelium, and therefore P1 promoter activity alone promotes the de novo generation of sufficient numbers of definitive hematopoietic cells to prevent embryonic lethality (Sroczynska et al., 2009, Bee et al., 2009b). Loss of P1 promoter activity, on the other hand, is less detrimental than P2 loss. P1-null mouse embryos have fewer hematopoietic clusters and produce fewer hematopoietic progenitors in the yolk sac and large arteries of the embryo proper compared to littermate controls, but the decrease in hematopoietic cells is not as severe as that caused by P2 attenuation, and loss of P1 is not lethal (Bee et al., 2010). However, the bone marrow and peripheral blood of P1-null adult mice does exhibit a significant decrease in white blood cells and platelets and an
increase in the percentage of bone marrow HSCs and hematopoietic progenitors (Bee et al., 2010). Interestingly, one functional P2-deleted Runx1 allele in the absence of P1-activity was sufficient to rescue embryonic lethality, but one functional P1 allele in the absence of P2 was not, suggesting that the dosage and timing of Runx1 expression is critical for the generation of definitive hematopoietic cells (Bee et al., 2010, Pozner et al., 2007).

The P1 and P2 promoters regulate the timing and dosage of Runx1 during hematopoiesis but they do not confer tissue specificity in mammalian embryos (Ghozi et al., 1996, Bee et al., 2009a). Hematopoietic specific expression is mediated by enhancers located within and upstream of the Runx1 gene locus (Schutte et al., 2016). The best known of these is a 531 bp enhancer located between P1 and P2, 23.4kb downstream of the ATG in exon 1 of Runx1 (Nottingham et al., 2007b, Ng et al., 2010). The +23 enhancer drives reporter expression at all sites of hematopoiesis in mouse embryos (Nottingham et al., 2007b, Ng et al., 2010). Specifically, the +23 enhancer is active in hemogenic endothelium, hematopoietic clusters and fetal liver hematopoietic cells. It is not, however, active in non-hematopoietic tissues that express Runx1, such as the mesenchyme beneath the dorsal aorta (Nottingham et al., 2007b, Ng et al., 2010). ChIP analysis of the +23 enhancer demonstrated association with Gata2, Runx1, Ets transcription factors, and the SCL/Lmo2/Lbd-1 complex in a myeloid progenitor cell line (Nottingham et al., 2007b). To determine if transcription factor binding was necessary for activity of the +23 enhancer in mouse embryos, Nottingham et al. assessed whether or not activity of the enhancer was disrupted after mutating the RUNX, ETS or GATA motifs. They found that the RUNX motif was not required for +23 enhancer activity but
the ETS and GATA motifs were required, therefore the +23 enhancer confers hematopoietic specific expression of Runx1 and is regulated, in part, through interaction with Gata and Ets transcription factors (Nottingham et al., 2007b).

Post-transcriptional control of Runx1 occurs through variations in translational efficiency and transcript attenuation via miRNAs. The translational efficiencies of P1 and P2-derived transcripts differ due to distinct 5' untranslated regions (UTR). P1-derived transcripts have a relatively short 5' UTR (452 bp) that directs efficient cap-dependent translation (Pozner et al., 2000). In contrast, P2-derived transcripts have a long 5' UTR (1,631bp) containing an internal ribosomal entry site (IRES), which mediates cap-independent translation (Pozner et al., 2000). It has been proposed that P2-derived transcripts are poorly transcribed due to the length of the UTR and cis-acting elements within it, including the IRES as well as multiple upstream AUG codons and GC-rich islands (Pozner et al., 2000, Levanon et al., 1996). A possible explanation for the presence of both IRES and cap-dependent translation of Runx1 mRNA is that IRES-containing transcripts are translated during mitosis and under stress conditions when cap-dependent translation is impaired (Levanon and Groner, 2004). Further post-transcriptional regulation of Runx1 occurs through miRNA transcript attenuation. In addition to distinct 5'UTRs, Runx1 mRNA isoforms have different 3' UTRs that range in size from 150 to 4,000 bp (Levanon et al., 2001b). Several putative miRNA binding sites were identified in the 3' UTR of Runx1, and the length of the 3'UTR was shown to change the susceptibility to miRNA targeting and attenuation (Ben-Ami et al., 2009). Although the role that translational regulation of Runx1 plays during embryonic
hematopoiesis has not been elucidated, it is plausible that it influences isoform, dose, timing and cell specific expression of Runx1 during development.

The past 10 years have seen the shaping of the roadmap of HSPC development from hemogenic endothelium: discrete cellular intermediates of the HSC lineage have been identified, along with the identification of distinct populations of HE giving rise to HPCs and HSCs. In addition, our understanding of the critical role Runx1 plays in this process has deepened with the identification of new target genes. The rapid developments in imaging and expression profiling technologies now enable taking the study of *de novo* HSPC generation to the single cell level, the level at which cell fate decisions are made. This will no doubt lead to more exciting insights into the role of the master regulator Runx1 in blood stem and progenitor cell generation.
Figure 1.1 Location of Runx1 expression and hemogenic endothelium in the mouse embryo
Figure 1.1 Location of Runx1 expression and hemogenic endothelium in the mouse embryo. Confocal Z-projections of mouse embryos between embryonic day (E) 8.5 and E11.5 immunostained for the endothelial and hematopoietic markers CD31 (red) and Runx1 (green). Runx1 is expressed in HE and hematopoietic cells in the yolk sac (YS) at E8.5. At E9.5, Runx1 expression is prominent in the vitelline artery (VA) and umbilical artery (UA). An E10.5 embryo (head removed) shows Runx1 protein in the vitelline artery, umbilical artery, dorsal aorta (DA), and the site of colonization, the fetal liver (FL). At E11.5 Runx1 expression in the fetal liver intensifies as hematopoietic cells colonize it. Conversely expression of Runx1 in the large arteries at E11.5 diminishes as the hemogenic endothelial cells give rise to hematopoietic cells that subsequently enter circulation. al allantois, pDA paired dorsal aortae. Scale bar = 500µm
CHAPTER 2: Materials and methods
General Protocols

**Mice.** All studies described herein were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All of the animals were handled according to approved institutional animal care and use committee (IACUC) protocol #803789 of the University of Pennsylvania.

**Superovulation.** 3-week-old B6C3F1 females ordered from Charles River and Jackson Laboratory were injected intraperitoneally with 5 IU of gonadotropin from pregnant mare serum (Sigma-Aldrich, St Louis, MO and the Los Angeles Biomedical Research Institute National Hormone & Peptide Program, Los Angeles, CA). 48 hours later the B6C3F1 females were injected intraperitoneally with 5 IU of human chorionic gonadotropin (Sigma-Aldrich and the Los Angeles Biomedical Research Institute National Hormone & Peptide Program) and placed in cages with males at a 1:1 ratio for mating. Superovulated females were euthanized no later than E10.5.

**Fluorescence-activated cell sorting and flow cytometry.** Embryos or tissues were harvested in dissecting media (see Table 2.1 for media recipes). Samples were dissociated in 0.125% collagenase Type I (Sigma-Aldrich, Catalog# C0130) at 37°C for 30 minutes. The samples were reduced to single cell solutions via manual trituration, and then rinsed two times in dissecting media before being immunostained (see Table
2.2 for antibody list). After a 1-hour incubation with antibodies at room temperature the samples were rinsed two times in dissecting media. DAPI was added to determine viability, the samples were filtered and then sorted using a BD Influx cell sorter, or analyzed on a BD LSR II flow cytometer. Data were analyzed using FlowJo (Tree Star, Ashland, OR).

**Whole mount immunofluorescence and confocal microscopy.** Embryos were prepared as previously described (Yokomizo et al., 2012). In brief, embryos or mouse organs were fixed in 2% paraformaldehyde, and dehydrated in methanol. Dehydrated samples were dissected then blocked in 1% goat serum (Jackson ImmunoResearch, Catalog# 005-000-001) for 1 hour on ice. Samples were then permeabilized with triton X-100 and immunostained (see Table 2.3 and 2.4 for primary and secondary antibodies). After staining, the samples were mounted in a fastwell and cleared using a 2:1 solution of benzyl benzoate and benzyl alcohol. A Zeiss LSM 710 AxioObserver inverted confocal microscope with ZEN 2011 software was used to acquire Z-projections and single optical projections. Images were processed using Fiji software (Schindelin et al., 2012). 3-dimensional reconstructions were produced using Volocity software (PerkinElmer).

**Erythro-myeloid progenitor assay.** Cell suspensions were filtered and plated in triplicate in MethoCult™ GF M3434 methylcellulose (STEMCELL Technologies, Vancouver, BC, Canada). Colonies were scored 7 days later. E10.5 mouse hearts were
plated at 1/2 dilution; E10.5 embryos were plated at 1/20 dilution and E10.5 yolk sac plated at 1/40 dilution.

**Lymphoid progenitor assay.** Lymphoid assays were performed as previously described (Schmitt and Zuniga-Pflucker, 2006). In brief, OP9 (stromal cells derived from mouse bone marrow, ATCC,CRL-2749) and OP9-DL1 cells (OP9 cells that ectopically express the Notch ligand, Delta-like 1) were plated in 96 well plates at a density of 4000 cells per well in OP9 media (Table 2.1). The next day the media was removed from the 96 well plates and B cell media was added to the OP9 plates, and T cell media was added to the OP9-DL1 plates (Table 2.1). Next, cell populations of interest were counted using a hemocytometer then plated on the OP9s or OP9-DL1s in limiting dilutions. T cell cultures were analyzed 9 days after plating and B cell cultures were analyzed 12 days after plating via flow cytometry. T cells were identified as CD45^+ CD25^+ CD90^+ cells and B cells as CD45^+ CD19^+ B220^+ cells. The progenitor frequencies were calculated using ELDA software (Hu and Smyth, 2009).
Protocols specific to chapter 3

Mice. Tg(Ly6a-GFP) mice [B6.Cg-Tg(Ly6a-GFP)G5Dzk/J] were described previously (Ma et al., 2002). Tg(Ly6a-GFP) and wild type conceptuses were generated by crossing B6C3F1 females with heterozygous Tg(Ly6a-GFP) males. B6C3F1 females were ordered from Charles River and Jackson Laboratory.

Quantification of cluster cells in the dorsal aorta via confocal microscopy. 950\(\mu\)m to 1mm of the dorsal aorta was imaged centered on the vitelline artery. Z-slices were acquired 2\(\mu\)m apart throughout the entire depth of the dorsal aorta to obtain a Z-stack. Hematopoietic cells distinguished based on morphology and Kit, CD31 and Runx1 expression were counted using the Fiji cell counter plugin (version 29 February 2008, Kurt De Vos; http://rsb.info.nih.gov/ij/plugins/cell-counter.html).
Protocols specific to chapter 5

**Immunoblotting.** E10.5 embryos were dissected free of the amniotic sac, frozen in liquid nitrogen, thawed, and disrupted by pipetting in Hank’s Balanced Salt Solution containing 5 mM EDTA. Total cell lysates were prepared by heating samples in boiling Laemli buffer (66 mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 10 mM EDTA). The samples were subjected to SDS-PAGE and immunoblotting analysis using anti-neurofibromin antibody (Abcam, Catalog# ab17963). Immunoreactive bands were visualized by chemiluminescence. Quantification of individual band intensities was performed using ImageJ. One-way analysis of variance (ANOVA) was used to assess statistical differences between band intensities. Significant ANOVA results were analyzed post hoc by the Tukey-Kramer multiple comparisons test.

**Histology and immunofluorescence analyses.** Whole mouse embryos or dissected hearts were fixed in 2 or 4% paraformaldehyde, dehydrated in ethanol, and embedded in paraffin for sectioning. Tissues were visualized with hematoxylin and eosin (H&E) stain or by immunofluorescent detection of marker proteins according to standard practices. Detailed protocols are available at http://www.pennmedicine.org/heart/. Antibodies used for immunofluorescence include rabbit polyclonal anti-tyrosine hydroxylase (EMD Millipore/Chemicon, Catalog# AB152), rabbit polyclonal anti-pERK (Cell Signaling Technology, Catalog# 9101) and mouse monoclonal anti-neurofilament (2H3, Developmental Studies Hybridoma Bank, Department of Biology, University of Iowa,
Images were adjusted using Adobe Photoshop using settings applied across the entirety of each image.

**Mice.** *Nf1*<sup>−/−</sup>, *Nf1*<sup>lox/lox</sup> and *Wnt1-Cre* mice have been described previously (Brannan et al., 1994, Jacks et al., 1994, Zhu et al., 2001, Danielian et al., 1998, Jiang et al., 2000). *Nf1*<sup>GRD/+</sup> and *Nf1*<sup>GRDCTL/+</sup> mice were produced by targeting C57BL/6 ES cells (Genoway, Lyon, France) with a targeting vector designed to replace arginine 1276 with proline (R1276P) or, in the case of *Nf1*<sup>GRDCTL/+</sup> to leave arginine 1276 as arginine. The selection strategy (Fig. 2.1) included a self-excising floxed neomycin resistance cassette that, after excision, leaves a single loxP site within intron 27. The *Nf1*<sup>GRDCTL/+</sup> mice were created in order to control for possible unpredicted effects related to the introduction of small changes in genomic sequence, other than those encoding the R1276P missense mutation, necessitated by the targeting strategy. *Nf1*<sup>GRD/+</sup> and *Nf1*<sup>GRDCTL/+</sup> mice were genotyped using PCR primers listed below, which produce a 175 bp wild type band and a 248 bp mutant band (Fig. 2.1). All mice were maintained on a C57BL/6 background.

**GRDF:** 5' - GAGGGGAGATGTCAAAGATGTATTGTAACTAC - 3'

**GRDR:** 5' - CAACCTTCAAACAGTACTAAAGTCCATCATGG - 3'
Protocols specific to chapter 6

**Mice.** Runx1 conditional knockin mice (Gt(Rosa)26Sor^{tm[RUNX1]Ma}) were provided by Quifa Ma at Harvard University. VEC-Cre-ERT2 (Tg(Cdh5-cre/ERT2)1Rha) mice have been described previously (Sörensen et al., 2009).

**Tamoxifen delivery.** Tamoxifen free base (MP Biomedicals, Santa Ana, CA) was dissolved in ethanol then diluted in corn oil. For embryonic and fetal experiments pregnant dams were injected intraperitoneally with 0.5mg to 2mg of tamoxifen. Neonates received 0.5mg tamoxifen intraperitoneally. 1-month-old mice received 2mg of tamoxifen via oral gavage.

**Limiting Dilution hemogenic endothelium (HE) assay.** OP9 stromal cells were maintained in OP9 media (Table 2.1). One day prior to the initiation of the HE assay, OP9 cells were plated at a concentration of 4000 cells/well in 96-well plates. On day 1, sorted endothelial cells were plated in limiting dilutions with 3 to10 replicates for each dilution on confluent OP9s in HE media (Table 2.1). On days 7-14, individual wells were inspected for hematopoietic cells via a phase contrast microscope; the cells were then harvested and analyzed by flow cytometry. Positive wells contained CD41^+^, CD45^+^ and/or Terr119^+^ cells. The HE frequency was calculated with ELDA software (Hu and Smyth, 2009).
Postnatal limiting dilution hemogenic endothelial cells assays. 1-month-old Cre;CKI/CKI and CKI/CKI littermate mice were fed 2mg of tamoxifen via oral gavage, 24 hours later they were euthanized and endothelial cells were sorted from their livers. The sorted endothelial cells were then plated in limiting dilution on endothelial cells transduced with the adenoviral E4ORF1 gene in HSC media (Table 2.1) as previously described (Sandler et al., 2014).

Lineage analysis. 5,000 Kit⁺ endothelial cells sorted from E9.5 or E13.5 mice were plated per well of a 12 well plate. Sorted endothelial cells were cocultured on a confluent monolayer of OP9s with HE media (Table 2.1) containing 10ng/mL of recombinant murine VEGF (Peprotech, catalog #450-32) to determine B cell, myeloid and erythroid potential. Sorted endothelial cells were cocultured on a confluent monolayer of OP9-DL1 stroma with HE media containing 10ng/mL of VEGF to determine T cell potential.

Stem cell transplantations. 5,000 endothelial cells sorted from the cranial region (above the fetal liver) of E9.5 or E13.5 mice were plated on a confluent monolayer of AKT-ECs (endothelial cells derived from mouse fetal liver engineered to constitutively express AKT). Cells were plated in 1 well of a 12 well plate in HSC media (Table 2.1) as previously described (Hadland et al., 2015). On day 7-10 of culture half of the media was carefully removed and replaced with fresh HSC media. Between 14 and 20 days of culture 800,000 cells were collected from each sample and transplanted via tail vein
injection into NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (45.1) recipients who had been treated 24 hours prior with 3mg per kg of busulfan. Donor engraftment was assessed (CD45.2) in peripheral blood at 4, 8 and 16 weeks post transplantation and in bone marrow 16 weeks post transplantation.

**Peripheral blood analysis.** 50 to 75µL of blood was collected in EDTA coated tubes from the retro orbital sinus of each recipient mouse. 700uL of red blood cell (RBC) lysing buffer (Sigma, Catalog# R7757) was added to each sample and placed on an agitator at room temperature for 10 minutes. Next the samples were washed in PBS and filtered. RBC lysing was repeated, and then the samples were washed twice in PBS. After the last wash, dissecting media (Table 2.1) containing 1µL/mL of Fc block (BD Biosciences catalog# 553141) was added to each sample followed by a 10-minute incubation at 4°C. An antibody cocktail was then added directly to each sample and incubated at room temperature for 30-minutes. Then the samples were rinsed 2 times in dissecting media (Table 2.1) and DAPI (Table 2.2) was added to each sample to determine viability.
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<th>Base media</th>
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<th>Cytokines*</th>
<th>Other</th>
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* all cytokines are from Peprotech and they are recombinant murine cytokines
### Table 2.2

#### Extracellular Flow Cytometry Antibodies

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<th>Antigen</th>
<th>Clone</th>
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#### Intracellular Flow Cytometry Antibodies

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<th>Conjugates</th>
<th>Vendor/Catalog#</th>
<th>Dilution</th>
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#### Viability markers

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41
<table>
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Figure 2.1. Generation of Nf1\textsuperscript{GRD} and Nf1\textsuperscript{GRDCTL} mouse lines
Figure 2.1. Generation of Nf1GRD and Nf1GRDCTL mouse lines. (A) Schematic diagram outlining the targeting strategy to develop the Nf1GRD mouse line by modifying the endogenous mouse Nf1 locus with a mutation corresponding to the human NF1 R1276P missense allele. This mutation abrogates neurofibromin GAP activity without impairing secondary or tertiary protein structure or reducing cellular levels of neurofibromin (Klose, et al., 1998). (B) Strategy to develop the Nf1GRDCTL knock-in “control” mouse by targeting the endogenous mouse Nf1 locus with a construct identical to that used to target the NF1 R1276P mutation in (A) with the exception that no mutation is introduced. Knock-in Nf1GRDCTL mice generated from this construct are a stringent control for Nf1GRD animals. For both (A) and (B) asterisks denote regions where additional DNA sequences are identically introduced into introns as part of the targeting process. The “ACN” cassette imparts G418 resistance and is self-excised in the male germ line. N = Ncol restriction endonuclease site. (C) Southern blots of genomic DNA from embryonic stem (ES) cell clones targeted with either the Nf1GRD or Nf1GRDCTL allele display a 13 kb wild type (WT) band as well as a 5.5 kb mutant (MT) band. Five and 3 positive clones were isolated with genotype Nf1GRD or Nf1GRDCTL, respectively, as shown. (D) DNA products from PCR reactions performed with primers specific for the Nf1 wildtype, Nf1 knockout (KO), or Nf1 GRD alleles using template DNA isolated from amniotic sacs of E10.5 embryos.
Abstract

Hematopoietic stem and progenitor cells (HSPCs) are generated *de novo* in the embryo in a process termed the endothelial to hematopoietic transition (EHT). EHT is most extensively studied in the yolk sac and dorsal aorta. Recently new sites of hematopoiesis have been described, including the heart, somites, head, and venous plexus of the yolk sac. We examined sites of HSPC formation in well-studied and in less well-known sites by mapping the expression of the key EHT factor Runx1 along with several other markers by means of confocal microscopy. We identified sites of HSPC formation in the head, heart and somites. We also identified sites of HSPC formation in both the arterial and venous plexuses of the yolk sac, and show that progenitors with lymphoid potential are enriched in hematopoietic clusters in close proximity to arteries. Furthermore, we demonstrate that many of the cells in hematopoietic clusters resemble monocytes or granulocytes based on nuclear shape.
Introduction

Hematopoietic cells are generated de novo during midgestation from a transient subset of endothelium called hemogenic endothelium (HE). HE is located within the endothelial layer, and undergoes a transition, autonomous of cell division, into hematopoietic progenitor and stem cells (HSPCs) (Kissa and Herbomel, 2010, Boisset et al., 2010, Bertrand et al., 2010, Lancrin et al., 2009, Eilken et al., 2009, Zovein et al., 2008). This endothelial to hematopoietic transition (EHT) is strictly dependent upon the transcription factor Runx1 (Lancrin et al., 2009, Chen et al., 2009, Boisset et al., 2010, Kissa and Herbomel, 2010, North et al., 1999, Yokomizo et al., 2001). When Runx1 is knocked out in the germ line, or ablated via endothelial cell specific Cre-recombinase-mediated excision, the EHT is completely blocked, preventing the development of all hematopoietic cells with the exception of primitive erythrocytes and diploid megakaryocytes (Cai et al., 2000b, Lancrin et al., 2009, Potts et al., 2014, Chen et al., 2009, North et al., 1999). When Runx1 is depleted in zebrafish embryos via morpholino knockdown, a small subset of endothelial cells begins the EHT process but the cells rapidly die upon leaving the endothelial layer, suggesting that in the absence of Runx1, HE is at least partially specified (Kissa and Herbomel, 2010). Transcription factors upstream of Runx1 that specify HE include Fli1, Gata2, and Tal1, which directly regulate Runx1 expression (Nottingham et al., 2007a).

Embryonic hematopoiesis occurs in multiple waves of HSPC differentiation from mesoderm or HE. The first wave of hematopoiesis begins in the yolk sac at embryonic day (E) 7.25 and produces primarily primitive erythrocytes but also megakaryocytes and macrophages (Palis et al., 1999, Tober et al., 2007). Primitive erythrocytes and
megakaryocytes appear to be generated directly from mesoderm, and their emergence is only partially dependent on Runx1 activity (Potts et al., 2014, Okuda et al., 1996, Wang et al., 1996a). The second wave of hematopoiesis, defined by the production of committed definitive hematopoietic progenitors prior to HSC formation (Lin et al., 2014), begins in the yolk sac at E8.75 as HE cells in the vascular plexus transition into erythromyeloid progenitors (EMPs) that are released into circulation (Palis et al., 1999, Palis et al., 2001, McGrath et al., 2015). Also in wave 2 at E9.5, lymphoid progenitors differentiate from endothelial cells in the yolk sac and in the major arteries of the embryo proper (Yoshimoto et al., 2011, Yoshimoto et al., 2012, Huang et al., 1994, Nishikawa et al., 1998). The third wave of hematopoiesis gives rise to hematopoietic stem cells (HSCs) that emerge between E10.5 and E11.5 from a subset of hemogenic endothelium in the dorsal aorta, vitelline artery and umbilical artery that expresses both Runx1 and Ly6a, the latter of which encodes the cell surface protein Sca-1 (Chen et al., 2011, de Bruijn et al., 2002, North et al., 2002). Both waves 2 and 3 hematopoiesis are dependent on Runx1 activity. In recent years additional sites of HSPC formation have been identified such as the endocardium of the heart and the endothelium of the head, which give rise to EMPs and HSCs, respectively (Nakano et al., 2013, Li et al., 2012).

EHT has been directly observed in live-imaging studies of midgestation embryos (Lam et al., 2010, Kissa and Herbmel, 2010, Boisset et al., 2010). These studies revealed that during the EHT, HE cells bend away from the lumen of the dorsal aorta leaving the endothelial layer as they transition into morphological hematopoietic cells independent of cell division (Kissa and Herbmel, 2010). In zebrafish the newly formed hematopoietic cells bud into the sub-aortic space and migrate towards the caudal
hematopoietic tissue (Murayama et al., 2006). In contrast, in mice the newly formed hematopoietic cells remain briefly attached to the endothelium where they accumulate in clusters of Kit+ cells on the luminal side of the vessel (Yokomizo and Dzierzak, 2010, Garcia-Porrero et al., 1995). Hematopoietic clusters are heterogeneous, but thought to largely consist of hematopoietic progenitor cells (Yokomizo and Dzierzak, 2010). Hematopoietic cluster cells eventually enter the circulation and seed the fetal liver where they undergo further maturation and proliferation before traveling to the thymus, spleen and bone marrow where they will reside throughout the lifetime of the animal (Kumaravelu et al., 2002, Ema et al., 1998, DeLuca et al., 1995).

Using whole mount immunofluorescence and confocal microscopy, we examined murine hematopoietic development between E7.5 and E10.5 by mapping the location of endogenous Runx1 and Kit protein, and also GFP expressed from a Ly6a-GFP transgene (de Bruijn et al., 2002) throughout the embryo. We describe several novel features of embryonic hematopoiesis in well-studied sites of hematopoietic cell formation, including the association of progenitors with lymphoid potential in the yolk sac with arterial endothelium. We also show that hematopoietic cluster cells have bean, round and ring-shaped nuclear morphology, the latter of which is suggestive of maturing myeloid lineage cells. Finally, we examined the vasculature of the head, heart and somitic region and found evidence of hematopoietic cluster formation in these sites.
Results and discussion

Hemogenic endothelium is present in both the arterial and venous yolk sac vasculature, but HSPCs with lymphoid potential are more proximal to arterial endothelium

We analyzed the embryo for sites of blood cell formation by examining the expression of several proteins, the presence or absence of which can be used to identify newly emerging blood cells and HE (Table 3.1). In brief, we identified HE cells by their integration in an endothelial layer, the expression of an endothelial marker (vascular endothelial cadherin (VEC) or CD31), Runx1, and low levels of Kit. Runx1, VEC/CD31, plus high Kit expression marks newly emerged hematopoietic cells, which may be either single dispersed cells, or cells located within hematopoietic clusters. The Ly6a-GFP$^+$ fraction of Runx1$^+$ VEC/CD31$^+$ Kit$^+$ cells marks lymphoid progenitors (beginning at E10.5), and HSCs (at E11.5) in the major arteries (dorsal aorta, umbilical and vitelline) (Li et al., 2014, de Bruijn et al., 2002). The Ly6a-GFP$^+$ fraction of Runx1$^+$ VEC/CD31$^+$ Kit$^+$/low cells contains hemogenic endothelium that gives rise to HSCs (Chen et al., 2011).

The antibody we used to detect Runx1 recognizes all three Runx proteins (Runx1, Runx2, Runx3), but as Runx2 and Runx3 are not expressed in endothelium or blood cells at the times of development we characterized (Levanon et al., 2001a, Otto et al., 1997), any observed signal is from Runx1. We obtained identical results using a Runx1 specific antibody (not shown), but used the pan-Runx antibody due to its stronger signal.
Multiple waves of hematopoietic cells are generated from the vasculature of the yolk sac as it develops from a primitive vascular plexus into an organized vascular tree consisting of arteries, veins and capillaries (Palis et al., 1999, Frame et al., 2013, Frame et al., 2015, Lin et al., 2014). The first wave of hematopoiesis, at E7.5, precedes the formation of the vasculature. Runx1 protein at E7.5 was present in mesoderm in the prospective yolk sac blood islands (not shown), as previously reported for Runx1 mRNA and reporter genes (Lacaud et al., 2002, Zeigler et al., 2006, North et al., 1999, Tanaka et al., 2014). At E8.0 (late headfold stage), an unorganized network of CD31\(^+\) endothelial cells is present in the proximal yolk sac, and contained within this endothelial network is a band of Runx1\(^+\) cells (Fig. 3.1 A, bracket). Additionally, small populations of Runx1\(^+\) cells are located distal to the discrete proximal band (Fig. 3.1 A). A higher magnification image of the distal Runx1\(^+\) population shows that the cells lack cell surface Kit and appear to be integrated in an endothelial layer, but have a rounded hematopoietic-like morphology (Fig. 3.1 B). The identity of these cells is unknown. In contrast, the proximal band of Runx1\(^+\) cells contains a population of CD31\(^+\) Runx1\(^{\text{high}}\) Kit\(^{\text{low}}\) cells with elongated endothelial-like morphology (Fig. 3.1 C, white arrowheads) representing yolk sac HE cells. The proximal band also has a CD31\(^+\) Runx1\(^{\text{low}}\) Kit\(^+\) population. The most abundant hematopoietic cells in the yolk sac at E8.5 are primitive erythrocytes (Palis et al., 1999) that express low levels of Runx1 compared to other hematopoietic lineages (North et al., 1999). Therefore the CD31\(^+\) Runx1\(^{\text{low}}\) Kit\(^+\) cells in the yolk sac are likely to be primitive erythrocytes (Fig. 3.1 C, yellow arrowheads). It was previously shown that endothelial cells in the posterior portion of the yolk sac have by E8.5 been specified as arterial, and express the arterial marker ephrin-B2, while venous endothelial cells are located in the anterior yolk sac (Wang et al., 1998). Runx1\(^+\) cells encircle the yolk sac between E7.5
and E8.5 (not shown), and therefore are located within both the anterior (venous) and posterior (arterial) portions.

To prepare and orient E9.5-E10.5 yolk sacs for confocal analysis we first fixed and dehydrated whole conceptuses within their yolk sacs. We removed the chorions and made three to four proximal to distal cuts so that the yolk sacs could be laid flat (Fig. 3.2 A). The yolk sacs were then separated from the embryo proper by severing the vitelline artery and vein, which enabled us to identify the arterial and venous vasculature based on their original relationship to the embryo. We determined that the arterial and venous yolk sac vasculature have several distinct morphological features that allowed us to distinguish them by confocal microscopy. For example, the vitelline vein has a lower intensity of CD31 staining compared to the artery (Fig. 3.2 B) (Hägerling et al., 2013). Furthermore, the Ly6a-GFP transgene is expressed more strongly in the vitelline artery compared to the vein (Fig. 3.2 B). An additional distinguishing feature is that at E10.5 the vascular plexus surrounding the vitelline vein consists of vessels with larger diameters than the plexus around the vitelline artery (Fig. 3.2 C). Thus by multiple independent criteria we are able to differentiate the arterial from the venous yolk sac vasculature.

At E9.5 the vitelline artery is very distinct; the large diameter vessel can be seen from its point of entry at the distal most portion of the yolk sac (Fig. 3.3 A, asterisk) all the way to the proximal yolk sac, where it branches several times (Fig. 3.3 A). In contrast, at E9.5 remodeling of the vitelline vein is less advanced, and a single large diameter vessel cannot be distinguished from the venous plexus (Fig. 3.3 A). Development of the vitelline artery has also been shown to precede development of the vein in the yolk sacs of chick embryos (Le Noble et al., 2004). The delayed development
of the vein may be due to lower shear stress in the vein relative to the artery, as shear stress due to blood flow has been shown to play a role in vascular remodeling (Lucitti et al., 2007, Culver and Dickinson, 2010).

E9.5 yolk sacs contained dispersed and tightly associated clusters of CD31⁺ Runx1⁺ Kit⁺ hematopoietic cells concentrated in the proximal region of the vascular plexus in both the arterial and venous vessels (Fig. 3.3 A-E). By E10.5 distinct clusters of hematopoietic cells are found throughout the proximal and distal regions of the yolk sac, and are located primarily in the small diameter vessels (Fig. 3.3 F, arrowheads). We could identify hematopoietic clusters in the small diameter vessels of both the arterial (posterior) and venous (anterior) yolk sac vasculature, as recently described by Frame et al. (Frame et al., 2015) (Fig. 3.3 F-J). Quantification of hematopoietic clusters containing 5 or more Kit⁺ cells in the yolk sac at E10.5 demonstrated that there is an average of 22.0 ± 7.9 clusters per E10.5 yolk sac, with 14.2 ± 5.1 residing in arteries, and 7.8 ± 3.0 residing in veins (mean ± SD, n=6). This differs from hematopoietic cluster formation in the embryo proper, which occurs primarily in the large arteries, and not in the venous vasculature. Notch signaling is required for hematopoietic cluster formation and arterial identity in the embryo proper (Marcelo et al., 2013b, Burns et al., 2005, Robert-Moreno et al., 2005, Kumano et al., 2003, Bigas et al., 2013). Yolk sac EMPs, on the other hand, can form in the absence of Notch1 signaling (Hadland et al., 2004). Notch signaling is repressed in venous endothelium by the transcription factor COUP-TFII (encoded by Nr2f2) (You et al., 2005). The distribution of hematopoietic clusters in both the small diameter arterial and venous yolk sac vessels, the latter of which would lack Notch signaling, suggests that many of these clusters contain EMPs (Hadland et al., 2004).
To determine the hematopoietic progenitor potential of HSPCs in Kit\textsuperscript{high} clusters we sorted VEC\textsuperscript{+} CD31\textsuperscript{+} Kit\textsuperscript{high} cells from E9.5 and E10.5 yolk sacs collected from superovulated mice for erythro-myeloid progenitor (EMP) and lymphoid progenitor assays (Fig. 3.4 A). To assess EMP potential, colony-forming assays in methylcellulose supplemented with cytokines were performed. The frequency of EMPs in yolk sac clusters at E9.5 and E10.5 were comparable (Fig. 3.4 B). However the percent of EMP colonies containing granulocytes and macrophages increases between E9.5 and E10.5, while mixed colonies containing granulocytes, erythroid cells, monocytes and megakaryocytes decrease (Fig. 3.4 C). To assess lymphoid potential, sorted hematopoietic cluster cells were plated in limiting dilutions on OP9 and OP9-DL1 stromal cells (Schmitt and Zuniga-Pflucker, 2006). Cells cultured on OP9 were analyzed for B lymphoid markers (CD45\textsuperscript+ CD19\textsuperscript+ B220\textsuperscript+), and cells cultured on OP9-DL1 for T markers (CD45\textsuperscript+ CD25\textsuperscript+ CD90\textsuperscript+ cells) one week later. The frequency of HSPCs with lymphoid potential increased more than 80 fold between E9.5 and E10.5 (Fig. 3.4 B). This suggests that lymphoid potential arises in Kit\textsuperscript{high} hematopoietic clusters in the yolk sac between E9.5 and E10.5.

In the embryo proper, HSPCs with lymphoid potential are enriched in the Ly6a-GFP\textsuperscript+ population of hematopoietic cluster cells (Li et al., 2014). To determine if hematopoietic clusters in the yolk sac contain Ly6a-GFP\textsuperscript+ cells we immunostained E9.5 and E10.5 Tg(Ly6a-GFP) yolk sacs for GFP, CD31 and Runx1 or Kit. Confocal analysis revealed that at E9.5 Ly6a-GFP is expressed in the endothelium of the vitelline artery and in rare Runx1\textsuperscript+ cells with hematopoietic morphology, but is not expressed in the venous plexus (Fig. 3.5 A-B). At E10.5, Ly6a-GFP is expressed most robustly in
endothelial cells of the vitelline artery, though rare GFP\(^+\) endothelial cells are also seen in the vitelline vein (Fig. 3.5 C). The hematopoietic clusters at E10.5 are heterogeneous, containing Ly6a-GFP\(^+\) and Ly6a-GFP\(^-\) cells, similar to what has been observed in the large arteries of the embryo proper (Chen et al., 2011) (Fig. 3.5 D). We examined whether the clusters containing Ly6a-GFP\(^+\) cells were associated with the arterial or the venous vasculature. Since we lacked a marker to distinguish between the two vascular beds, we measured the distance of the hematopoietic clusters to the nearest distinguishable artery or vein. Clusters in closer proximity to an artery than to a vein were classified as being associated with arteries or the arterial plexus, and vice versa. Hematopoietic clusters that lack Ly6a-GFP\(^+\) cells localize to both the arterial and venous plexuses (Fig. 3.5 C, white arrowheads), whereas hematopoietic clusters containing Ly6a-GFP\(^+\) cells are found primarily in arteries and their surrounding plexus (Fig. 3.5 C, green arrowheads). Five-fold more clusters containing Ly6a-GFP\(^+\) cells were closer to arteries than to veins (P \(\leq 0.0065\)), whereas 40% of clusters containing only Ly6a-GFP\(^-\) cells were found in the vitelline vein or the surrounding plexus (Fig. 3.5 E). A majority of hematopoietic clusters containing Ly6a-GFP\(^+\) cells were located within small diameter vessels in the arterial plexus (Fig. 3.5 F) but they could also be found in larger diameter arterial vessels (Fig 3.5 G). This suggests that hematopoietic clusters that contain Ly6a-GFP\(^+\) cells are associated primarily with the arterial vasculature of the yolk sac at E10.5.

As HSPCs with B and T lymphoid potential are enriched within the Ly6a-GFP\(^+\) population of hematopoietic cluster cells in the major arteries (Li et al., 2014), we examined whether this was also true in the yolk sac. We sorted hematopoietic cluster cells from E10.5 yolk sacs and separated them based on Ly6a-GFP expression (Fig. 3.5
H), and performed progenitor assays to determine the frequency of HSPCs with T and B potential in each population. The frequency of HSPCs with B and T potential within yolk sac hematopoietic cluster cells was significantly enriched in the Ly6a-GFP* population (Fig. 3.5 I). Previous studies found that the EMP potential is enriched in the Ly6a-GFP yolk sac population (Li et al., 2014), therefore Ly6a-GFP expression appears to segregate HSPCs with lymphoid potential from those with restricted erythro-myeloid potential in the E10.5 yolk sac. As Ly6a-GFP* cells are localized primarily in the yolk sac artery and arterial plexus, we conclude that, unlike EMPs that form from both arterial and venous vasculature, lymphoid progenitors in the yolk sac are more closely associated with arterial vasculature.

New insights into blood cell formation in the major arteries

Runx1 expression in the major arteries initiates at the late headfold stage in the vessel of confluence (VOC), which is the vascular intersection between the placenta, yolk sac and embryo proper (Daane and Downs, 2011) (Fig. 3.6 A, arrowheads). Concentrated in the area surrounding the VOC are Runx1^- CD31^+ Kit^ primordial germ cells (PGCs) (Fig. 3.6 A). The VOC and its immediate surroundings is a location of high levels of bone morphogenic protein (BMP) signaling (Rhee and Iannaccone, 2012), which is required for both Runx1 expression in hemogenic endothelium (Wilkinson et al., 2009), and for PGC specification (Lawson et al., 1999). The Kit ligand Steel, or stem cell factor is also expressed in the ventral hindgut and visceral endoderm in the vicinity of the
VOC (Gu et al., 2009), which may explain, in part, why the first aortic hemogenic endothelial cells and the PGCs co-localize to that particular anatomic site.

Beginning at E8.5 the major arteries undergo extensively remodeling, which is described in detail elsewhere (Walls et al., 2008, Drake and Fleming, 2000). In brief, at E8.5 the paired dorsal aorta (pDA) in the embryo loop from the heart tube, and at the distal most point of the conceptus connect to the vitelline artery (VA) via the VOC (Fig. 3.6 B). By the 7sp stage, the umbilical artery (UA), which arises from de novo vasculogenesis in the allantois (A), also fuses to the pDA at the VOC (Walls et al., 2008, Inman and Downs, 2007). The pDA, aside from the VOC contain neither Runx1⁺ nor Ly6a-GFP⁺ endothelial cells at this stage (Fig. 3.6 C). However the VA contains both Runx1⁺ Ly6a-GFP⁻ CD31⁺ endothelial cells (Fig. 3.6 D, yellow arrowhead) and a smaller number of Runx1⁺ Ly6a-GFP⁺ CD31⁺ endothelial cells (Fig. 3.6 D, white arrowhead). By E9.5 the embryo has turned and active angiogenesis has created a more complex vascular network (Fig. 3.7 A). Runx1 expression at E9.5 is concentrated in the endothelium of the VA, UA, and the pDA (Fig. 3.7 A-D). Clusters of CD31⁺ Runx1⁺ Kit⁺ cells, some of which are also Ly6a-GFP⁺ (not shown) line the entire length of the vitelline artery, but clusters are still largely absent in the UA and dorsal aorta (DA) (Fig. 3.7 A-D), and completely absent from the vitelline and umbilical vein (not shown). Garcia-Porrero et al. also noted the appearance of hematopoietic clusters first in the VA (Garcia-Porrero et al., 1995). Many CD31⁺ Kit⁺ PGCs cells are also present in the vicinity of the dorsal aorta at E9.5, in the process of migrating from the base of the allantois towards the gonadal ridge (Fig. 3.7 B, magnified image of boxed region on the right).
At E10.5 the number of hematopoietic clusters peaks (Yokomizo and Dzierzak, 2010), with hundreds lining the lumens of the DA, the UA and the VA. Numerous studies have shown that hematopoietic cluster cells are heterogeneous with respect to the expression of cell surface markers (Yokomizo and Dzierzak, 2010, Bertrand et al., 2005, Chen et al., 2011). We found that the shape of nuclei in cells within hematopoietic clusters, highlighted by nuclear Runx1 protein, is also heterogeneous (Fig. 3.8 A-H). In hematopoietic clusters in the UA, some cells have round Runx1+ nuclei (Fig. 3.8 A, asterisks). However most hematopoietic cluster cells within the large arteries have nuclei that are ring (yellow arrow) or bean-shaped (Fig. 3.8 A-B, Movie 3.4-3.5). Hemogenic endothelial cells with ring-shaped nuclei were not identified, suggesting that hematopoietic cells acquire the ring-shaped nuclei while in clusters. Hematopoietic cluster cells within the yolk sac are similar to embryonic clusters in that they contain a heterogeneous mix of round, ring and bean-shaped nuclei (Fig. 3.8 C-D, Movie 3.6-3.7). In adult mice, cells with ring-shaped nuclei make up 50% of total bone marrow and can also be found in the peripheral blood (Biermann et al., 1999). Hematopoietic cells with ring-shaped nuclei in adult blood comprise both mature myeloid cells and myeloid progenitor cells (Biermann et al., 1999), thus the hematopoietic cluster cells with ring-shaped nuclei are likely myeloid cells. Interestingly, Ly6a-GFP+ hematopoietic cluster cells in both the embryo proper and the yolk sac have bean-shaped or round nuclei, and no ring-shaped nuclei were observed within that population (Fig. 3.8 E-H, Movie 3.8-3.11).

Hematopoietic cluster formation in the heart
A subset of endocardial cells has hemogenic potential and gives rise to a transient population of erythroid and myeloid cells at E9.5, prior to the emergence of hematopoietic clusters in the dorsal aorta (Nakano et al., 2013). We examined hearts at this stage for the presence of Runx1\(^+\) endocardial cells. Confocal analysis of E8.5 (9sp), E9.0 (16sp) and E9.5 (22sp) embryos identified disperse Runx1\(^+\) cells with hematopoietic morphology in the ventricle and atrium (Fig. 3.9 A). However we could find no Runx1\(^+\) endocardial cells at this stage of development. One possible explanation for this is that hematopoietic cell formation from hemogenic endocardium may not require Runx1. Two lineages of hematopoietic cells in the embryo do not require Runx1 for their formation, primitive erythrocytes and diploid megakaryocytes, which appear to differentiate directly from mesoderm (Okuda et al., 1996, Wang et al., 1996a, Mucenski et al., 1991, Potts et al., 2014). However, the hematopoietic cells that differentiate from endocardium were reported to be definitive erythroid and myeloid progenitors, the equivalents of which in the embryonic yolk sac differentiate from HE in a process that is strictly Runx1 dependent. It is possible that hemogenic endocardium and HE rely on different transcriptional pathways during their specification and transition into hematopoietic cells. For example, Nakano et al. demonstrated that the transcription factor Nkx2-5 is required for the hemogenic potential of endocardium, whereas endothelial cells in the major arteries of the embryo proper do not express Nkx2-5. However, a more likely explanation for the lack of Runx1+ endocardial cells is that Runx1 may not be expressed until the endocardial cell has almost completed its transition into a hematopoietic cell, in which case it would be difficult to identify a hemogenic endocardial precursor.
A second wave of blood formation from the heart occurs at E10.5, and at this time rare Runx1+ endocardial cells were detectable in the ventricular trabeculae (Fig. 3.9 B, arrowhead) and the atrioventricular canal (Fig. 3.9 C, arrowhead). Large clusters of Kit+ CD31+ cells were found in the ventricular cavity that were morphologically identical to the hematopoietic clusters that form in the major arteries at E10.5 (Fig. 3.9 D, arrowheads). The hematopoietic clusters were also found in the atrioventricular canal, and expressed Runx1 (Fig. 3.9 E, arrowheads). These clusters may be equivalent to CD41+ Flk1+ hematopoietic clusters that were previously observed in the atrium, ventricular cavity and outflow tract as late as E11.5 (Jankowska-Steifer et al., 2015b). A third wave of hematopoiesis in the heart occurs between E11 and E14, and involves the formation of cardiac blood islands from the ventricular endocardium (Jankowska-Steifer et al., 2015b, Red-Horse et al., 2010, Ratajska et al., 2009b, Ratajska et al., 2006b). Cardiac blood islands balloon out from the ventricular endocardium near the interventricular sulci and are associated with Runx1+ hematopoietic cells (Fig. 3.9 F, arrowheads). Endocardial cells at the base of cardiac blood islands express Runx1, suggesting that rather than simply trapping hematopoietic cells, cardiac blood islands generate hematopoietic cells de novo (Fig. 3.9 F, lower panels). Thus the heart, like the yolk sac, appears to have multiple waves of a hematopoiesis; an initial wave described by Nakano et al. characterized by disperse erythroid and myeloid cells, a second wave characterized by hematopoietic cluster formation, and a third wave characterized by the formation of cardiac blood islands.

**Hematopoietic cluster formation in the head**
In a previous study, lineage tracing from the cerebrovasculature using Cre recombinase driven from the surfactant protein A (Sftpa1) promoter demonstrated that endothelium within the head has hemogenic potential and generates HSCs de novo (Li et al., 2012). To locate sites of hematopoietic cell formation in the head we analyzed E9.5, E10 and E10.5 mouse heads immunostained for CD31, Runx1 and Kit. At E9.5, Runx1+ cells with hematopoietic morphology are scattered throughout the vasculature, but very few Runx1+ cells express Kit, and those that do are not found within clusters, suggesting that they are either in the circulation or represent an early stage of cluster formation (Fig. 3.10 A-B). By E10 there is an increase in Runx1+ Kit− and Runx1+ Kit+ cells with hematopoietic morphology in circulation (Fig. 3.10 C). The Runx1+ Kit+ cells are predominantly located in the periphery of the cephalic plexus and do not form clusters of closely associated cells (Fig. 3.10 D). Kit+ CD31− Runx1− cells not associated with the vasculature are present in the midbrain and maxillary arch and likely correspond to neuronal cells since Kit is widely expressed in the brain (Zhang and Fedoroff, 1997, Orr-Urtreger et al., 1990) (Fig. 3.10 A, C, E, arrowheads). By E10.5 very rare small clusters of CD31+ Runx1+ Kit+ cells are present in the peripheral cephalic plexus (Fig. 3.10 E-F), but the larger diameter internal carotid artery did not contain CD31+ Runx1+ hemogenic endothelial cells or Kit+ Runx1+ clusters suggesting that hematopoietic cell formation may be restricted to the cephalic plexus (Fig. 3.10 G). Interestingly, concentrated regions of Runx1+ hematopoietic cells could be identified within the plexus however the cells did not have the typical polygonal shape of cluster cells and they heterogeneously expressed Kit, therefore a majority of the Runx1+ hematopoietic cells in the head are morphologically distinct from the hematopoietic clusters that form in the large diameter arteries, heart and yolk sac (Fig. 3.10 H). Similar to the heart and yolk sac, there were
no obvious regions of hemogenic endothelium (defined here as CD31⁺ Runx1⁺ Kit<sub>low/-</sub> cells) in the cerebrovasculature. Expression of Runx1 in endothelium for long periods of time prior to the initiation of the EHT seems to be a unique characteristic of hemogenic endothelial cells in the major arteries (umbilical artery, vitelline artery and dorsal aorta) of the embryo proper. Our findings illustrate a lack of hematopoietic clusters and hemogenic endothelium in the cerebrovasculature and are consistent with recent studies (Iizuka et al., 2016, Li et al., 2016).

**Hematopoietic cluster formation in the somitic region**

A recent study in zebrafish embryos has demonstrated that the somitic vasculature contains hemogenic potential (Qiu et al., 2016). Using whole mount immunofluorescence we identified CD31⁺ Runx1⁺ Kit⁺ hematopoietic clusters in the intersomitic vessels (ISVs) and the dorsal longitudinal anastomotic vessels (DLAVs) of E10.5 embryos suggesting that the somitic region of mouse embryos may also contain hemogenic potential (Fig. 3.11 A-B). Beginning at 8 somite pairs, sprouting angiogenesis from the dorsal aorta gives rise to ISVs that then fuse and interconnect to form the DLAVs (Walls et al., 2008). Since ISVs and DLAVs arise from a hemogenic vessel it follows that they too would harbor hemogenic potential. However, another explanation could be that the hematopoietic clusters entered the ISVs and DLAV via the circulation. At E10.5 the most abundant cells in circulation are CD31<sub>low</sub> Runx1⁻ Kit⁻ erythroid cells, therefore it is not likely that the rare circulating CD31⁺ Runx1⁺ Kit⁺ cells would be found concentrated together unless CD31⁺ Runx1⁺ Kit⁺ cells enter the circulation clustered
together and subsequently become trapped in narrow capillaries. Further analysis aimed at determining the hemogenic potential of ISV and DLAV endothelium are necessary to confirm the hypothesis that mouse ISVs and DLAVs give rise to hematopoietic cells de novo.
<table>
<thead>
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<td></td>
<td>9.5</td>
<td>HE, allantoic mesothelium, endothelium of the placenta, hematopoietic clusters, circulating hematopoietic cells except erythrocytes</td>
<td>(Zeigler et al., 2006, North et al., 1999)</td>
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<td>10.5</td>
<td>HE, hematopoietic clusters, circulating hematopoietic cells except erythrocytes, placental labyrinth and mesenchymal cells surrounding DA</td>
<td>(Zeigler et al., 2006, North et al., 1999, Rhodes et al., 2008, Levanon et al., 2001a)</td>
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<td>Tg(Ly6a-GFP)</td>
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<td>Posterior yolk sac endothelium and chorion</td>
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<td>9.5</td>
<td>Endothelial cells in the DA, VA, some hematopoietic cluster cells and tail</td>
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<td>10.5</td>
<td>Endothelial cells in the DA, VA, UA, heart and liver, some hematopoietic cluster cells, tail, mesenephros and some hematopoietic cells in the fetal liver</td>
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Table 3.1. Embryonic expression of markers used in the analysis
Figure 3.1. Runx1 and Kit expression in the yolk sac at late head fold stage
**Figure 3.1. Runx1 and Kit expression in the yolk sac at late head fold stage.** (A-C)

A late head fold stage (E8.0) mouse embryo immunostained for CD31 (i), Runx1 (i,ii) and Kit (i,iii). (A) Confocal Z-projection. Proximal (P) and distal (D) axes are indicated in i. Bracket in ii indicates band of Runx1\(^+\) cells in the proximal yolk sac. Arrowheads in ii point to Runx1\(^+\) cells located distal of the proximal band of Runx1\(^+\) cells. Scale bar = 500\(\mu\)m (see Movie 3.1 for animation of Z-stack). (B) Magnified image of Runx1\(^+\) cells in the distal yolk sac. Scale bar = 10\(\mu\)m. (C) Single optical projection showing hematopoietic cells (yellow arrowheads) and hemogenic endothelial cells (white arrowheads) in the proximal band of Runx1\(^+\) cells in the yolk sac. Scale bar = 10\(\mu\)m.
Figure 3.2. Distinguishing features of arteries and veins in the yolk sac
Figure 3.2. Distinguishing features of arteries and veins in the yolk sac. (A) Scheme demonstrating removal of the yolk sac prior to imaging to preserve orientation of the vitelline artery and vein. (B) Z-projection of an E10.5 Tg(Ly6a-GFP) yolk sac immunostained for CD31 (i) and GFP (i,ii). Scale bar = 500µm, VV = vitelline vein and VA = vitelline artery. (C) Z-projection of arterial vascular plexus surrounding the vitelline artery (left) and venous plexus surrounding the vitelline vein (right) at E10.5 in samples immunostained for CD31. Scale bar = 100µm. The diameters of capillary vessels surrounding the vitelline artery and vein were measured using Image J software. Five E10.5 yolk sacs and 60 capillary vessels per yolk sac were measured. The diameter of arterial capillary vessels is 15.3µm ± 0.7µm and the diameter of venous capillary vessels is 22.0µm ± 0.5µm (mean ± SEM). Unpaired 2-tailed Student’s t-test was applied to determine significance. *** indicates that P ≤ 0.001.
Figure 3.3. Hematopoietic clusters in the vitelline artery and vein of the yolk sac from E9.5 to E10.5.
Figure 3.3. Hematopoietic clusters in the vitelline artery and vein of the yolk sac from E9.5 to E10.5. (A) Confocal Z-projection of a 22sp yolk sac immunostained for CD31, Runx1 and Kit. Dotted line roughly demarcates the venous and arterial sides of the yolk sac. VA = vitelline artery, VV = vitelline vein. Scale bar = 1mm. (B-E) Z-projection of the vascular plexus near the vitelline artery and vein immunostained for CD31 (i), Runx1 (i,ii) and Kit (i,iii). Scale bars = 50μm. (C) Single optical projection of a hematopoietic cluster in the vascular plexus in close proximity to the vitelline artery. (D) Z-projection of the venous plexus. (E) Z-projection of a hematopoietic cluster in the vascular plexus in close proximity to the vitelline vein. (F) Z-projection of an E10.5 yolk sac. Dotted blue lines demarcate the vitelline artery, dotted yellow lines demarcate the vitelline vein, and arrowheads point to CD31+ Runx1+ Kit+ clusters containing 5 or more cells. Scale bar = 1mm. (G-H) Immunostaining for CD31 (i), Runx1 (i,ii) and Kit (i,iii). (G) Z-projection of an E10.5 yolk sac near the vitelline artery. Scale bar = 250 μm. (H) Z-projection of a hematopoietic cluster near the vitelline artery. Scale bar = 50μm. (I) Z-projection of the venous vasculature of an E10.5 yolk sac. Scale bar = 250μm. (J) Magnified image of hematopoietic cluster from (I). Scale bar = 50μm.
Figure 3.4. Hematopoietic progenitor potential of Kit high cluster cells in the yolk sac at E9.5 and E10.5
Figure 3.4. Hematopoietic progenitor potential of Kit$^{\text{high}}$ cluster cells in the yolk sac at E9.5 and E10.5. (A) Representative scatter plots of CD31$^+$ VEC$^+$ Kit$^{\text{high}}$ hematopoietic cluster cells collected from E9.5 and E10.5 wild type yolk sacs for progenitor assays. (B) The frequency of erythro-myeloid progenitors (EMP), T cell progenitors, and B cell progenitors in the VEC$^+$ CD31$^+$ Kit$^{\text{high}}$ cluster population in the yolk sac at E9.5 and E10.5 (mean ± SD). Progenitor frequency is indicated above columns. Data are from three experiments using pooled cells from superovulated litters of E10.5 and E9.5 wild type embryos. Biological replicates are as follows: E9.5 EMP, n=8; E9.5 T progenitors, n=5; E9.5 B progenitors, n=4; E10.5 EMP, n=7; E10.5 T progenitors, n=7; E10.5 B progenitors, n=4. (C) Percent of EMP colony type derived from sorted VEC$^+$ CD31$^+$ Kit$^{\text{high}}$ cells. Mk: megakaryocyte; CFU-GEMM: granulocyte-erythroid-monocyte-megakaryocyte; BFU-E: burst forming unit-erythroid; G/M: granulocyte-macrophage colonies. Unpaired 2 tailed Student’s t-test applied to determine significance. ** indicates that P ≤ 0.01.
Figure 3.5. Yolk sac lymphoid progenitors are enriched in the Ly6a-GFP\(^+\) population of hematopoietic cluster cells that reside primarily in the arteries.
Figure 3.5. Yolk sac lymphoid progenitors are enriched in the Ly6a-GFP+ population of hematopoietic cluster cells that reside primarily in the arteries. (A-D, F-G) Immunostaining for CD31 (i), Runx1 (i, ii) and Ly6a-GFP (i, iii) (A) Confocal Z-projection of the vitelline artery (VA) and surrounding vascular plexus of a 28sp (E9.5) Tg(Ly6a-GFP) yolk sac. Scale bar = 100μm. (B) Z-projection of the vitelline vein (VV) and surrounding vascular plexus of a 28sp (E9.5) Tg(Ly6a-GFP) yolk sac. Scale bar = 100μm. (C) Z-projection of an E10.5 Tg(Ly6a-GFP) yolk sac immunostained for CD31 (i), GFP (i,ii) and Kit (i). White arrowheads point to hematopoietic clusters that contain 5 or more CD31+ Kit+ cells and no Ly6a-GFP+ cells, and green arrowheads point to hematopoietic clusters that contain 3 or more CD31+ Kit+ cells and at least 1 GFP+ cell. Scale bar = 1mm. (D) Magnified image of a hematopoietic cluster found within the vascular plexus of an E10.5 Tg(Ly6a-GFP) yolk sac immunostained for CD31 (i), Kit (i,iii) and GFP (i,ii). Scale bar = 50μm. (E) Quantification of CD31+ Kit+ Ly6a-GFP- hematopoietic clusters in arteries (7.7 ± 0.9) and veins (7.0 ± 3.6), and CD31+ Kit+ hematopoietic clusters containing Ly6a-GFP+ cells in arteries (5.0 ± 3.0) and veins (0.7 ± 0.6). Mean ± SD, n = 3. (F) Z-projection of a hematopoietic cluster within the vascular plexus of an E10.5 Tg(Ly6a-GFP) yolk sac. Scale bar = 100μm. (G) Z-projection of a hematopoietic cluster in the vitelline artery of an E10.5 Tg(Ly6a-GFP) yolk sac. Scale bar = 100μm. (H) Representative scatter plots of CD31+ VEC+ Kit$^{high}$ Ly6a-GFP+ and CD31+ VEC+ Kit$^{high}$ Ly6a-GFP- hematopoietic cluster cells collected from Tg(Ly6a-GFP) E10.5 yolk sacs for progenitor assays. (I) Frequency of T and B cell progenitors in the VEC+ CD31+ Kit$^{high}$ Ly6a-GFP+ and VEC+ CD31+ Kit$^{high}$ Ly6a-GFP- cluster populations from E10.5 yolk sacs (mean ± SD). Progenitor frequency is indicated above columns.
Data represent 2 biological replicates using pooled cells from superovulated litters of E10.5 Tg(Ly6a-GFP) embryos.
Figure 3.6. Expression of Runx1 in the major arteries at E8.0 and E8.5
Figure 3.6. Expression of Runx1 in the major arteries at E8.0 and E8.5. (A) Confocal Z-projection of the vessel of confluence (VOC) and surrounding primordial germ cells (PGCs) in a late head fold stage embryo (E8.0) immunostained for CD31 (i), Runx1 (i,ii) and Kit (i,iii). White arrowheads point to CD31⁺ Runx1⁺ endothelial cells in the VOC. Scale bar = 100 µm. (B-D) Immunostaining for CD31 (i), Runx1 (i,ii) and Ly6a-GFP (i,iii). (B) Confocal Z-projection of a 6 sp (E8.5) Tg(Ly6a-GFP) embryo. The top and bottom Z-sections containing the yolk sac were removed to make the vasculature in the embryo proper visible. A = allantois, VA = vitelline artery, pDA = paired dorsal aortae, VOC = vessel of confluence. Scale bar = 500 µm. See Movie 3.2 for animation of Z-stack. (C) Z-projection of one of the two vessels that make up the paired dorsal aortae in an E8.5 Tg(Ly6a-GFP) embryo. Scale bar = 100 µm. (D) Z-projection of the vitelline artery; white arrowhead points to a CD31⁺ Runx1⁺ Ly6a-GFP⁺ endothelial cell and yellow arrowhead points to a CD31⁺ Runx1⁺ Ly6a-GFP⁻ endothelial cell. Scale bar = 100 µm.
Figure 3.7. Expression of Runx1 and Kit in the major arteries at E9.5
Figure 3.7. Expression of Runx1 and Kit in the major arteries at E9.5. (A-D) Immunostaining for CD31 (i), Runx1 (i,ii) and Kit (i,iii). (A) Confocal Z-projection of a 25sp mouse embryo partially enveloped in its yolk sac. Scale bar = 500µm. See Movie 3.3 for animation of Z-stack (B) Z-projection of the paired dorsal aortae (pDA) and vitelline artery (VA). Scale bar = 100µm. Magnified view of boxed region on the right shows primordial germ cells (PGCs) in between the DA and VA. (C) Single optical projection through the pDA shown in (B). (D) Single optical projection through the VA shown in (B); clusters are visible on both the ventral and dorsal sides of the VA.
Figure 3.8. Confocal analysis of hematopoietic cluster cell nuclear shape at E10.5
Figure 3.8. Confocal analysis of hematopoietic cluster cell nuclear shape at E10.5. (A-D) Immunostaining for CD31 (i), Runx1 (i,ii) and Kit (i,iii). (A-B) Confocal Z-projections of hematopoietic clusters in the umbilical artery of an E10.5 embryo. Arrowhead points to a cluster cell with a round nucleus (see Movies 3.4-3.5 for 3D reconstruction) (C-D) Z-projections of hematopoietic clusters in the yolk sac of an E10.5 embryo (see Movies 3.6-3.7 for 3D reconstruction). (E-H) Immunostaining for CD31 (i), Runx1 (i,ii) and Ly6a-GFP (i,iii) (E-F) Z-projections of hematopoietic clusters in the umbilical arteries of E10.5 Tg(Ly6a-GFP) embryos (see Movies 3.8-3.9 for 3D reconstruction). (G-H) Z-projections of hematopoietic clusters in the yolk sacs of E10.5 Tg(Ly6a-GFP) embryos (see Movies 3.10-3.11 for 3D reconstruction).
Figure 3.9. Hematopoietic cluster formation in the heart
Figure 3.9. Hematopoietic cluster formation in the heart. (A-C) Immunostaining for CD31 (i) and Runx1 (i,ii). (A) Confocal Z-projections of the hearts of E8.5 (9sp), E9 (16sp) and E9.5 (22sp) embryos. VA = vitelline artery. (B) Z-projection of the ventricle of an E10.5 embryo. Arrowhead points to a Runx1+ endocardial cell. Scale bar = 50µm. (C) Z-projection of the atrioventricular canal of an E10.5 embryo. Arrowhead points to Runx1+ endocardial cell. Scale bar = 50µm. (D) Ventricle of an E10.5 mouse embryo immunostained for CD31 and Kit. Arrowheads point to hematopoietic clusters in the ventricular cavity in both the Z-projection and the single optical projection. All scale bars = 100µm. (E) Confocal Z-projection of the heart of an E10.5 embryo immunostained for CD31 and Runx1. Arrowheads point to hematopoietic clusters in the ventricular cavity and atrioventricular canal. Inset represents a single optical projection showing the hematopoietic cluster in the ventricular cavity. A = atrium, V = ventricle, FL = fetal liver. (F) Z-projection of the heart of an E11.5 embryo immunostained for CD31 and Runx1. Arrowheads point to cardiac blood islands. A = atrium, V = ventricle; scale bars = 250µm. Lower panels are magnified images of cardiac blood islands. Scale bars in lower panels = 50µm.
Figure 3.10. Hematopoietic cluster formation in the head
**Figure 3.10. Hematopoietic cluster formation in the head.** (A-E) Immunostaining for CD31 (i), Runx1 (i,ii) and Kit (i,iii). (A) Confocal Z-projection of the head of an E9.5 embryo. Scale bar = 500µm. Arrowheads point to Runx1⁺ Kit⁺ neurons. (B) Magnified view of the boxed region in (A) demonstrating the presence of Runx1⁺ Kit⁺ and Runx1⁻ Kit⁻ cells with hematopoietic morphology within the cephalic vascular plexus. Scale bar = 50µm. (C) Confocal Z-projection of the head of an E10.0 embryo. Arrowheads point to Runx1⁻ Kit⁺ neurons. Scale bar = 500µm. (D) Magnified view of the boxed region in (C). Scale bar = 50µm. (E) Confocal Z-projection of the head of an E10.5 embryo. Arrowheads point to Runx1⁻ Kit⁺ neurons. Scale bar = 500µm. (F) Single optical projection of a hematopoietic cluster found in the peripheral cephalic plexus of the E10.5 head in (E). Scale bar = 50µm.
Figure 3.11. Hematopoietic cluster formation in the somitic region at E10.5
Figure 3.11. Hematopoietic cluster formation in the somitic region at E10.5. (A) Confocal Z-projection of the dorsal aorta (DA) and somitic region of an E10.5 mouse immunostained for CD31 (i), Runx1 (i,ii) and Kit (i,iii). Arrowheads point to hematopoietic clusters in the dorsal longitudinal anastomotic vessels (DLAV) and intersomitic vessels (ISV). Scale bar = 500µm (B) Magnified view of the hematopoietic cluster within the DLAV shown in (A).
**Movie 3.1.** Animation of a Z-stack of a late head fold stage embryo immunostained for CD31 (red), Runx1 (grey) and Kit (green). Z-sections collected at 5µm intervals. Z-projection of this sample is shown in Fig. 3.1A.

**Movie 3.2.** Animation of a Z-stack of a 6sp Tg(Ly6a-GFP) embryo immunostained for CD31 (red), Runx1 (grey) and GFP (green). Z-sections collected at 5µm intervals. Z-projection of this sample is shown in Fig. 3.6B.

**Movie 3.3.** Animation of a Z-stack of a 25sp embryo immunostained for CD31 (red), Runx1 (grey) and Kit (green). Z-sections collected at 5µm intervals. Z-projection of this sample is shown in Fig. 3.7A.

**Movie 3.4-3.5.** 3-dimensional reconstruction of a hematopoietic cluster in the umbilical artery of an E10.5 embryo immunostained for CD31 (red), Runx1 (grey) and Kit (green). Z sections collected at 0.5 µm.

**Movie 3.6-3.7.** 3-dimensional reconstruction of a hematopoietic cluster in the yolk sac of an E10.5 embryo immunostained for CD31 (red), Runx1 (grey) and Kit (green). Z sections collected at 0.5 µm.
**Movie 3.8-3.9.** 3-dimensional reconstruction of a hematopoietic cluster in the umbilical artery of an E10.5 Tg(Ly6a-GFP) embryo immunostained for CD31 (red), Runx1 (grey) and GFP (green). Z sections collected at 0.5 µm. Yellow arrowheads indicate Ly6a-GFP⁺ hematopoietic cluster cells.

**Movie 3.10-3.11.** 3-dimensional reconstruction of a hematopoietic cluster in the yolk sac of an E10.5 Tg(Ly6a-GFP) embryo immunostained for CD31 (red), Runx1 (grey) and GFP (green). Z sections collected at 0.5 µm. Yellow arrowheads indicate Ly6a-GFP⁺ hematopoietic cluster cells.
CHAPTER 4: Extravascular endothelial and hematopoietic islands form through multiple pathways in midgestation mouse embryos
Abstract

The \textit{de novo} generation of hematopoietic cells occurs during midgestation when a population of endothelial cells called hemogenic endothelium transitions into hematopoietic progenitors and stem cells. In mammalian embryos, the newly formed hematopoietic cells form clusters in the lumens of the major arteries in the embryo proper and in the vascular plexus of the yolk sac. Small clusters of hematopoietic cells that are independent of the vasculature (referred to here as extravascular islands) were shown to form in the mesentery during vascular remodeling of the vitelline artery. Using three-dimensional imaging of whole mouse embryos we demonstrate that extravascular budding of hematopoietic clusters is a more widespread phenomenon that occurs from the vitelline and the umbilical arteries both proximal to the embryo proper and distal in the extraembryonic yolk sac and placenta. Furthermore, we show that there are several mechanisms by which hematopoietic clusters leave the arteries, including vascular remodeling and extrusion. Lastly, we provide static images suggesting that extravascular islands contribute to the formation of new blood vessels. Thus, extravascular islands may represent a novel mechanism of vasculogenesis whereby established vessels contribute endothelial and hematopoietic cells to developing vascular beds.
Introduction

During embryogenesis, definitive hematopoietic progenitors and stem cells (HSPCs) are derived de novo from a transient population of endothelial cells called hemogenic endothelium (HE). HE is located in the large diameter arteries including the vitelline artery, umbilical artery and dorsal aorta, and is also found in the heart, yolk sac and the head (Li et al., 2012, Nakano et al., 2013, Frame et al., 2015). HE cells are the precursors to hematopoietic cells, and as such transition into the full repertoire of definitive hematopoietic cells including erythro-myeloid progenitors, lymphoid progenitors, and hematopoietic stem cells (HSCs) during a process termed the endothelial to hematopoietic transition (EHT). Live-imaging studies demonstrated that during the EHT, HE cells bend away from the endothelial layer eventually releasing contact with neighboring endothelial cells and acquiring hematopoietic morphology in a cell-division independent process (Kissa and Herbomel, 2010, Lam et al., 2010, Boisset et al., 2010). The EHT is dependent upon expression of the transcription factor Runx1 (Lancrin et al., 2009, Chen et al., 2009, Boisset et al., 2010, North et al., 1999, Kissa and Herbomel, 2010, Yokomizo et al., 2001). Runx1 expression initiates a hematopoietic transcriptional program and inhibits an endothelial program in HE allowing the EHT to progress (Lancrin et al., 2012b, Lichtinger et al., 2012, Hoogenkamp et al., 2009). Runx1 is widely considered the master regulator of hematopoiesis and is the most reliable marker of HE identity.

After the EHT, the newly formed hematopoietic cells take different paths in different species. In zebrafish, hematopoietic cells bud away from the lumen of the artery into the sub-aortic space, and enter the circulation via intravasation through the axial
vein (Murayama et al., 2006, Kissa et al., 2008b). In chick embryos the EHT occurs both into the aortic lumen and also out of the aorta into the subaortic mesenchyme (Jaffredo et al., 2000, Jaffredo et al., 1998). In mammalian embryos the newly formed hematopoietic cells bud into the lumen of the artery where they remain briefly attached to the endothelium forming clusters of hematopoietic cells (Yokomizo and Dzierzak, 2010, Garcia-Porrero et al., 1995). Hundreds of hematopoietic clusters line the major arteries of the midgestation mouse embryo and dozens can be found within the vascular plexus of the yolk sac (Yokomizo and Dzierzak, 2010, Frame et al., 2015). Hematopoietic clusters are heterogeneous, as individual cells within a single cluster can differ in gene expression and function (Yokomizo and Dzierzak, 2010). For example, the Ly6a-GFP transgene (Ly6a encodes Sca-1) was shown to mark a subset of hematopoietic cluster cells that are enriched for HSCs and lymphoid progenitors (Li et al., 2014, de Bruijn et al., 2002).

Clusters of hematopoietic cells wrapped in endothelial cells were observed in the mesentery of chick embryos one hundred years ago (Miller, 1913). These structures became known as mesenteric blood islands owing to their separation from the cardiovascular system. Mesenteric blood islands were observed in the vicinity of the aortic arches and along the aorta as far as the superior mesenteric artery (the structure that supersedes the vitelline artery) in 100 to 110 hour chick embryos (Miller, 1913). In the 1990’s mesenteric blood islands were also observed in mouse embryos between embryonic day (E) 9.5 and E11.5 (Garcia-Porrero et al., 1998, Garcia-Porrero et al., 1995). At the time, the origin of mesenteric blood islands was unknown, but immunohistochemistry of E11 mouse sections illustrated that mesenteric blood islands
expressed the endothelial/hematopoietic markers CD31, CD34 and vWf, similar to intra-arterial clusters (Garcia-Porrero et al., 1998). Further histological analysis of sectioned mouse embryos revealed three types of mesenteric blood islands: type I consisting of tightly arranged, highly basophilic, electron-dense cells with no clear endothelial component, type II characterized by undifferentiated hemocytoblasts that are tightly wrapped by endothelial cells, and type III consisting of erythroblasts and hemocytoblasts that are loosely arranged and circumscribed by a single layer of endothelial cells (Garcia-Porrero et al., 1995). The vitelline artery was later identified as the source of mesenteric blood islands (Zovein et al., 2010). During midgestation the vitelline artery undergoes extensive remodeling, and during this process clusters of hematopoietic cells leave the artery and migrate through the mesentery as aggregates of endothelial and hematopoietic cells (Zovein et al., 2010). However, the extravascular emergence of hematopoietic clusters was called into question when studies using three-dimensional imaging of whole mouse embryos only found hematopoietic clusters within arterial lumens (Yokomizo et al., 2011).

Using confocal microscopy, we examined hematopoiesis in midgestation mouse embryos by mapping the expression of hematopoietic and endothelial-specific proteins. We provide additional evidence that extravascular clusters of endothelial and/or hematopoietic cells (referred to here as extravascular islands) emerge from the vitelline artery during vascular remodeling. We also demonstrate that the umbilical artery is another source of extravascular islands. Furthermore, we observed extravascular islands in both the embryo proper as well as in the extraembryonic yolk sac and placenta. We propose two mechanisms by which extravascular islands leave the
umbilical and vitelline arteries including extrusion and vascular remodeling. We also demonstrate that extravascular islands elongate and contribute endothelial and hematopoietic cells to newly forming blood vessels. Finally, we show that neither the hematopoietic nor endothelial component of extravascular islands express the transcription factor Prox1, and are therefore unlikely to contribute to the lymphatic vascular system.
Results and discussion

Extravascular islands are localized near the umbilical and vitelline arteries both proximal to the embryo proper and distal in the placenta and yolk sac

To localize extravascular islands in midgestation (E10.5-E11.5) embryos we immunostained for CD31 to mark the entire vasculature, Runx1 to mark hemogenic endothelial (HE) and hematopoietic cells, and Kit to identify hematopoietic cluster cells (Fig. 4.1 A). We also analyzed Tg(Ly6a-GFP) embryos to determine if endothelial and hematopoietic cells in extravascular islands express Ly6a-GFP, which would suggest lymphoid/HSC potential (Chen et al., 2011, Li et al., 2014) (Fig. 4.1 A). In our analysis of whole embryos, we observed all three types of the previously described extravascular islands (referred to in Garcia-Porrero et al. as mesenteric blood islands) (Garcia-Porrero et al., 1995). In addition, we identified a fourth type (Type IV) that consists of a sphere of endothelial cells without associated hematopoietic cells (Fig. 4.1 B-C). By analyzing whole, as opposed to sectioned embryos, we were able to pinpoint the location of the extravascular islands to the region between the vitelline and umbilical arteries in the embryo proper (Fig. 4.2 A-B, arrowheads).

Confocal analysis of the umbilical artery of a 40 somite pair (sp) embryo reveals a large type II extravascular island consisting of 25-35 tightly arranged Runx1⁺ CD31⁺ Ly6a-GFP⁺ hematopoietic cells (Fig. 4.3 A, arrowhead) wrapped in a single layer of endothelial cells (Fig. 4.3 B, arrows). The endothelial cells do not express Runx1 or Ly6a-GFP and are therefore unlikely to be hemogenic (Fig. 4.3 B). Individual Z-slices
through the extravascular island demonstrate that it is circumscribed by endothelium but independent of the vascular network (Fig. 4.3 B and Movie 4.1). Currently, it is not possible to separate extravascular islands from intravascular clusters, so gauging the function and potential of these cells is difficult. However, the lack of Ly6a-GFP expression suggests that they do not contain lymphoid progenitors or HSCs as these populations are enriched in the Ly6a-GFP* fraction of hematopoietic cluster cells (de Bruijn et al., 2002, Li et al., 2014).

We also found extravascular islands in extra-embryonic locations, at the distal ends of the umbilical and vitelline arteries near the chorion and yolk sac, respectively. Confocal analysis of a 36 sp yolk sac revealed a large type III extravascular island containing 75-100 Runx1* cells located between the vitelline artery and the vascular plexus (Fig. 4.4 A-B, arrowhead). Single optical projections through the extravascular island reveals that the hematopoietic cells are heterogeneous; most Runx1* cells express Kit but several near the periphery of the island lack Kit expression (Fig. 4.4 C, arrows). Confocal analysis of the chorion from a 40sp Tg(Ly6a-GFP) embryo immunostained for CD31 and GFP demonstrates that type I extravascular islands also form near the distal end of the umbilical artery near the chorionic vascular plexus (Fig. 4.4 D and E, arrowheads). Although we found no intravascular hematopoietic clusters in the chorion, we identified a type III extravascular island at the base of the umbilical artery where it connects to the placenta in an E11.5 (46sp) embryo (Fig. 4.4 F, arrowhead). The type III extravascular island was heterogeneous containing 32 CD31* Runx1* Kit* cells and 29 CD31* Runx1* Kit* cells (Fig. 4.4 F). Since extravascular islands are usually located near arteries that contain intravascular hematopoietic clusters, the
type III extravascular island near the base of the umbilical artery may have migrated to
the chorion from a more proximal location in the umbilical artery where clusters are
abundant.

**Extravascular islands exit blood vessels via vascular remodeling and ballooning**

Between E9.0 and E11.0 the vitelline artery (VA) undergoes an extensive
remodeling process. At E9.0 the VA runs parallel to the paired dorsal aorta (DA),
connecting to the DA at the caudal end of the embryo. By E11 the connection of the VA
to the DA has moved in the rostral direction and is located at the level of the midgut. The
rostral movement of the intersection between the VA and DA occurs via a tributary of
smaller connecting vessels that are established then lost during the remodeling process.
The tributary vessels are associated with a large concentration of CD31⁺ Runx1⁺ Kit⁺
hematopoietic clusters at E10.5 (Fig. 4.5 A). As reported by Zovein et al. (Zovein et al.,
2010) hematopoietic clusters in the small tributary vessels of the remodeling vitelline
artery may become extravascular as the vessels surrounding them retract during the
remodeling process. This idea is supported by the observation that CD31⁺ Runx1⁺ Kit⁺
hematopoietic clusters in these small vessels are often connected to the large diameter
vessels via endothelial projections that do not appear to contain lumens, thus preventing
the hematopoietic cluster cells from entering the circulation (Fig. 4.5 B, arrows).

During lymphangiogenesis, lymphatic endothelial cells balloon out from the
cardinal vein to create primitive lymphatic sacs (François et al., 2012). We observed a
similar phenomenon in the umbilical and vitelline arteries as hematopoietic clusters
extruded ("ballooned") from the vessels, eventually pinching off to form extravascular islands. Single optical projections of a 34sp embryo revealed CD31⁺ Runx1⁻ Kit⁻ endothelial cells beneath a hematopoietic cluster egressing away from the lumen of the umbilical artery as the cluster is extruded (Fig. 4.6 A, arrowhead). Ballooning also occurred in the vitelline artery, as illustrated in a confocal Z-projection of a 32 sp embryo (Fig. 4.6 B,C). The balloon-shaped extrusion of CD31⁺ endothelial cells from the vitelline artery has entrapped part of a cluster, the rest of which remains intra-arterial (Fig. 4.6 C and Movie 4.2). These images suggest that extravascular islands emerge from the vitelline artery both through ballooning and vascular remodeling. We analyzed the dorsal aortas of 14 E10.5 embryos between 32 and 39sp and 6 E11.5 embryos between 42 and 49sp and did not observe ballooning of endothelium beneath hematopoietic clusters in the dorsal aorta (Fig. 4.6 A).

Since type I extravascular islands do not contain an endothelial component, their mechanism of vascular exit may be more akin to transendothelial migration, or similar to the EHT that occurs in zebrafish embryos which is oriented away from the vessel lumen. This demonstrates that there are at least three mechanisms by which extravascular islands form: 1) extrusion, in which endothelial cells balloon from the vessel trapping hematopoietic clusters as they pinch off from the vessel (Fig. 4.7 A); 2) the retraction of vessels surrounding hematopoietic clusters due to vascular remodeling (Fig. 4.7 B); and 3) transendothelial migration of type I hematopoietic cluster cells or EHT away from rather than into the vessel lumen.
Extravascular islands contribute to vasculogenesis

An identical process to extravascular budding of hematopoietic clusters has previously been described in the heart. Between E11 and E14 ventricular endocardial cells near the interventricular sulci balloon outwards into the myocardium (Jankowska-Steifer et al., 2015b, Red-Horse et al., 2010, Ratajska et al., 2009b, Ratajska et al., 2006b). These protrusions, called cardiac blood islands consist primarily of erythroblasts and megakaryocytes circumscribed by a single layer of endocardial cells (Jankowska-Steifer et al., 2015b). Cardiac blood islands are thought to pinch off from the ventricular endocardium and fuse with the coronary vessels, contributing to the development of the coronary vasculature (Ratajska et al., 2009b, Red-Horse et al., 2010). We also noted the apparent merging of extravascular islands with the vasculature in the embryo proper. A confocal Z-projection of the umbilical artery of a 32sp Tg(Ly6a-GFP) embryo shows extravascular islands on the cranial side of the umbilical artery. They included a spherical type II extravascular island consisting of CD31⁺ Runx1⁺ GFP⁻ hematopoietic cells circumscribed by CD31⁺ Runx1⁻ GFP⁻ endothelial cells (Fig. 4.8 A, white boxed region, 8B, and Movie 4.4), as well as several type IV extravascular islands that lack hematopoietic cells (Fig. 4.8 A, yellow boxed region and C). Between 32 and 38 sp, large, elongated type II, III and IV extravascular islands are visible on the cranial side of the umbilical artery (Fig. 4.8 D-F, Movie 4.5). Some extravascular islands at this stage are connected to small diameter vessels running parallel to the umbilical artery, suggesting that they contributed to the formation of those vessels (Fig. 4.8 F and H, arrowheads). More well-developed small diameter vessels running parallel to the umbilical vessels were also observed in 32sp to 40sp embryos (Fig. 4.8 G and J).
Confocal analysis of these small vessels demonstrates that they are often associated with a large concentration of Runx1\(^+\) hematopoietic cells, but do not contain Runx1\(^+\) CD31\(^+\) hemogenic endothelial cells (Fig. 4.8 G and J, Movies 4.5 and 4.6). As the majority of hematopoietic cells in circulation at this stage are CD31\(^{low}\) Runx1\(^-\) Kit\(^-\) erythroid cells, the numerous CD31\(^+\) Runx1\(^+\) Ly6a-GFP\(^{+/-}\) hematopoietic cells in the small vessels are most likely derived from extravascular islands. This is confirmed by 3D reconstruction of the umbilical artery of a 40sp Tg(Ly6a-GFP) embryo; the small vessel above the umbilical artery does not appear to be connected to the rest of the vasculature, indicating that the hematopoietic cells are unlikely to have arrived there via the circulation (Fig. 4.8 G, Movie 4.6). A Z-projection of the umbilical artery of a 38sp embryo immunostained for CD31, Runx1 and Kit confirms the absence of Runx1\(^+\) hemogenic endothelial cells in the small parallel vessel located cranial to the umbilical artery (Fig. 4.8 J). Interestingly, a cluster of CD31\(^+\) Runx1\(^+\) Kit\(^+\) hematopoietic cells connected to the small vessel above the umbilical artery has a diameter larger than that of the vessel (Fig. 4.8 J, arrowhead). Therefore it is unlikely that this hematopoietic cluster was derived from the vessel, and more likely that it was an extravascular island that fused to the vessel. These observations suggest that extravascular islands derived from established large diameter vessels contribute to the development of nearby blood vessels.

Extravascular islands are unlikely to contribute to the lymphatic vasculature
Development of the lymphatic vasculature begins with expression of the transcription factor Sox18, and following that the lineage specifying transcription factor Prox1 in endothelial cells in the cardinal vein (Oliver and Srinivasan, 2010, Wigle and Oliver, 1999, Wigle et al., 2002, Hong et al., 2002, Petrova et al., 2002). The Prox1-expressing endothelial cells then balloon out to form primary lymphatic sacs (François et al., 2012). However, recently it was proposed that hemogenic endothelium in the major arteries also contributes to the lymphatic vasculature (Stanczuk et al., 2015). This hypothesis was based on lineage tracing experiments in which Cre recombinase driven from the Kit regulatory sequences (Kit is expressed at low levels on hemogenic endothelium (Marcelo et al., 2013b)) was shown to mark cells in the lymphatic vasculature (Stanczuk et al., 2015). We reasoned that if hemogenic endothelium contributes to the formation of the lymphatic vasculature, that extravascular islands might be involved. To determine whether a subset of extravascular islands contained prospective lymphatic endothelium we determined whether they contained Prox1⁺ endothelial cells. Prox1 is expressed starting at E9.5 in endothelial cells in the cardinal vein, that at E10.5 begin delaminating from the vessel (Wigle and Oliver, 1999). At E10.5 (32-38sp) we observed Prox1⁺ (Kit⁺) lymphatic endothelial cells in the cardinal vein, as expected (Fig. 4.9 A) (François et al., 2012, Wigle and Oliver, 1999, Yang et al., 2012, Hägerling et al., 2013). In the same embryos, we observed several extravascular islands including a small type I island near the vitelline artery consisting of 4-5 Kit⁺ CD31⁺ Prox1⁻ hematopoietic cells with no endothelial component (Fig. 4.9 B-C, arrowhead), and a type III island near the umbilical artery, which consisted of 12-15 loosely arranged CD31⁺ Kit⁺ Prox1⁻ hematopoietic cells and CD31⁺ Kit⁻ Prox1⁺ endothelial cells (Fig. 4.9 D-E, arrowhead). Also shown are two type II extravascular islands near the vitelline artery in
the yolk sac of a 38 sp embryo (Fig. 4.9 F-G, arrowheads). Neither the hematopoietic nor the endothelial components of the extravascular islands expressed Prox1. In total we identified 7 extravascular islands in 7 E10.5 embryos, and found none with Prox1+ cells, suggesting that extravascular islands do not contribute to the lymphatic vasculature.

**Conclusion**

Whole mount confocal microscopy revealed that extravascular islands are common in midgestation mouse embryos. The islands are released from the arteries via several mechanisms including extrusion (ballooning), the retraction of vessels during vascular remodeling, and transendothelial migration or EHT away from the vessel lumen. Although extravascular islands could simply be byproducts of vascular remodeling and EHT, we instead posit that they actively contribute to the formation of new vessels, similar to what has been described for the coronary vasculature (Red-Horse et al., 2010). Our data suggest that extravascular islands do not, however, contribute to the lymphatic vasculature, based on the absence of Prox1 expression. It is formally possible, though, that Prox1 is expressed after extravascular islands have fused with the lymphatic vessels.

Although the budding of hematopoietic cluster cells away from the arterial lumen was not believed to occur in mammalian embryos, our data suggest that this process, which is common in chick embryos, is also conserved in mammalian embryos, although not in the dorsal aorta. Our pictures are necessarily static. Nevertheless, we believe that they capture a dynamic aspect of vascular remodeling in the embryo.
Figure 4.1. Four types of extravascular islands
Figure 4.1. Four types of extravascular islands. (A) Schematic illustrating the cell types identified by the combination of markers used in the analysis. (B) The four types of extravascular islands. Type I consists of tightly arranged hematopoietic cells with no endothelial component. Type II islands contain tightly arranged hematopoietic cells wrapped in a single layer of endothelial cells. Type III has loosely arranged hematopoietic cells wrapped in an endothelial layer. Type IV consists of a sphere of endothelial cells that are not associated with hematopoietic cells. (C) Z-projection of a type IV extravascular island that has been immunostained for CD31 (i,ii), Runx1 (i,iii) and Kit (i,iv). Panel on the right is a single optical projection illustrating the lack of Runx1+ Kit+ hematopoietic cells within a type IV extravascular island. Z-interval = 2µm and scale bar = 50µm.
Figure 4.2. Extravascular islands localize between the remodeling vitelline artery and umbilical artery at E10.5
Figure 4.2. Extravascular islands localize between the remodeling vitelline artery and umbilical artery at E10.5. (A) Z-projection of an E10.5 embryo immunostained for CD31 (i, ii) and Runx1 (i, iii). The embryo is oriented with its cranial end on the top and dorsal edge on the left. Lower panels are split channel views of the boxed region. Arrowheads in bottom panels indicate extravascular islands. Z-interval = 1.5µm, scale bar = 100µm. (B) Z-projection of an E10.5 Tg(Ly6a-GFP) embryo immunostained for CD31 (i, ii) and GFP (i, iii). Lower panels are split channel views of the boxed region. Arrowheads indicate extravascular islands. Z-interval = 5µm, scale bar = 100µm. DA = dorsal aorta, VA = vitelline artery, UA = umbilical artery.
Figure 4.3. Extravascular islands do not contain Runx1$^+$ hemogenic endothelial cells
Figure 4.3. Extravascular islands do not contain Runx1⁺ hemogenic endothelial cells. (A) Whole-mount Z-projection of the umbilical artery and vein of a 40sp Tg(Ly6a-GFP) embryo immunostained for CD31, Runx1 and GFP. Arrowhead indicates a Type II extravascular island on the cranial side of the umbilical artery. The extravascular island is located in between the embryo proper (which would be located to the left) and the chorion; inset is a magnified view of the extravascular island. Z-interval = 2µm, scale bar = 100µm; Z-interval of inset image = 1.17µm. UA = umbilical artery and UV = umbilical vein. (B) Montage of Z-slices through the extravascular island in (A). Each slice is 1.17µm apart. White arrows indicate endothelial cells.
Figure 4.4. Extravascular islands in the extraembryonic yolk sac and placenta
Figure 4.4. Extravascular islands in the extraembryonic yolk sac and placenta. (A-C) Immunostaining for CD31 (i,ii) Runx1 (i,iii) and Kit (i,iv). (A) Confocal Z-projection of a 36sp yolk sac. Arrowhead indicates a large type III extravascular island near the vitelline artery (VA). Z-interval= 2µm and scale bar = 100µm. (B) Magnified view of the extravascular island in (A). Z-interval =2µm and scale bar = 50µm. (C) Single optical projection from Z-projection in (B), arrows indicate representative CD31⁺ Runx1⁺ Kit⁻ cells within the island. (D-E) Immunostaining for CD31 (i) and Ly6a-GFP (i,ii). (D) Whole-mount confocal Z-projection of the umbilical artery, vein and chorionic vessels of a 40sp Tg(Ly6a-GFP) placenta. Z-interval = 5µm, scale bar = 500µm. (E) Montage of Z-slices from the boxed in region in (D). Z-slices are 5µm apart and arrowheads indicate type IV extravascular islands near the intersection of the umbilical artery and the chorion. (F) Z-projection of umbilical artery and chorionic vessels of a 46sp placenta immunostained for CD31 (i), Runx1 (i,ii) and Kit (i,iii). Arrowhead points to a type III extravascular island. Z-interval = 5µm and scale bar = 100µm. Inset is a magnified image of the type III extravascular island. Z-interval of the inset = 2µm and scale bar = 10µm.
Figure 4.5. Hematopoietic clusters in the remodeling vitelline artery
Figure 4.5. Hematopoietic clusters in the remodeling vitelline artery. (A-B) Immunostaining for CD31 (i), Runx1 (i,ii) and Kit (i,iii). (A) Confocal Z-projection of the tributary vessels of the remodeling vitelline artery (VA) of a 34sp embryo. Z-interval = 5µm, scale bar = 100µm, DA = dorsal aorta. The embryo is oriented with its cranial end on the top and dorsal edge on the left. (B) Single optical projection of a hematopoietic cluster in the small tributary vessels of the vitelline artery of a 32sp embryo. Arrows indicate small endothelial projections that do not contain lumens. Scale bar = 50µm.
Figure 4.6. Extrusion of hematopoietic clusters from the umbilical and vitelline artery through ballooning.
Figure 4.6. Extrusion of hematopoietic clusters from the umbilical and vitelline artery through ballooning. (A) Single optical projection of hematopoietic clusters immunostained for CD31 (i, ii), Runx1 (i, iii) and Kit (i, iv) in the umbilical artery (top panels) and dorsal aorta (bottom panels) of 34-36sp embryos. Outline highlights endothelial layer beneath the hematopoietic clusters. Arrowhead points to extruding cluster. Scale bar = 50µm. (B) Z-projection of a 32sp embryo immunostained for CD31. Scale bar = 500µm. Z-interval = 11.04µm. DA = dorsal aorta, VA = vitelline artery and UA = umbilical artery. (C) Magnified view of boxed area in (B). Dotted line outlines cranial side of the vitelline artery to highlight the ballooning hematopoietic cluster (arrowhead). Z-interval = 2.34µm.
Figure 4.7. Schematics illustrating the different mechanisms of the extravascular budding of clusters
Figure 4.7. Schematics illustrating the different mechanisms of the extravascular budding of clusters. (A) Schematic illustrating ballooning of a hematopoietic cluster from an artery. (B) Vascular remodeling as a mechanism that promotes the formation of extravascular islands.
Figure 4.8. Extravascular islands contribute endothelial and hematopoietic cells to nearby vascular beds.
Figure 4.8. Extravascular islands contribute endothelial and hematopoietic cells to nearby vascular beds. (A-C) Immunostaining for CD31, Runx1 and Ly6a-GFP. (A) Z-projection of the umbilical artery (UA) of a 32sp embryo. Z-interval = 2µm and scale bar = 100µm. White boxed region contains a type II, and yellow box a type IV extravascular island) (see Movie 4.3 for 3D reconstruction) (B) Magnified image of a type II extravascular island on the cranial side of the UA in the white boxed region in (A). Scale bar = 10µm and Z-interval = 1.5µm. (C) Magnified image of an elongated type IV extravascular island in the yellow boxed region in (A). Scale bar = 10µm and Z-interval = 1.5µm. (D-F) Immunostaining for CD31, Runx1 and Kit. (D) Z-projection of the UA of 36sp embryo. Arrowhead points to a large elongated type III extravascular island. Scale bar = 100µm and Z-interval = 2µm (see Movie 4.4 for 3D reconstruction). (E) Magnified image of a type III elongated extravascular island in (D). Scale bar = 10µm and Z-interval = 1.5µm. (F) Z-projection of an extravascular island near the UA. Arrowhead points to endothelial cells sprouting from one end of the extravascular island. Scale bar = 50µm and Z-interval = 1.5µm. (G-I) Immunostaining for CD31, Runx1 and Ly6a-GFP. (G) Z-projection of the UA of a 40sp embryo. Arrowhead points to a type II hematopoietic island that appears to have merged with a small diameter vessel running parallel to the UA. Z-interval = 2µm and scale bar = 100µm (see Movie 4.5 for 3D reconstruction). (H) Magnified image of the extravascular island on the cranial side of the UA indicated by an arrowhead in (G). Arrowhead points to endothelial cells projecting from the extravascular island. Scale bar = 10µm and Z-interval = 1.5µm. (I) Magnified image of a type IV extravascular island behind the UA in (G) (not visible in panel G, see Movie 4.5). Scale bar = 10µm and Z-interval = 1.5µm. (J) Z-projection of the UA of a 32sp embryo.
immunostained for CD31, Runx1 and Kit. Arrowhead points to an extravascular island that is incorporated into a small diameter vessel running parallel to the UA. Scale bar = 100µm and Z-interval = 2µm. (See Movie 4.6 for 3D reconstruction)
Figure 4.9. Extravascular islands do not express the lymphatic endothelial marker Prox1
Figure 4.9. Extravascular islands do not express the lymphatic endothelial marker Prox1. (A-G) Immunostaining for CD31 (i), Prox1 (i,ii) and Kit (i,iii). (A) Z-projection of the cardinal vein (CV) and intersomitic vessels (ISV) of a 32sp embryo. Scale bar = 100µm and Z-interval = 2µm. (B) Z-projection of the vitelline artery (VA) of a 32sp mouse embryo. The arrowhead indicates an extravascular island. Scale bar = 100µm and Z-interval = 2µm. (C) Montage of Z-slices of the type I extravascular island seen in (B). Z-slices are 2µm apart. (D) Z-projection of the umbilical artery of a 36 sp mouse. Arrowhead indicates a type III extravascular island. Scale bar = 50µm and Z-interval = 2µm. (E) Montage of Z-slices through the type III extravascular island seen in (D). Z-slices are 2µm apart. (F) Z-projection of the yolk sac of a 38sp embryo. Arrowheads indicate extravascular islands near the VA. Scale bar = 500µm and Z-interval = 2µm. (G) Montage of Z-slices of the type II extravascular islands seen in (F). Z-slices are 2µm apart.
**Movie 4.1.** 3-dimensional reconstruction of a type II extravascular island on the cranial side of the umbilical artery in a 40sp Tg(Ly6a-GFP) embryo. Immunostaining for CD31 (red), Runx1 (cyan) and GFP (green). Z-projection of this sample is shown in Figure 4.3.

**Movie 4.2.** Animation of a Z-stack of a 32 sp embryo immunostained for CD31 (red), showing an extruding (ballooning) extravascular island (arrow). The Z-projection of this sample is shown in Fig. 4.6 C.

**Movie 4.3.** 3-dimensional reconstruction of extravascular islands on the cranial side of the umbilical artery in a 32sp Tg(Ly6a-GFP) embryo. Immunostaining for CD31 (red), Runx1 (grey) and GFP (green). A Z-projection of this sample shown in Figure 4.8 A.

**Movie 4.4.** 3-dimensional reconstruction of an elongated type III extravascular island on the cranial side of the umbilical artery of a 36sp embryo. Immunostaining for CD31 (red), Runx1 (grey) and Kit (green). Z-projection of this sample is shown in Figure 4.8 D.

**Movie 4.5.** 3-dimensional reconstruction of a small diameter vessel on the cranial side of the umbilical artery in a 40sp Tg(Ly6a-GFP) embryo. Immunostaining for CD31 (red), Runx1 (grey) and GFP (green). Z-projection of this sample is shown in Figure 4.8 G.
**Movie 4.6.** 3-dimensional reconstruction of extravascular islands connected to a small diameter vessel (arrow) on the cranial side of the umbilical artery of a 36sp embryo. Immunostaining for CD31 (red), Runx1 (grey) and Kit (green). Z-projection of this sample is shown in Figure 4.8 J.
CHAPTER 5: Loss of neurofibromin Ras-GAP activity enhances the formation of cardiac blood islands in murine embryos
Abstract

Type I Neurofibromatosis (NF1) is caused by mutations in the NF1 gene encoding neurofibromin. Neurofibromin exhibits Ras GTPase activating protein (Ras-GAP) activity that is thought to mediate cellular functions relevant to disease phenotypes. Loss of murine Nf1 results in embryonic lethality due to heart defects, while mice with monoallelic loss of function mutations, or with tissue-specific inactivation have been used to model NF1. Here, we characterize previously unappreciated phenotypes in Nf1−/− embryos, which are inhibition of hemogenic endothelial specification in the dorsal aorta, enhanced yolk sac hematopoiesis, and exuberant cardiac blood island formation. We show that a missense mutation engineered into the active site of the Ras-GAP domain is sufficient to reproduce ectopic blood island formation, cardiac defects and overgrowth of neural crest-derived structures seen in Nf1−/− embryos. These findings demonstrate a role for Ras-GAP activity in suppressing hemogenic potential of the heart, and restricting growth of neural crest-derived tissues.
Introduction

NF1 is a common human disorder characterized by benign and malignant tumors of neural crest origin, pigmentation defects, learning disorders, cardiovascular abnormalities and a wide spectrum of other abnormalities including a predilection for leukemia (especially juvenile myelomonocytic leukemia, JMML) and vascular defects (Cichowski and Jacks, 2001, Friedman et al., 2002). Some of these phenotypes, including JMML and vascular defects, are shared by patients with related disorders associated with activation of the Ras signaling pathway, which together have been termed the “RAS-opathies” (Rauen et al., 2010). Neurofibromin contains a protein domain termed the GAP-related domain or GRD that is homologous to yeast IRA proteins. The NF1 GRD is able to complement yeast IRA mutants and hydrolyze GTP bound to active Ras, thereby down-regulating Ras signaling (Ballester et al., 1990, Xu et al., 1990). Interestingly, however, missense mutations in humans with NF1 have been identified that alter amino acids throughout the protein, suggesting functional domains outside of the GRD (Mattocks et al., 2004). Additional cellular functions for neurofibromin have also been identified, including modulation of protein kinase A (PKA) and cyclic adenosine monophosphate (cAMP) pathways (Brown et al., 2010, The et al., 1997, Wolman et al., 2014). A C-terminal region of neurofibromin has also been shown to interact with a major class of heparan sulfate proteoglycans (Hsueh et al., 2001) while full-length neurofibromin can bind to the scaffolding domain of caveolin-1 (Boyanapalli et al., 2006). Therapeutic strategies for the treatment of NF1 have focused on modulation of the Ras pathway, but the degree to which Ras dysregulation accounts for the diverse aspects of the human disease, or for the equally diverse features of various animal models of NF1, remains a critical question in the field.
Mouse models of NF1 have demonstrated critical developmental functions for
neurofibromin in multiple tissues, including neural crest, endothelium, and hematopoietic
stem and progenitor cells (HSPCs) (Brossier and Carroll, 2012, Costa and Silva, 2003,
Gitler et al., 2003, Bollag et al., 1996, Zhang et al., 1998). HSPCs arise during
midgestation from a transient population of endothelial cells called hemogenic
endothelium (HE) located in the yolk sac, the dorsal aorta, vitelline and umbilical arteries
(Bertrand et al., 2010, Boisset et al., 2010, Chen et al., 2009, Kissa and Herbomel, 2010,
Lam et al., 2010, Oberlin et al., 2010, Zovein et al., 2008). HSPCs form from HE through
a direct transition of endothelial cells into hematopoietic cells, independent of cell
division (Kissa and Herbomel, 2010, Eilken et al., 2009). This endothelial to
hematopoietic transition (EHT) was thought to occur only in the major arteries of the
embryo, placenta and yolk sac, but recent studies identified the heart and the head as
sites of de novo hematopoiesis (Dzierzak and Speck, 2008, Nakano et al., 2013, Li et
al., 2012). In the heart, hemogenic endocardial cells are integrated into the outflow
cushion and atria and undergo EHT at embryonic day (E) 9.5. Unlike arterial HE cells
that give rise to the full repertoire of hematopoietic cells, hemogenic endocardial cells
produce a transient population of hematopoietic cells restricted to the erythroid/myeloid
lineage, similar in potential to an early wave of erythroid/myeloid progenitors (EMP.s) that
emerge starting at E8.5 in the yolk sac (Nakano et al., 2013, Palis et al., 1999).

Later in gestation the heart is associated with a less-defined second wave of
hematopoiesis characterized by aggregates of endothelial and hematopoietic cells called
blood islands. Cardiac blood island formation is a prevalent physiological process that
has been identified in embryonic mice, chicks, quails and humans, but surprisingly little
is known about the formation of these structures (Hiruma and Hirakow, 1989, Hutchins et al., 1988, Kattan et al., 2004, Ratajska et al., 2006a, Red-Horse et al., 2010, Wu et al., 2013, Jankowska-Steifer et al., 2015a). What is known about blood islands comes primarily from histological studies. Blood islands form in the subepicardial space near the interventricular sulci between E11 and E14 and consist primarily of erythroblasts, but have also been associated with megakaryocytes, platelets, and leukocytes (Ratajska et al., 2009a, Red-Horse et al., 2010). Clonal and histological analysis suggests that blood islands bud from the endocardium, protruding into the myocardium where they pinch off, forming blood filled spheres or tubules that join the coronary plexus (Red-Horse et al., 2010, Jankowska-Steifer et al., 2015a). It has been suggested that hematopoietic cells enter cardiac blood islands by diapedesis, but other routes such as circulation or the de novo generation of hematopoietic cells from the endocardium in situ have not been ruled out (Ratajska et al., 2006). Cardiac blood island formation was found to be more robust in Tbx18 null mouse embryos, and thought to be an indirect consequence of aberrant signaling (Wu et al., 2013). Here we show that hyperactive Ras signaling increases cardiac blood island formation, and that endocardial cells of the blood islands have functional characteristics of HE and express Runx1, a marker of HE.
Results

**Nf1 deficiency increases yolk sac hematopoiesis but decreases specification of hemogenic endothelium in the dorsal aorta**

Embryonic day (E) 12.5-13.5 Nf1-deficient fetuses were reported to have increased numbers of committed hematopoietic progenitors in the fetal liver (Largaespada et al., 1996, Bollag et al., 1996, Zhang et al., 1998). Since many fetal liver progenitors in the midgestation embryo originate in the yolk sac (Lux et al., 2008), we examined the number of erythroid/myeloid progenitors (EMPs) in the yolk sac of E10.5 Nf1-deficient embryos. Nf1<sup>−/−</sup> yolk sacs (Fig. 5.1 A) contained significantly more EMPs, specifically due to an increased number of erythroid progenitors (Fig. 5.1 B), suggesting that Ras signaling positively regulates EMP numbers. We next examined the impact of Nf1 deficiency on hematopoiesis in the major arteries. The majority of HE cells in the major arteries (dorsal aorta, vitelline and umbilical) undergo EHT between E9.0-E10.5, resulting in the formation of Kit<sup>+</sup> CD31<sup>+</sup> Runx1<sup>+</sup> hematopoietic cells that remain briefly attached as clusters to the luminal wall of the arteries. In contrast to the increase in EMPs observed in the yolk sac, CD31<sup>+</sup> Kit<sup>+</sup> Runx1<sup>+</sup> hematopoietic cluster cells were decreased in the dorsal aortas of E10.5 Nf1-deficient embryos (Fig. 5.1 C,D). The decrease in CD31<sup>+</sup> Kit<sup>+</sup> Runx1<sup>+</sup> hematopoietic cluster cells appears to be due to decreased *de novo* generation, as fewer Runx1<sup>+</sup> CD31<sup>+</sup> Kit<sup>low</sup> HE cells were present in the dorsal aortas (Fig. 5.1 C,D). These data suggest that disruption of neurofibromin function augments the formation of EMPs in the yolk sac, but inhibits the specification of hemogenic endothelium in the dorsal aorta.
Nf1 deficiency results in ectopic cardiac blood island formation

Nf1 deficiency results in embryonic lethality by midgestation (approximately E13) due to cardiac defects. These defects include enlarged endocardial cushions and a malformed outflow tract (Brannan et al., 1994, Jacks et al., 1994, Lakkis and Epstein, 1998). Despite midgestation lethality, E11.5 Nf1\(^{-/-}\) embryos appeared grossly normal (Fig. 5.2 A). However, blood filled protrusions were often visible on the ventricles of Nf1\(^{-/-}\) embryos (Fig. 5.2 B). Whole-mount confocal analyses revealed that the protrusions are blood island-like structures budding from the ventricular endocardium, as they express CD31 and the hematopoietic markers CD41 and Runx1 (Fig. 5.2 C arrowheads). The blood islands are concentrated laterally on both ventricles of Nf1\(^{-/-}\) embryos (Fig. 5.2 C), in contrast to wild type embryos, in which it was reported that blood islands are generally located on the dorsal surface in the interventricular sulcus (Jankowska-Steifer et al., 2015a). Cardiac blood island formation was more robust in Nf1-deficient embryos compared to Nf1\(^{+/+}\) and Nf1\(^{+-}\) littermates; an average of 63.7 ± 7.6 blood islands could be identified via confocal microscopy on the ventricles of E11.5 Nf1\(^{-/-}\) embryos, whereas Nf1\(^{+/+}\) and Nf1\(^{+-}\) littermates averaged 0.3 ± 0.6 and 1.2 ± 1.3 blood islands, respectively (Fig. 5.2 D). At E10.5, only 25% (1/4) of Nf1-deficient embryos displayed small budding cardiac blood islands, whereas 92% (11/12) of E11.5 embryos had robust blood island formation, indicating that cardiac blood islands arise between E10.5 and E11.5 in Nf1\(^{-/-}\) embryos.
To determine if the ectopic cardiac blood islands harbored functional hematopoietic progenitors we performed colony-forming assays. To eliminate circulating blood cells, the atrium was removed and circulating blood flushed from the ventricles before the ventricles were dissociated and plated in methylcellulose supplemented with cytokines. Nf1<sup>−/−</sup> ventricles contained significantly more erythroid/myeloid progenitors than their Nf1<sup>+/+</sup> and Nf1<sup>+/−</sup> littermates (Fig. 5.2 E). This suggests that the phenotypic hematopoietic cells in the blood islands are functional erythroid and myeloid progenitors.

We used CD31, Runx1, Kit and CD41 whole mount immunofluorescence and confocal microscopy to examine the structure of cardiac blood islands in Nf1-deficient ventricles at E11.5. Single optical projections through the blood islands indicate that they are cystic structures that consist of a layer of CD31<sup>+</sup> endocardial cells that is continuous with the endocardium lining the ventricular trabeculae (Fig. 5.3 A, C). A layer of 3-4 CD31 bright cells with morphology between a flat endocardial cell and a rounded hematopoietic cell lined the base of most blood islands (Fig. 5.3 A-D). Some of these cells express the hemogenic endothelial marker Runx1 but they do not express high levels of the early hematopoietic markers CD41 and Kit, suggesting that they are hemogenic endocardial cells that have not yet initiated the transition into hematopoietic cells (Fig. 5.3 B, D, arrows). Within the cystic structure of most blood islands, there are also rounded cells that are CD31<sup>+</sup> Kit<sup>+</sup> Runx1<sup>+</sup> or CD31<sup>+</sup> CD41<sup>+</sup> Runx1<sup>+</sup>; these cells are phenotypic and morphological HSPCs (Fig. 5.3 B, D, arrowhead). These data suggest that blood islands are derived from the endocardium of the ventricle, and that the endocardium of blood islands has a latent HE potential that is held in check by Ras-GAP activity.
In addition to robust cardiac blood island formation, E11.5 Nf1$^{-/-}$ embryos have enlarged fetal livers populated by Runx1$^{+}$ and CD41$^{+}$ hematopoietic cells (Fig. 5.2 C), consistent with previous studies that found significantly higher numbers of fetal liver clonogenic progenitors (Zhang et al., 1998, Largaespada et al., 1996, Bollag et al., 1996). Furthermore, competitive repopulation assays comparing Sca1$^{+}$lin$^{-/}$dim cells isolated from the fetal livers of Nf1$^{-/-}$ and Nf1$^{+/+}$ embryos demonstrated that Nf1$^{-/-}$ cells have a growth advantage, particularly in the myeloid compartment (Bollag et al., 1996). Thus, the enlargement of the fetal liver may be due to elevated proliferation of Nf1$^{-/-}$ hematopoietic cells.

**Creation of Nf1 R1276P GRD mice**

In order to determine if the increase in cardiac blood islands seen in Nf1$^{-/-}$ embryos is due specifically to loss of the Ras-GAP activity of neurofibromin, we engineered a missense mutation within the GAP-related domain (GRD). Arginine 1276 has been shown to be the “arginine finger” of the GRD and is critical for catalytic activity. Mutation of this residue to proline was identified in a family with NF1, and crystal structures of related GAP domains are consistent with empiric studies showing loss of GAP activity following R1276P mutagenesis (Ahmadian et al., 1997, Scheffzek et al., 1997, Klose et al., 1998, Hiatt et al., 2004). We generated “knockin” mice in which arginine 1276 is mutated to proline (R1276P) and designate these mice Nf1$^{GRD/+}$ (Fig. 5.4). We generated an additional line of engineered mice in order to control for minor changes to intronic genomic sequences necessitated by the gene targeting and
selection strategy (see Methods, Fig. 5.4). For these control mice, designated Nf1\textsubscript{GRDCTL/+}, we utilized the identical targeting strategy but arginine 1276 was left intact. Appropriate targeting in several ES cell clones for each of the Nf1\textsubscript{GRD} or Nf1\textsubscript{GRDCTL} constructs was demonstrated by Southern blotting (Fig. 5.4). These were used to generate chimeric animals that were then bred for germ line transmission.

Heterozygous Nf1\textsubscript{GRD/+} mice appeared normal and were able to breed, but heterozygous intercrosses failed to produce any viable homozygous Nf1\textsubscript{GRD/GRD} pups (Table 5.1). One out of 61 embryos genotyped at E12.5 was Nf1\textsubscript{GRD/GRD}, and 11 of 63 (17.5%) were Nf1\textsubscript{GRD/GRD} at E11.5 (Table 5.1). Hence, homozygous R1276P mutation of Nf1 causes midgestation embryonic lethality with most embryos succumbing by E12.5.

Total cell lysates from Nf1\textsubscript{GRD/+} and Nf1\textsubscript{GRD/GRD} embryos exhibited similar levels of neurofibromin protein of expected apparent molecular weight of 250-280 kDa (Fig. 5.4 A). The relative neurofibromin protein expression was similar to that of wild-type embryos and was increased relative to Nf1\textsuperscript{+/-} embryos (Fig. 5.4 B).

To assess whether the introduced R1276P mutation within the GAP domain of neurofibromin disrupts Ras-GAP activity \textit{in vivo}, tissues were examined for elevated phosphorylated extracellular-signal regulated kinase (pERK), a downstream effector of Ras, as evidence of up-regulated Ras pathway activity. Nf1\textsubscript{GRD/flox} newborns in which Nf1 was deleted by Wnt1-Cre, displayed elevated pERK staining in neural crest-derived tissues such as peripheral nerves (Fig. 5.4 C), within hyperplastic adrenal medullary tissue (Fig. 5.4 D), and in enteric ganglia (Fig. 5.4 and Fig. 5.5). Wnt1-Cre; Nf1\textsubscript{GRD/flox} newborns showed prominent pERK staining in the axons and cell bodies of peripheral...
nerves (Fig. 5.4 and Fig. 5.5) that was not observed in control animals. Elevated pERK staining was also seen in the enlarged cardiac cushions of Nf1\textsuperscript{GRD/GRD} embryos, indicating the R1276 mutation is sufficient to elevate pERK levels (Fig. 5.4 E). Multiple reports showed that mutation of the conserved “arginine finger” within the GAP domain decreases neurofibromin GAP function while leaving the domain structurally intact (Ahmadian et al., 1997, Scheffzek et al., 1997, Klose et al., 1998, Hiatt et al., 2004). These observations indicate that inactivation of neurofibromin GAP activity elevates the phosphorylation of the Ras pathway effector ERK \textit{in vivo}.

Nf1\textsuperscript{GRDCTL} mice either heterozygous or homozygous for the control allele in which arginine 1276 was left intact, appeared normal. Intercrosses of Nf1\textsuperscript{GRDCTL/+} mice produced 6 of 26 Nf1\textsuperscript{GRDCTL/GRDCTL} offspring (23%). These control mice were not examined further, and we conclude that embryonic lethality observed in Nf1\textsuperscript{GRD/GRD} embryos is due specifically to the R1276P mutation.

\textbf{Nf1\textsuperscript{GRD/GRD} embryos exhibit cardiac endocardial cushion and neural crest defects}

Histologic analysis of E11.5 Nf1\textsuperscript{GRD/GRD} embryos revealed abnormal cardiac outflow tract morphology and enlarged endocardial cushions, similar to those seen in Nf1\textsuperscript{+/-} embryos (Fig. 5.6 A), which have been previously described in detail (Brannan et al., 1994, Jacks et al., 1994, Lakjis and Epstein, 1998). Atrioventricular (AV) endocardial cushions were also enlarged and ventricular septal defects were present, similar to the phenotype seen in Nf1\textsuperscript{+/-} embryos (Fig. 5.6 B). Sympathetic ganglia, derived from neural crest, were enlarged in both Nf1\textsuperscript{GRD/GRD} and Nf1\textsuperscript{+/-} embryos (Fig. 5.6 C). Enlargement of
sympathetic ganglia in Nf1\textsuperscript{GRD/GRD} mutants was confirmed by immunofluorescence staining for neurofilament and tyrosine hydroxylase (Fig. 5.7 A,B).

**Neural crest specific loss of Nf1 Ras GAP function leads to tissue overgrowth**

Tissue-specific loss of Nf1 in neural crest results in late gestation lethality, bypassing the midgestation cardiac defects seen in Nf1 null mutants (Gitler et al., 2003). In order to examine in more detail if the Ras-GAP function of neurofibromin is necessary in developing neural crest in embryos surviving past midgestation, we crossed Wnt1-Cre; Nf1\textsuperscript{GRD/+} mice with Nf1\textsuperscript{flox/flox} mice to generate Wnt1-Cre; Nf1\textsuperscript{GRD/+} offspring. At E18.5-P0, no viable Wnt1-Cre; Nf1\textsuperscript{GRD/+} offspring were identified out of 80 genotyped, although 12 non-viable pups (15%) were stillborn or died shortly after birth (Table 5.2). Live Wnt1-Cre; Nf1\textsuperscript{GRD/+} embryos were recovered between E12.5 and E16.5 at the expected frequency (Table 5.2).

Histologic examination of Wnt1-Cre; Nf1\textsuperscript{GRD/+} embryos revealed overgrowth of the adrenal medulla when compared to control Nf1\textsuperscript{GRD/+} embryos that phenocopied adrenal medullary defects seen in Wnt1-Cre; Nf1\textsuperscript{flox/flox} embryos (Fig. 5.8 A), described previously (Gitler and Epstein, 2003). Massive enlargement of paraspinal neural crest derived ganglia was also noted in both Wnt1-Cre; Nf1\textsuperscript{GRD/+} and Wnt1-Cre; Nf1\textsuperscript{flox/flox} embryos (Fig. 5.8 B,C). These findings suggest that loss of neurofibromin Ras-GAP function in neural crest is sufficient to reproduce the late-gestation lethality and tissue overgrowth that results from by tissue-specific deletion of Nf1 in neural crest.
Ectopic cardiac blood island formation is due to loss of NF1 Ras GAP activity

We examined E11.5 Nf1^{GRD/GRD} embryos for evidence of cardiac blood island formation to determine if this results from the loss of Ras GAP activity. Nf1^{GRD/GRD} embryos appeared grossly normal at E11.5 (Fig. 5.9 A), but blood filled protrusions were often visible on the ventricles (Fig. 5.9 B). Whole mount immunofluorescence revealed that the blood filled protrusions were phenotypically identical to the ectopic cardiac blood islands that formed on the ventricles of Nf1^{-/-} embryos (Fig. 5.9 C, arrowheads). Furthermore, Nf1^{GRD/GRD} embryos had enlarged fetal livers populated with Runx1^{+} CD41^{+} hematopoietic cells, similar to Nf1^{-/-} embryos (Fig. 5.9 C). Ventricular blood islands were evident in histologic sections after hematoxylin and eosin staining of Nf1^{GRD/GRD} and Nf1^{-/-} embryos and had similar structural characteristics (Fig. 5.9 D). Single optical projections through Nf1^{GRD/GRD} blood islands confirm that they were associated with CD31^{+} CD41^{+} Runx1^{+} phenotypic hematopoietic cells (Fig. 5.9 E). An average of 26.3 ± 9.2 blood islands could be identified via confocal microscopy on the ventricles of E11.5 Nf1^{GRD/GRD} embryos, whereas Nf1^{+/+} and Nf1^{GRD/+} ventricles contained no cardiac blood islands (Fig. 5.9 F). However, Nf1 deficient E11.5 embryos had, on average, > 2 fold more morphological blood islands as compared to Nf1^{GRD/GRD} embryos (compare Figure 5.9 F and 5.2 D, P ≤ 0.022), suggesting that the Nf1^{GRD} is a hypomorphic Nf1 allele, at least in regard to blood island formation. Flushed Nf1^{GRD/GRD} ventricles contained significantly more erythroid/myeloid progenitors than Nf1^{+/+} and Nf1^{GRD/+} littermates (Fig. 5.9 G), but there was a trend towards fewer progenitors than in Nf1^{-/-} embryos.
Discussion

In this study we identify ectopic cardiac blood island formation as a novel phenotype that arises in Nf1-deficient embryos. Furthermore, using a mouse that expresses a mutant form of neurofibromin with decreased Ras GAP activity, we demonstrate that the phenotype is a direct result of dysregulation of the Ras signaling pathway. We also show that some endocardial cells in the ectopic blood islands express Runx1, a master regulator of hematopoiesis and a marker of HE. This, in addition to the enrichment of both phenotypic and functional hematopoietic progenitors in the ventricles of E11.5 Nf1-deficient embryos, suggests that the endocardial cells are producing hematopoietic cells de novo.

We also observed dysregulation of de novo hematopoietic progenitor formation in Nf1-deficient embryos in normal sites of hematopoiesis. A previous study in zebrafish embryos found that the downstream effector of the Ras signaling pathway, pERK, has a biphasic role in blood cell formation from endothelium (Zhang et al., 2014). When zebrafish embryos were treated with an ERK signaling inhibitor prior to artery-vein specification, runx1 and myb expression in the dorsal aorta decreased, however when treated with an ERK signaling inhibitor after artery-vein specification but before EHT, runx1 and myb expression increased (Zhang et al., 2014). Thus, early in development pERK is necessary for de novo generation of hematopoietic cells, but after artery-vein specification, pERK inhibits the specification of HE cells (Zhang et al., 2014). Consistent with a role for ERK signaling in HE specification increased signaling through the fibroblast growth factor (FGF) receptor, which is upstream of ERK and regulated by Ras-GAP, decreases runx1 expression in the dorsal aorta of zebrafish (Pouget et al., 2014).
The mechanism by which increased FGF signaling decreases runx1 expression in the HE involves inhibition of bone morphogenic protein (BMP) signaling, which is required for runx1 expression (Pouget et al., 2014, Wilkinson et al., 2009, Pimanda et al., 2007). Consistent with these findings, we show that loss of Nf1, which is associated with activation of the Ras-pERK pathway, results in fewer Runx1+ HE cells in the dorsal aorta at E10.5, as well as fewer CD31+ Kit+ Runx1+ hematopoietic cluster cells. In contrast, the yolk sac of Nf1-/- embryos produced more EMPs when compared to littermate controls, consistent with the positive role for FGF signaling in regulating erythropoiesis and myelopoiesis at a similar earlier stage in the zebrafish embryo (Yamauchi et al., 2006, Walmsley et al., 2008). Thus the level of Ras activity must be carefully titrated, as elevating Ras signaling in the dorsal aorta limits HE specification, but it enhances EMP formation in the yolk sac, and unleashes the hematopoietic potential of the endocardium.

It was previously reported that hematopoietic cells derived from the fetal livers of Nf1-deficient mice are hyperproliferative and cause a juvenile myelomonocytic leukemia (JMML)-like myeloid proliferative disorder when transplanted into irradiated recipients (Birnbaum et al., 2000, Largaespada et al., 1996, Zhang et al., 2001, Zhang et al., 1998). Based on immunofluorescence, it appears that hematopoiesis is elevated as early as E11.5 in fetal livers of both Nf1-/- and Nf1GRD/GRD embryos compared to controls, thus implicating activated Ras in hyperproliferation of hematopoietic cells (at E11.5, primarily EMPs) that populate the fetal liver. Likewise, our results implicate loss of neurofibromin Ras GAP function within neural crest cells as sufficient to result in overgrowth of sympathetic and dorsal root ganglia and of the adrenal medulla.
The ability of the Nf1 gene product to act as a Ras GAP has been known for a quarter of a century (Ballester et al., 1990, Xu et al., 1990), but the degree to which this function accounts for some or all Nf1 phenotypes has been an ongoing topic of research with relevance for therapeutic strategies. We and others have provided evidence for the ability of neurofibromin to affect alternate signaling pathways, including PKA and cAMP (Guo et al., 1997, The et al., 1997, Hegedus et al., 2007, Brown et al., 2010, Wolman et al., 2014). Prior work has suggested that midgestation embryonic lethality resulting from loss of Nf1 can be rescued by transgenic expression of the isolated neurofibromin GRD, but this was not sufficient for rescue of neural crest overgrowth (Ismat et al., 2006). Failure to rescue neural crest overgrowth could have been the result of inadequate transgenic expression of GRD in this tissue, or because of the necessity for an additional function of neurofibromin outside of the GRD. The findings reported here for Nf1GRD/GRD embryos do not rule out the existence of critical non-GRD functions of neurofibromin in neural crest or other tissues. In fact, the Nf1GRD has characteristics of a hypomorphic allele, which could be explained by non-GRD related functions. Rather, we demonstrate the necessity of GRD function for normal embryonic development. The development of the Nf1GRD/+ mouse line described here will allow researchers to determine the necessity of GRD function across the spectrum of developmental and tumor phenotypes observed in mouse models of neurofibromatosis.
Figure 5.1. Nf1 deficiency increases yolk sac hematopoiesis but decreases specification of hemogenic endothelium in the dorsal aorta at E10.5
Figure 5.1. Nf1 deficiency increases yolk sac hematopoiesis but decreases specification of hemogenic endothelium in the dorsal aorta at E10.5. (A) Quantification of erythroid and myeloid progenitors (EMPs) in the yolk sacs of E10.5 Nf1<sup>+/+</sup>, Nf1<sup>−/−</sup> and Nf1<sup>−/−</sup> conceptuses (Nf1<sup>+/+</sup> n=7; Nf1<sup>−/+</sup> n=8; Nf1<sup>−/−</sup> n=3). 1-way ANOVA and Bonferroni’s multiple comparison test was applied to determine significance, error bars represent the standard deviation (SD). (B) Percent of EMP colony type. Mk: megakaryocyte; Mix: granulocyte-erythroid-monocyte-megokaryocyte; BFU-E: burst forming unit-erythroid; G/M: granulocyte-macrophage colonies. There were significantly more BFU-E progenitors in the yolk sacs of Nf1<sup>−/−</sup> compared to Nf1<sup>+/+</sup> and Nf1<sup>−/+</sup> littermates, P ≤ .0001. (C) Confocal Z-projections (Z intervals = 2µm) of Nf1<sup>+/+</sup>, Nf1<sup>−/+</sup> and Nf1<sup>−/−</sup> dorsal aortas at E10.5, immunostained for CD31 (red) Runx1 (green) and Kit (cyan). Scale bars = 100µm. Aortas are oriented with the ventral aspect on the left. (D) Quantification of CD31<sup>+</sup> Runx1<sup>+</sup> Kit<sup>+</sup> hematopoietic cluster cells and CD31<sup>+</sup> Runx1<sup>+</sup> Kit<sup>−/low</sup> hemogenic endothelial cells within the dorsal aorta at E10.5, 1-way ANOVA and Bonferroni’s multiple comparison test applied to determine significance, error bars represent the SD and n = 3 for all genotypes. ** indicates that P ≤ 0.01.
Figure 5.2. Ectopic formation of cardiac blood islands in Nf1−/− embryos
Figure 5.2. Ectopic formation of cardiac blood islands in Nf1<sup>+/−</sup> embryos. (A) Gross view of E11.5 Nf1<sup>+/+</sup>, Nf1<sup>+/−</sup> and Nf1<sup>−/−</sup> embryos (B) Isolated hearts from embryos in A. Arrowheads point to two examples of blood filled protrusions. (C) Confocal Z-projections (Z interval = 5µm) of CD31 (red), CD41 (cyan) and Runx1 (green) immunostained Nf1<sup>+/+</sup>, Nf1<sup>+/−</sup> and Nf1<sup>−/−</sup> E11.5 embryos. Blood island-like structures (arrowheads) are visible on the ventricle of the Nf1<sup>−/−</sup> embryo. Scale bars = 500µm. V, ventricle; A, atrium; FL, fetal liver. (D) Quantification of blood islands on the ventricles of E11.5 embryos, 1-way ANOVA and Bonferroni’s multiple comparison test applied to determine significance, error bars represent SD. (E) Number of erythroid and myeloid progenitors per flushed E11.5 ventricles. Mk: megakaryocyte; Mix: granulocyte-erythroid-monocyte-megakaryocyte; BFU-E: burst forming unit-erythroid; G/M: granulocyte-macrophage colonies. 1-way ANOVA and Bonferroni’s multiple comparison test applied to determine significance, error bars represent the standard SD. Nf1<sup>+/+</sup> n = 10, Nf1<sup>+/−</sup> n = 33, and Nf1<sup>−/−</sup> n = 6. ** indicates that P ≤ 0.001.
Figure 5.3. Nf1⁺ cardiac blood islands
**Figure 5.3. Nf1⁻/⁻ cardiac blood islands.** (A) Single optical projection through the ventricle of an E11.5 Nf1⁻/⁻ embryo immunostained for CD31 (red), CD41 (cyan) and Runx1 (green). Blood islands (arrowheads) are visible sprouting from the ventricles of Nf1⁻/⁻ embryos. (B) Single optical projection through blood islands on the ventricles of E11.5 Nf1⁻/⁻ embryos. Runx1⁺ endocardial cells are visible in the blood islands (arrows). Arrowheads indicate examples of CD31⁺ CD41⁺ Runx1⁺ hematopoietic cells. (C) Single optical projection through the ventricle of an E11.5 Nf1⁻/⁻ embryo immunostained for CD31 (red), Kit (cyan) and Runx1 (green). (D) Single optical projection through blood islands. Arrows indicate Runx1⁺ endocardial cells. Arrowheads indicate examples of CD31⁺ CD41⁺ Runx1⁺ hematopoietic cells. Scale bars = 50µm.
Figure 5.4. Neurofibromin protein expression and activity from the Nf1 alleles.
Figure 5.4. Neurofibromin protein expression and activity from the Nf1 alleles. (A) Total cell lysates from E10.5 Nf1\(^{+/+}\), Nf1\(^{+/}\), Nf1\(^{−/−}\), Nf1\(^{GRD/+}\), and Nf1\(^{GRD/GRD}\) embryos were analyzed by SDS-PAGE followed by immunoblotting with either anti-neurofibromin (top panel) or anti-beta tubulin (bottom panel) antibodies as indicated. (B) Band intensities from 5 immunoblots as in (A) were quantified by ImageJ. The relative neurofibromin expression for each genotype compared to wild-type is indicated. All data are represented as the mean ± S.E. **, p < 0.05; ***, p < 0.001; NS = not significant (p < 0.001, one-way ANOVA between groups, post hoc multiple comparisons, Tukey’s test). (C) A cross-section of a peripheral nerve (demarcated in white and indicated by an arrow) from each of Nf1\(^{GRD/flox}\) and Wnt1-Cre ; Nf1\(^{GRD/flox}\) P0 animals shows elevated expression of pERK, a downstream indicator of Ras activity, in Wnt1-Cre ; Nf1\(^{GRD/flox}\) animals (right panel). An adjacent blood vessel (BV) is indicated. (D) Adrenal medullary tissue within an adrenal gland from either a Nf1\(^{GRD/flox}\) or Wnt1-Cre ; Nf1\(^{GRD/flox}\) animal shows increased pERK expression in a hyperplastic area from the Wnt1-Cre ; Nf1\(^{GRD/flox}\) animal (right panel). pERK-positive cells are marked by arrowheads. Background fluorescence from non-neural-crest-derived adrenal cortical cells and blood cells is evident in the Nf1\(^{GRD/flox}\) sample. (E) Cardiac cushions from E11.5 embryos show elevated pERK staining in Nf1\(^{GRD/GRD}\) embryos compared to Nf1\(^{+/+}\) animals as indicated within the dashed oval. Scale bars = 50\(\mu\)m.
Table 5.1

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Table 5.1. Genotypes from Nf1<sup>GRD/+</sup> x Nf1<sup>GRD/+</sup> intercrosses
Figure 5.5. Increased pERK staining in neural crest derivatives of Nf1<sup>GRD/flox</sup> newborn animals following deletion by Wnt1-Cre
Figure 5.5. Increased pERK staining in neural crest derivatives of Nf1GRD/flox newborn animals following deletion by Wnt1-Cre. (A) pERK staining was observed in neural crest-derived enteric ganglia within the intestinal wall that was more evident in Wnt1-Cre ; Nf1GRD/flox animals (right panel). Arrowheads indicate cells exhibiting positive staining. (B) Both axons (arrows) and nerve cell bodies (arrowheads) were readily visualized in Wnt1-Cre ; Nf1GRD/flox newborns but not in control animals (data not shown).
Figure 5.6. Inactivation of Nf1 GRD function affects heart and sympathetic ganglia development
Figure 5.6. Inactivation of Nf1 GRD function affects heart and sympathetic ganglia development. (A) Sections of hearts from E12.5-E13.5 Nf1+/+, Nf1GRD/GRD, and Nf1−/− embryos. The enlarged endocardial cushions in hearts from Nf1GRD/GRD embryos (arrowheads) are similar to the oversized cushions of Nf1−/− embryos. (B) Enlarged atrioventricular endocardial cushions (arrowheads) and ventricular septa defects (arrows) in Nf1GRD/GRD and Nf1−/− embryos. (C) Sympathetic ganglia (arrowheads) are similarly enlarged in Nf1GRD/GRD and Nf1−/− embryos. Scale bars = 500 µm.
Figure 5.7. Enlarged sympathetic ganglia in E11.5 Nf1^{GRD/GRD} embryos
Figure 5.7. Enlarged sympathetic ganglia in E11.5 Nf1^{GRD/GRD} embryos. (A) Transverse sections of E11.5 Nf1^{+/+}, Nf1^{GRD/+}, and Nf1^{GRD/GRD} embryos stained with antibodies against neurofilament. Arrowheads indicate sympathetic ganglia. (B) Transverse sections of E11.5 Nf1^{+/+}, Nf1^{GRD/+}, and Nf1^{GRD/GRD} embryos stained with antibodies against tyrosine hydroxylase. Scale bars = 100\mu m.
Table 5.2.

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*2 non-viable Wnt1-Cre; Nf1^{GRD/flox} embryos were recovered at E12.5-16.5

**12 non-viable Wnt1-Cre; Nf1^{GRD/flox} pups were recovered at E18.5-P

Table 5.2. Genotypes from Wnt1-Cre; Nf1^{GRD/+} X Nf1^{floxflox} crosses
Figure 5.8. Hyperplasia of neural crest derivatives is similar in $\text{Nf1}^{\text{GRD/flox}}$ and $\text{Nf1}^{\text{flox/flox}}$ newborn animals in which Nf1 is deleted in neural crest cells with Wnt1-Cre
Figure 5.8. Hyperplasia of neural crest derivatives is similar in Nf1\textsuperscript{GRD/flox} and Nf1\textsuperscript{flox/flox} newborn animals in which Nf1 is deleted in neural crest cells with Wnt1-Cre. (A) Adrenal medullary tissue (demarcated in white and indicated with an arrowhead) contained within an adrenal gland of P0 wild-type, P0 Wnt1-Cre; Nf1\textsuperscript{GRD/flox}, or E16.5 Wnt1-Cre; Nf1\textsuperscript{flox/flox} animals. The tissue is similarly overgrown in Wnt1-Cre; Nf1\textsuperscript{GRD/flox} and Wnt1-Cre; Nf1\textsuperscript{flox/flox} newborns/fetuses. Scale bars = 100µm. The arrow indicates a tumor-like medullary protrusion. (B) Sagittal sections showing peripheral ganglia (arrowheads) in Nf1\textsuperscript{GRD/flox} newborn pups, and abnormally enlarged ganglia and tumor-like overgrowth of nerve tissue adjacent to the lumbar spine in a Wnt1-Cre; Nf1\textsuperscript{GRD/flox} newborn pup and a E16.5 Wnt1-Cre; Nf1\textsuperscript{flox/flox} fetus. Scale bars = 500µm. (C) Magnifications of images in (B), with hyperplastic tissue demarcated in black and marked by arrowheads. Lu, lung; Li, liver; Scale bars = 500µm.
Figure 5.9. E11.5 Nf1^{GRD/GRD} embryos form ectopic cardiac blood islands
Figure 5.9. E11.5 Nf1^{GRD/GRD} embryos form ectopic cardiac blood islands. (A) Gross view of E11.5 Nf1^{+/+}, Nf1^{GRD/+} and Nf1^{GRD/GRD} littermates. (B) Isolated hearts from E11.5 Nf1^{+/+}, Nf1^{GRD/+} and Nf1^{GRD/GRD} embryos. Arrowheads indicate blood filled protrusions on the ventricle of the Nf1^{GRD/GRD} heart. (C) Confocal Z-projections (Z interval = 5μm) of CD31 (red), CD41 (cyan) and Runx1 (green) immunostained E11.5 Nf1^{+/+}, Nf1^{GRD/+} and Nf1^{GRD/GRD} embryos. Arrowheads point to blood islands on the ventricle of the Nf1^{GRD/GRD} embryo. Scale bars = 500μm. V, ventricle; A, atrium; FL, fetal liver. (D) Cell aggregates resembling blood islands (arrowheads) in hearts of E12.5 Nf1^{+/+}, Nf1^{−/−} and Nf1^{GRD/GRD} embryos. Lower panels, are magnifications of images in top panels. Scale bars = 100μm. (E) Single optical projection through the cardiac blood islands of an E11.5 Nf1^{GRD/GRD} embryo immunostained for CD31 (red), Runx1 (green) and CD41 (cyan). Scale bars = 50μm. (F) Quantification of blood islands on the ventricles of E11.5 embryos. 1-way ANOVA and Bonferroni’s multiple comparison test applied to determine significance, error bars represent the SD. (G) Number of erythroid and myeloid progenitors per flushed E11.5 ventricle. Mk: megakaryocyte; Mix: granulocyte-erythroid-monocyte-megakaryocyte; BFU-E: burst forming unit-erythroid; G/M: granulocyte-macrophage colonies. 1-way ANOVA and Bonferroni’s multiple comparison test applied to determine significance; error bars represent SD. Nf1^{+/+} n = 21, Nf1^{GRD/+} n = 22 and Nf1^{GRD/GRD} n = 8. * indicates that P ≤ 0.05 and *** indicates that P ≤ 0.001.
CHAPTER 6: Respecification of endothelium towards the hematopoietic lineage by Runx1
Abstract

The *de novo* generation of hematopoietic stem and progenitor cells (HSPCs) occurs during embryogenesis from a rare population of endothelial cells called hemogenic endothelium (HE). HE cells transition into HSPCs during midgestation in a process called the endothelial to hematopoietic transition (EHT). The transcription factor, Runx1 orchestrates the transcriptional switch from an endothelial to hematopoietic signature and is required for the EHT to occur. However, whether Runx1 activity alone is sufficient to respecify endothelial cells into the rare and transient HE is unknown.

To address this question, we activated an endothelial-specific conditional Runx1 allele at embryonic, fetal and postnatal time points. We found that both embryonic and fetal endothelial populations are competent to respecification into hematopoietic cells by Runx1, but fetal endothelium requires a higher dose of Runx1 compared to embryonic endothelium. In contrast, adult endothelium is not competent to respecification by Runx1 even when exposed to high Runx1 levels. This data suggest that as development progresses endothelium becomes less competent to respecification into HE by Runx1.
Introduction

The de novo generation of hematopoietic progenitors and stem cells (HSPCs) occurs only during embryogenesis from a transient and rare population of endothelial cells called hemogenic endothelium (HE) (Zovein et al., 2008, Chen et al., 2009). In mouse embryos, during early to mid-gestation HE cells can be found in the arterial and venous plexus of the yolk sac, the chorionic vasculature, the large diameter arteries of the embryo proper and the endocardium (Rhodes et al., 2008, Nakano et al., 2013, Yzaguirre and Speck, 2016b, Frame et al., 2015). HE cells can be distinguished from their non-hemogenic endothelial neighbors based on expression of the transcription factor, Runx1 (North et al., 1999, Yzaguirre and Speck, 2016b). Runx1 initiates the up-regulation of a hematopoietic specific transcriptional program and the down regulation of endothelial genes, resulting in the transdifferentiation of HE cells into hematopoietic cells in a process called the endothelial to hematopoietic transition (EHT) (Kissa and Herbomel, 2010, Lancrin et al., 2012b, Chen et al., 2009, Yokomizo et al., 2001, North et al., 1999). When Runx1 or it’s non-DNA binding partner, CBFβ are knocked out in the germ line, or ablated via endothelial specific Cre-recombinase-mediated excision, the EHT is completely abrogated and definitive hematopoietic cells, including HSCs, do not form (Sasaki et al., 1996, Bresciani et al., 2014, Wang et al., 1996b, Niki et al., 1997, Wang et al., 1996a, Okuda et al., 1996, Li et al., 2006, Chen et al., 2009). In contrast, when Runx1 is deleted ubiquitously at E11.5, HSCs and hematopoietic progenitors persist (Tober et al., 2013). Hence, Runx1 is specifically required for the EHT but whether Runx1 is sufficient to induce EHT during embryogenesis is not known.
Epigenetic and transcriptional studies in HE differentiated from embryonic stem cells have established that Runx1 is a master regulator of the de novo generation of hematopoietic cells (Lichtinger et al., 2012). During the specification of HE, Runx1 binds promoter distal sites previously marked by little or no H3K9Ac, and strongly increases H3K9Ac influencing the epigenetic state of various downstream factors (Lichtinger et al., 2012). Furthermore, Runx1 recruits hematopoietic regulators, including Tal1 and Fli1, to its target sites in HE acting as a linchpin for the hematopoietic program (Lichtinger et al., 2012). Direct downstream targets of Runx1, Gfi1 and Gfi1b downregulate endothelial markers and promote the physical transition from flat cells integrated in the vessel wall into round mobile cells (Lancrin et al., 2012b). In HE Runx1 initiates the activation of a hematopoietic program and the concurrent downregulation of the endothelial signature thereby promoting EHT and the de novo generation of HSPCs.

Endogenous HE is specified during early to mid-gestation, then undergoes EHT by mid-gestation and is depleted prior to birth. However postnatal endothelial cells can be reprogrammed into HE through ectopic expression of Fosb, Gfi1, Runx1 and Spi1 so long as the cells are in a permissive in vitro serum-free vascular niche system (Sandler et al., 2014). Interestingly, both Gfi1 and Spi1 are direct downstream targets of Runx1 (Lancrin et al., 2012b, Huang et al., 2008, Hoogenkamp et al., 2009) suggesting that Runx1 may drive reprogramming. Direct reprogramming of adult endothelial cells into patient-specific HSC-generating HE has the potential to cure hematological malignancies. However, to increase the efficiency of reprogramming and to gain a better understanding of the reprogrammed cells it is imperative to understand what defines a competent cellular state.
Here we show that Runx1 alone is sufficient to respecify embryonic and fetal endothelium into hemogenic endothelium in a permissive culture system. Furthermore, the respecified endothelial cells can generate multilineage hematopoietic cells. In contrast, postnatal endothelium is not competent to respecification by Runx1 alone. Interestingly, when endothelial-specific ectopic expression of Runx1 is activated \textit{in vivo} during embryogenesis the total number of hematopoietic progenitor cells in the conceptus increases significantly but only when Runx1 is activated prior to E8.5. This data suggests that despite being the endogenous site of \textit{de novo} hematopoiesis, the embryo is not a permissive environment for the respecification of endothelium into HE.
Results

Embryonic and fetal endothelial cells are competent to respecification into hemogenic endothelium by Runx1 but postnatal endothelium is not.

To ectopically express Runx1 in endothelial cells at embryonic and fetal time points we crossed mice with conditional Runx1 knocked (CKI) into the Rosa26 locus preceded by a floxed stop cassette (Gt(Rosa)26Sortm(RUNX1)Ma) to mice with an endothelial-specific tamoxifen inducible Cre driven from the VE-cadherin (Cdh5) promoter (Tg(Cdh5-cre/ERT2)1Rha) referred to here as Cre. We crossed WT or CKI/CKI female mice to Cre;CKI/+ or Cre;CKI/CKI males to obtain heterozygous and/or homozygous knockin progeny (Cre;CKI/+ and/or Cre;CKI/CKI) and littermate controls (CKI). To activate ectopic endothelial-specific expression of Runx1 during development, we injected pregnant mice with 2mg of tamoxifen intraperitoneally (IP) at E8.5 for the embryonic time point and E12.5 for the fetal time point; 24 hours later embryos or fetuses were harvested for analysis (Fig. 6.1 B). To confirm that Runx1 was ectopically expressed in the endothelium of Cre;CKI/+ embryos (E9.5) and fetuses (E13.5) we used flow cytometry to compare the percentage of Runx1+ endothelium in Runx1 knockins and littermate controls (Fig. 6.1 A). At E9.5, 28.23% ± 8.05% (mean ± SD) of total Cre;CKI/+ endothelium (CD45− CD41− Ter119− CD31+ VEC+) expressed Runx1 in comparison to 3.28% ± 1.02% for littermate controls (Fig. 6.1 A). At E13.5 35.74% ± 13.30% of Cre;CKI/+ endothelium expressed Runx1, 32.85% ± 9.64% of Cre;CKI/CKI endothelium expressed Runx1 and only 0.66% ± 0.59% of CKI endothelium expressed Runx1 (Fig. 6.1 A).
To determine if embryonic Cre;CKI/+ endothelium has elevated hemogenic potential we sorted endothelium from E9.5 Cre;CKI/+ and CKI/+ littermates 24 hours after the initiation of ectopic Runx1 expression \textit{in utero} and then plated the endothelium in limiting dilutions in 96-well plates on a confluent monolayer of OP9 stromal cells (Nakano et al., 1994) in conditions that support hematopoiesis (Fig. 6.1 B-C). Endogenous HE is enriched in the Kit$^+$ population of endothelial cells (Nadin et al., 2003, Goldie et al., 2008, Marcelo et al., 2013b) therefore to determine if Kit expression affected respecification competency we assayed both Kit$^+$ and Kit$^-$ endothelium to determine if either were competent to respecification into HE by Runx1 (Fig. 6.1 C). 5 days after plating the embryonic Cre;CKI/+ Kit$^+$ endothelium, semi-adherent cells with hematopoietic morphology began to appear (Fig. 6.2). 10 days after plating the endothelium we assayed the populations by flow cytometry to determine the number of replicates at each dilution that generated hematopoietic cells (CD41$^+$, CD45$^+$ and/or Ter119$^+$) in order to calculate the frequency of EHT in each population assayed (Fig. 6.1 B). We found that the frequency of EHT was significantly higher in both the Cre;CKI/+ Kit$^+$ (1/54) and Cre;CKI/+ Kit$^-$ (1/171) populations compared to CKI/+ Kit$^+$ (1/851) and CKI/+ Kit$^-$ (1/8,372) populations (Fig. 6.1 D). Since the Runx1 expressing endothelium cannot be separated from the Runx1$^-$ endothelium in this system, the frequency of EHT was calculated based on the total number of endothelial cells plated. To determine if the sorted endothelial populations contained contaminating hematopoietic progenitors we plated freshly sorted endothelial cells in colony forming assays and found that the frequency of contaminating hematopoietic progenitors was much lower than the frequency of HE we observed in the limiting dilution HE assays (Table 6.1). This data suggests that embryonic endothelium is competent to respecification into HE by Runx1.
Endogenous HE is first observed in the embryo proper at E8, by E9.5 the population of HE expands throughout the large diameter arteries of the embryo and begins to undergo EHT (Yzaguirre and Speck, 2016b), by the time fetal development begins (~E13.5) most, if not all, of the HE has transitioned into hematopoietic cells and very few Runx1 expressing endothelial cells remain. To determine if fetal endothelium can be respecified into HE we initiated ectopic Runx1 expression at E12.5 in utero, then sorted endothelial cells for limiting dilution HE assays 24 hours later (Fig. 6.1 B). Cre;CKI/+ Kit+ and Kit− endothelium had a significantly higher frequency of EHT compared to CKI/+ littermate endothelium but the frequency was not much higher than the frequency of contaminating hematopoietic progenitors in the sorted populations (Fig. 6.1 E and Table 6.1). Therefore, Cre;CKI/+ fetal endothelial cells were not or very weakly respecified into HE in response to ectopic Runx1 expression. Next we wanted to determine if fetal endothelial cells could be respecified into HE by increasing the dose of ectopic Runx1 expression. To increase the dose of Runx1 we used homozygous Runx1 knockin (Cre;CKI/CKI) fetuses. Cre;CKI/CKI endothelial cells ectopically express Runx1 from both Rosa26 loci in contrast to Cre;CKI/+ endothelial cells which ectopically express Runx1 from one Rosa26 loci. To confirm that Runx1 levels were higher we compared the mean fluorescent intensity (MFI) of Runx1 in the endothelium (CD45−CD41−Ter119−CD31+ VEC+) of E13.5 CKI, Cre;CKI/+ and Cre;CKI/CKI fetuses 24 hours after the initiation of ectopic Runx1 expression and found that the MFI is significantly higher in Cre;CKI/CKI endothelium compared to Cre;CKI/+ endothelium (Fig. 6.1 F). The frequency of EHT in the Cre;CKI/CKI Kit+ (1/117) and Kit− (1/526) endothelial populations was significantly higher when compared to endothelium from littermate controls (CKI/CKI) and Cre;CKI/+ fetuses (Fig. 6.1 E). These data suggests that both fetal and
embryonic endothelium is competent to respecification into HE by Runx1 but fetal endothelium requires a higher dose of Runx1 than embryonic endothelium.

HE is a transient population of cells that exist solely during development; therefore the de novo generation of HSPCs occurs strictly during embryogenesis. However, adult dermal endothelial cells can be reprogrammed into HSPC-generating HE through forced expression of Fosb, Gfi1, Runx1 and Spi1 (Sandler et al., 2014). Interestingly, both Gfi1 and Spi1 (Pu.1) are direct downstream targets of Runx1 (Lancrin et al., 2012b, Huang et al., 2008, Hoogenkamp et al., 2009) suggesting that Runx1 may drive the reprogramming of adult endothelial cells into HE. To determine if Runx1 alone is sufficient to reprogram adult endothelial cells we initiated ectopic Runx1 expression in 1-month-old Cre;CKI/CKI mice and CKI/CKI littermate controls, then removed their livers and lungs 24-hours later for analysis (Fig. 6.3 A, scheme). First, to confirm that Runx1 was being ectopically expressed in the Cre;CKI/CKI tissues we immunostained the livers and lungs for CD31, Runx1 and Kit and imaged them via confocal microscopy (Fig. 6.3 A). We found that Runx1 was ectopically expressed in the vascular beds of the livers and lungs of 1-month-old Cre;CKI/CKI mice but not CKI/CKI controls (Fig. 6.3 A). In addition, small clusters of ectopic CD31+ Runx1+ Kit+ phenotypic hematopoietic cells formed on the periphery of Cre;CKI/CKI livers (Fig. 6.3 A, boxed region and lower panel arrowheads). To determine if the endothelium of the liver and lung contain hemogenic potential after ectopic Runx1 expression we sorted the Kit+ and Kit- endothelium from 1-month-old Cre;CKI/CKI and CKI/CKI mice and performed limiting dilution HE assays on OP9 stromal cells. We did not detect EHT in any of the populations assayed (not shown). However, the OP9 culture system, while sufficient for the promotion of EHT,
does not phenocopy the endogenous niche of HE cells. To more closely phenocopy the endogenous niche of HE cells we cocultured sorted endothelium from the livers and lungs of Cre;CKI/CKI and CKI/CKI mice on endothelial cells transduced with the adenovirus E4ORF1 in the absence of serum, as previously described (Sandler et al., 2014). Despite the supportive vascular induction niche, hematopoietic cells were not generated from any of the populations assayed (not shown). The small ectopic clusters of phenotypic hematopoietic cells that form on the periphery of the liver after ectopic Runx1 expression may represent a population of endothelial cells that are competent to respecification but are too rare to detect in limiting dilution HE assays, alternatively they may be only partially reprogrammed and undergo the physical but not functional transition into hematopoietic progenitor cells. These data suggest that, in large part, postnatal endothelium is not competent to respecification into HE by Runx1 alone.

EHT and angiogenesis occur simultaneously during embryogenesis and rely on many of the same signaling pathways such as BMP, Notch and Retinoic acid (Marcelo et al., 2013a). To determine if angiogenesis promotes competency to respecification in postnatal endothelium we used the retina as a model because in mice, the retinal vasculature undergoes angiogenesis postnatally from postnatal day (P)1 to P8 and is a well established model of angiogenesis (Stahl et al., 2010). We initiated ectopic Runx1 expression at P2 in Cre;CKI/CKI and CKI/CKI littermates and collected retinas for analysis 72 hours later at P5 (Fig. 6.3 B, scheme). Retinas were immunostained for CD31, Runx1 and Kit and imaged via confocal microscopy. We found that Runx1 was ectopically expressed in the vasculature of Cre;CKI/CKI retinas but ectopic EHT indicated by Kit$^+$ expression and rounded morphology was not observed (n=3) (Fig. 6.3
B). This suggests that the retinal vasculature, despite undergoing active angiogenesis, is not competent to respecification into HE by Runx1.

**Respecified embryonic and fetal endothelial cells give rise to multilineage hematopoietic cells.**

In previous studies we demonstrated that HE is heterogeneous. Specifically, HE that expresses Tek (encodes Tie2) generates erythroid/myeloid (EMP) progenitors and HE that expresses Ly6a (encodes Sca1) generates lymphoid progenitors and HSCs (Li et al., 2014, Chen et al., 2011). To determine if respecified HE has the potential to generate multilineage hematopoietic cells we plated Kit+ E9.5 Cre;CKI/+ and Kit+ E13.5 Cre;CKI/CKI as well as littermate control (CKI/+ or CKI/CKI) endothelium in culture conditions that support either erythroid/myeloid and B cell development or T cell development (Fig. 6.4 A). We found that both embryonic Cre;CKI/+ and fetal Cre;CKI/CKI respecified endothelial cells gave rise to Gr1+, Mac1+ and Ter11+ cells (Fig. 6.4 B, D). Furthermore, respecified embryonic and fetal endothelium also generated phenotypic B (CD45+ B220+) and T cells (CD45+ CD90+ CD25+) suggesting that both embryonic and fetal respecified endothelial cells generate multilineage hematopoietic cells (Fig. 6.4 C, D).

To determine if respecified endothelium gives rise to phenotypic HSCs we plated Kit+ endothelium sorted from Cre;CKI/CKI fetuses, CKI/CKI fetuses, Cre;CKI/+ embryos and CKI/+ embryos on a confluent monolayer of Akt-ECs (endothelial cells derived from the fetal liver that ectopically express AKT) in serum-free media supplemented with
cytokines (Fig. 6.5 A). To exclude endogenous hemogenic endothelium and limit contaminating hematopoietic cells we sorted endothelium from the region superior to the fetal liver in both embryonic and fetal samples (Fig. 6.5 A). By day 10 in culture, E9.5 Cre;CKI/+ and E13.5 Cre;CKI/CKI endothelium but not the control endothelium generated colonies of round semi-adherent cells, that in contrast to the cells plated on OP9s were homogenous in size and complexity (Fig. 6.5 B compare to Fig. 6.2). Flow analysis of the semi-adherent cells at day 12-14 revealed that both fetal and embryonic respecified endothelial cells gave rise to lineage⁻ (Gr1, CD3e, B220, Ter119, Mac1) Kit⁺ Sca1⁺ Mac1⁻⁺ phenotype HSCs (Fig. 6.5 C).

The embryo is not a permissive environment for the respecification of endothelial cells into HE

The niche, which consists of cell-cell interactions, signaling factors and biomechanical forces is one of the most important components of successful cellular reprogramming. Establishing a niche that promotes successful reprogramming involves recapitulation of the natural niche that exists during the specification of the cell type of interest. Since the embryo is the endogenous site of de novo hematopoiesis it is, theoretically, the best niche for the respecification of endothelial cells into HE. Therefore, we attempted to respecify endothelial cells into HE in vivo during embryogenesis to determine if the embryonic niche facilitated the respecification of endothelium into HE.

HE is a very small percentage of endothelium (~1%) that is specified in the embryo between E7.5 and E10.5 and transitions into HSPCs between E8 and E11.5.
Therefore to determine if Runx1 is sufficient to respecify endothelial cells into HE in vivo, we initiated ectopic expression of endothelial-specific Runx1 at E7.5, E8.5 or E9.5 than collected the embryos for analysis at E10.5, the peak of EHT (Fig. 6.6 A). If endothelium within the conceptus (embryo and yolk sac) is competent to respecification into HE by Runx1 we would anticipate an increase in EHT and thus an increase in the total number of hematopoietic cells and a concurrent loss of endothelial cells. When ectopic Runx1 expression was initiated at E7.5 embryonic lethality due to cardiovascular defects including a loss of vascular density resulted but no apparent increase in kit+ hematopoietic cells was observed (not shown). To prevent E10.5 lethality we reduced the percentage of endothelial cells ectopically expressing Runx1 by reducing the dose of tamoxifen from 1mg to 0.5mg. When ectopic Runx1 expression was induced at E7.5 via 0.5mg of tamoxifen, embryos survived until E10.5 but had some hemorrhaging in the head and dorsal region, indicative of vascular defects (Fig. 6.6 C, arrowheads). When Runx1 expression was initiated at E8.5 or E9.5 via 1mg of tamoxifen, embryos remained viable by E10.5 despite hemorrhaging when Runx1 was induced at E8.5 (Fig. 6.6 C, arrowheads). Therefore, to achieve both viability and the highest percentages of endothelium ectopically expressing Runx1 at each time point we used 0.5mg of tamoxifen to initiate ectopic Runx1 expression at E7.5 and 1mg at E8.5 and E9.5. To confirm that Runx1 was in fact being ectopically expressed in the endothelium of E10.5 Cre;CKI/+ embryos and yolk sacs after IP injections of tamoxifen at E7.5, E8.5 or E9.5 we did FACs analysis and found that the percentage of endothelium (CD45-CD41-Ter119-CD31+VEC+) expressing Runx1 is significantly higher in Cre;CKI/+ samples compared to littermate controls at all time points (Fig. 6.6 B). However, as expected, the percentage of Runx1+ endothelium in Cre;CKI/+ embryos and yolk sacs is much lower
when Runx1 is initiated at E7.5 (embryo, 4.31 ± 1.99 and yolk sac, 5.92 ± 2.51) compared to E8.5 (embryo, 20.31 ± 4.8 and yolk sac 22.34 ± 7.8) and E9.5 (embryo, 23.63 ± 6.19 and yolk sac 16.54 ± 8.04) due to the lower dose of tamoxifen used (Fig. 6.6 B). To examine the vasculature for signs of ectopic EHT, we immunostained E10.5 Cre;CKI/CKI, Cre;CKI/+ (not shown) and CKI/CKI embryos and yolk sacs for CD31, Runx1 and kit and analyzed the samples via confocal microscopy. Confocal Z-projections of the embryos and yolk sacs confirmed that Runx1 is ectopically expressed throughout the vasculature of Runx1 knockin samples after initiation of ectopic expression at E7.5, E8.5 and E9.5 compared to CKI/CKI littermate controls (Fig. 6.6 C). A dramatic increase in Kit+ phenotypic hematopoietic cells was not observed after the initiation of Runx1 expression in the embryos (Fig. 6.6 C). In contrast, an increase in phenotypic Runx1+ Kit+ CD31+ hematopoietic cluster cells was observed in the vasculature of the yolk sac after initiation of ectopic Runx1 expression at E7.5 and E8.5 but not E9.5 (Fig. 6.6 C). To confirm the observed increase in Kit+ cells we did flow cytometry analysis to determine the percentage of VEC+ Kit+ cells in the embryo and yolk sac of Cre;CKI/+ and Cre;CKI/CKI littermate controls (CKI). We found that there is a significant increase in the percentage of VEC+ Kit+ cells in Cre;CKI/+ and Cre;CKI/CKI embryos after ectopic Runx1 expression is initiated at E7.5 but not E8.5 or E9.5 (Fig. 6.6 D). The yolk sac has a significantly higher percentage of VEC+ Kit+ cells after Runx1 is ectopically initiated at E7.5 in both Cre;CKI/+ and Cre;CKI/CKI samples or E8.5 but only in Cre;CKI/CKI samples (Fig. 6.6 D). Since we did not observe a dramatic increase in phenotypic hematopoietic cells in the embryo via confocal microscopy and VEC+ Kit+ cells include both hemogenic endothelium and hematopoietic cells we further analyzed the cells to determine if the Kit+ cells express the hematopoietic markers CD41 and
CD45 and found that there is a significant increase in CD41$^+$ and CD45$^+$ populations in the embryo after the initiation of Runx1 at E7.5 (Fig. 6.7 A, B). These data suggests that the initiation of Runx1 at E7.5 or E8.5 increases the percentage of phenotypic hematopoietic cells by E10.5.

To more precisely localize the ectopic hematopoietic cells within the embryo proper after the initiation of Runx1 expression at E7.5 we examined the embryos via confocal microscopy. Quantification of Kt$^+$ CD31$^+$ Runx1$^+$ hematopoietic cluster cells in the dorsal aorta of Cre;CKI/+, Cre;CKI/CKI, CKI controls and Cre controls did not reveal an increase in total hematopoietic cells, however there was a mislocalization of hematopoietic cluster cells in knockin embryos (Fig. 6.8 A-B). Normally the majority of hematopoietic cluster cells form on the ventral side of the dorsal aorta (de Bruijn et al., 2000) but we found that only 41% ± 5.54 (mean ± SD) of hematopoietic cluster cells localized to the ventral side of the dorsal aorta in Cre;CKI/+ embryos and 45% ± 8.42 in Cre;CKI/CKI embryos compared to 67.11% ± 7.41 in CKI controls and 77.61% ± 8.24 in Cre controls (Fig. 6.8 B). In addition, we observed ectopic angiogenic sprouting from the Cre;CKI/+ and Cre;CKI/CKI dorsal aortas and the ectopic sprouts were often associated with hematopoietic cells (Movies 6.1, 6.2 and 6.3). Hematopoietic cells have been demonstrated to influences angiogenesis during embryogenesis through the secretion of the chemoattractant angiopoietin-1 (Ang-1) (Takakura et al., 2000, Witzenbichler et al., 1998). Therefore, the mislocalization of hematopoietic cells in the dorsal aorta of Cre;CKI/+ and Cre;CKI/CKI embryos may explain the ectopic angiogenic sprouting.

Next we examined the hearts of Cre;CKI/+, Cre;CKI/CKI, CKI controls and Cre controls for ectopic hematopoietic cells after the initiation of Runx1 expression at E7.5. Like the
dorsal aorta the heart is an endogenous site of hematopoiesis but unlike the dorsal aorta it is thought to generate only transient erythroid/myeloid progenitors (EMPs) (Nakano et al., 2013). We found that all CreCKI/+ and Cre;CKI/CKI hearts examined (n=6) had more Kit\textsuperscript{+} phenotypic hematopoietic cells when compared to littermate controls (n=4) (Fig. 6.8 C, arrowheads). In addition, ectopic angiogenic sprouts were observed near the atrioventricular canal in 83% Runx1 knockin hearts (Fig. 6.8 C, arrows). Interestingly, most of the endothelial cells within the angiogenic sprouts were not ectopically expressing Runx1 suggesting that the sprouts are a secondary effect of ectopic Runx1 expression (Fig. 6.8 C, arrows). To quantify the increase in the hematopoietic cells within the knockin hearts we isolated the hearts and did colony-forming assays to determine the total number of EMPs in each heart. We determined that Cre;CKI/+ and Cre;CKI/CKI hearts had significantly more EMPs than littermate controls (Fig. 6.8 D). Furthermore, when ectopic Runx1 expression was initiated at E7.5 on a Runx1 null background Kit\textsuperscript{+} hematopoietic cells formed in the dorsal aorta, heart and yolk sac suggesting that endogenous Runx1 expression is not necessary for the induction of EHT in these sites (Fig. 6.9 A-C).

To determine if ectopic expression of Runx1 in the endothelium increases the total number of functional hematopoietic progenitors in the whole conceptus we did colony forming assays to quantify EMPs in the conceptuses of E10.5 Cre;CKI/+, Cre;CKI/CKI and CKI littermates after the initiation of ectopic Runx1 expression at E7.5, E8.5 or E9.5 (Fig. 6.10 A). We found significantly more EMPs in Cre;CKI/+ and Cre;CKI/CKI conceptuses after the initiation of ectopic Runx1 expression at E7.5 but no later (Fig. 6.10 B). Most of the ectopic EMPs were derived from the yolk sac (Fig. 6.10
However, since circulation between the yolk sac and embryo is established by E8.5 it is unclear if the EMPs originated in the yolk sac or embryo. Despite the increase in phenotypic hematopoietic cells in the yolk sacs of Cre;CKI/CKI conceptuses after the initiation of ectopic Runx1 expression at E8.5 there was no increase in functional EMPs in either the yolk sac or embryo (Fig. 6.10 B). These data suggest that in vivo, ectopic expression of Runx1 increases the total number of EMPs but only when ectopic expression of Runx1 is initiated before E7.5.

To determine the lymphoid potential of the Kit⁺ population in the yolk sacs and embryos of Cre;CKI/+ conceptuses after initiation of ectopic Runx1 expression at E7.5 we did limiting dilution lymphoid assays on OP9-DL1 stroma to determine T cell potential and OP9 stroma to determine B cell potential (Fig. 6.10 C). We found that despite the higher percentage of Kit⁺ cells in Cre;CKI/+ yolk sacs compared to CKI/+ littermates (Fig. 6.10 D) there was no significant difference in the total number of lymphoid progenitors in the yolk sacs and embryos of Cre;CKI/+ and CKI/+ littermates (Fig. 6.10 E).

A possible explanation for the loss of respecification when Runx1 is initiated after E7.5 is the length of time Runx1 is ectopically expressed in the endothelium. When Runx1 is initiated at E7.5 and the embryos are analyzed at E10.5 there is 72 hours between initiation of expression and analysis. Whereas when Runx1 is initiated at E9.5 there is only 24 hours between initiation and analysis. Therefore, we increased the time between initiation and analysis to 72 hours by inducing expression at E9.5 and collecting the embryos at E12.5 (Fig. 6.11 A). When we used 1mg of tamoxifen to induce ectopic Runx1 expression at E9.5 the embryos died by E12.5 (not shown). To prevent lethality we reduced the dose of tamoxifen to 0.5mg. We found that despite the low dose of
tamoxifen Cre;CKI/+ embryos hemorrhaged and had flaky yolk sacs but nevertheless, survived (Fig. 6.11 B). Flow analysis of the embryos and yolk sacs indicated no significant difference in the percentage of VEC* Kit* phenotypic hematopoietic/HE cells (Fig. 6.11 C-D). Furthermore, there were significantly fewer EMPs in Cre;CKI/+ conceptuses compared to littermate controls (Fig. 6.11 E). These data confirm that the in vivo environment is not permissive to respecification after E7.5. This is in contrast to the respecification of endothelial cells in vitro. In vitro, when Runx1 is initiated as late as E12.5 the endothelium is competent to respecification. Therefore, contrary to our initial hypothesis the embryo is not the ideal niche for the respecification of endothelial cells into HE.
Discussion

In this study, we show that Runx1 expression drives the respecification of embryonic and fetal endothelial cells into HE with the potential to generate multilineage hematopoietic cells. Furthermore, we demonstrate that *in vivo* respecification within the vasculature of the developing embryo is inefficient relative to respecification *in vitro*.

*In vitro versus in vivo respecification of endothelial cells into HE*

One difference between the *in vivo* embryonic niche and the *in vitro* system that may influence respecification efficiency is the presence of tight junctions. Tight junctions help maintain the vascular structure *in vivo* but are dissolved prior to *in vitro* culture. The dissolution of tight junctions between endothelial cells is a critical step in the physical transition of endothelial cells into hematopoietic cells. Studies in zebrafish and mice have demonstrated that the BMP-Smad1/5 pathway increases endothelial pERK levels which results in strengthened tight junctions between endothelial cells ultimately inhibiting EHT (Zhang et al., 2014). In addition, the F2r-RhoA/ROCK pathway inhibits EHT by promoting endothelial identity and preventing the dissolution of tight junctions in zebrafish and mESC differentiation systems (Yue et al., 2012). The inhibitory affect of tight junctions on EHT was directly addressed by knocking down a component of the tight junction, ZO-1, in cultured ES-derived endothelium (Yue et al., 2012). Knocking down ZO-1 resulted in accelerated EHT and a significant increase in CD41+ hematopoietic cells. Dissolution of tight junctions is a necessary step in the EHT but
whether tight junction formation and dissolution are tied to the specification of hemogenic endothelium or simply a hurdle to EHT has not been established.

Other factors that may affect the efficiency of \textit{in vivo} respecification during embryogenesis are the availability of cytokines and the presence of inhibitory cell-cell interactions or signaling pathways. During \textit{in vitro} respecification, endothelial cells are provided with relatively high concentrations of hematopoietic cytokines and cell-cell interactions known to support hematopoiesis. \textit{In vivo}, cytokines and cell-cell interactions necessary for specification of HE may be more spatially and temporally restricted thus preventing ectopic specification of HE.

\textbf{The \textit{in vitro} respecification of embryonic, fetal and postnatal endothelial populations}

Our results demonstrate that Runx1 alone is sufficient to respecify embryonic and fetal endothelium into HE that can generate multilineage hematopoietic cells. However, a higher dose of Runx1 is required to respecify fetal endothelium compared to embryonic endothelium. In contrast, postnatal endothelium is not competent to respecification even when exposed to a high dose of Runx1. This data suggests that as endothelium matures it becomes less competent to respecification into HE. The loss of respecification competency in maturing endothelial cells may be due to changes in the chromatin landscape associated with cellular maturation. As maturation progresses, chromatin condenses and the transcribed portion of the genome is reduced (Ugarte et al., 2015, Efroni et al., 2008, Stergachis et al., 2013). Heterochromatin formation,
specifically through H3K9me3 represses lineage-inappropriate genes and impedes cellular reprogramming thereby “locking in” cellular identity (Ugarte et al., 2015, Soufi et al., 2012). Global epigenetic changes in endothelial cells may render imperative Runx1 targets inaccessible in maturing endothelial cells, thereby preventing respecification. To test this hypothesis future studies will be aimed at comparing chromatin accessibility of the embryonic, fetal and postnatal endothelial populations by ATAC-seq and Runx1 ChIP-seq.

Another explanation for the loss of competency during cell maturation is the progressive loss of Runx1-independent cofactors necessary for respecification. A previous study reported that postnatal dermal endothelial cells are reprogrammed into HE via the ectopic expression of FosB, Gfi1, Runx1 and Spi1 (Sandler et al., 2014). When any of the four factors were removed from the reprogramming cocktail the efficiency of reprogramming plummeted (Sandler et al., 2014). Both Gfi1 and Spi1 are direct downstream targets of Runx1 and known regulators of embryonic hematopoiesis (Lancrin et al., 2012b, Huang et al., 2008, Hoogenkamp et al., 2009). In contrast, Fosb1 is important in cell cycle regulation but has no known role in either the specification of HE or progression of the EHT. Therefore, factors independent of Runx1 that are required for respecification, such as Fosb, may be expressed in embryonic and fetal endothelial populations but not postnatal endothelium contributing to the failure of postnatal respecification. To test this hypothesis future studies will compare the transcriptome of competent cell populations (E9.5 Cre;CKI/+ and E13.5 Cre;CKI/CKI endothelium) to cell populations that are resistant to respecification (E13.5 CreCKI/+ and postnatal Cre;CKI/CKI endothelium).
In conclusion, defining a cellular state that is competent to respecification and establishing methods to achieve competency with minimal manipulation of the cells will be imperative to increasing the efficiency and safety of cellular reprogramming. The ability to reprogram cells into HSC producing-HE that can be expanded in vitro will aid in disease modeling, drug discovery and clinical applications.
Figure 6.1. Ectopic expression of Runx1 in embryonic and fetal endothelium increases the frequency of endothelial to hematopoietic transitions (EHT)
Figure 6.1. Ectopic expression of Runx1 in embryonic and fetal endothelium increases the frequency of endothelial to hematopoietic transitions (EHT). A) Representative scatter plots of Runx1 expression in the endothelium of CKI/+ and Cre;CKI/+, embryos and fetuses 24 hours after the initiation of conditional Runx1 expression. Dot plot on the right indicates percentage of Runx1+ endothelium. Error bar represents mean ± SD. Unpaired 2-tailed Student’s t-test was applied to E9.5 samples to determine significance. 1-way ANOVA and Tukey’s multiple comparison test applied to determine significance of E13.5 samples. Each point represents a single embryo or fetus and data are from two E9.5 litters and six E13.5 litters. B) Scheme illustrating limiting dilution hemogenic endothelial assays for quantification of EHT frequency. C) Representative scatter plot for the isolation of Kit+ and Kit- endothelium (CD41- CD45- Ter119- CD31+ VEC+). D) Frequency of EHT in Kit+ and Kit- embryonic E9.5 endothelial cells (EHT frequency ± upper and lower 95% CI). EHT frequency indicated above the floating bars. ELDA software (Hu and Smyth, 2009) was applied to determine EHT frequencies and P values. Data represents four biological replicates using pooled cells from superovulated litters of E9.5 CKI/+ and Cre;CKI/+ embryos collected in four independent experiments. E) Frequency of EHT in Kit+ and Kit- fetal E13.5 endothelial cells (EHT frequency ± upper and lower 95% CI). EHT frequency indicated above the floating bars. ELDA software (Hu and Smyth, 2009) was applied to determine EHT frequencies and P values. Data represents three biological replicates using pooled cells from E13.5 litters collected in three independent experiments. F) Scatter plot indicating the mean fluorescent intensity of Runx1 expression in the endothelium (CD41- CD45- Ter119- CD31+ VEC+) of E13.5 CKI, Cre;CKI/+ and Cre;CKI/CKI fetuses. 1-way ANOVA
and Tukey’s multiple comparison test applied to determine significance. *** indicates that 
$P \leq 0.001$ and ** indicates that $P \leq 0.01$. 

Figure 6.2. Respecified embryonic and fetal endothelium generates round semi-adherent cells in culture.
Figure 6.2. Respecified embryonic and fetal endothelium generates round semi-adherent cells in culture. E9.5 CKI/+, E9.5 Cre;CKI/+, E13.5 CKI/CKI and E13.5 Cre;CKI/CKI endothelium 3, 5, 7 and 10 days after being plated in coculture with OP9 stromal cells. 5,000 endothelial cells were plated in each well of a 12 well plate and ectopic Runx1 expression was initiated in the embryonic and fetal endothelium 24 hours prior to plating.
### Sorted Endothelium CFU-Cs

<table>
<thead>
<tr>
<th>Stage</th>
<th>Genotype</th>
<th>Kit</th>
<th>Region</th>
<th>CFU-C frequency in sorted endothelium</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E9.5</strong></td>
<td>CKI</td>
<td>+</td>
<td>Embryo</td>
<td>1:21,803</td>
</tr>
<tr>
<td></td>
<td>CKI</td>
<td>-</td>
<td>Embryo</td>
<td>0:26,350</td>
</tr>
<tr>
<td></td>
<td>Cre;CKI/+</td>
<td>+</td>
<td>Embryo</td>
<td>1:43,600</td>
</tr>
<tr>
<td></td>
<td>Cre;CKI/+</td>
<td>-</td>
<td>Embryo</td>
<td>1:63,350</td>
</tr>
<tr>
<td><strong>E13.5</strong></td>
<td>CKI</td>
<td>+</td>
<td>Fetus</td>
<td>1:4,988</td>
</tr>
<tr>
<td></td>
<td>CKI</td>
<td>-</td>
<td>Fetus</td>
<td>1:6,222</td>
</tr>
<tr>
<td></td>
<td>Cre;CKI/+</td>
<td>+</td>
<td>Fetus</td>
<td>1:5,700</td>
</tr>
<tr>
<td></td>
<td>Cre;CKI/+</td>
<td>-</td>
<td>Fetus</td>
<td>1:23,000</td>
</tr>
<tr>
<td></td>
<td>Cre;CKI/CKI</td>
<td>+</td>
<td>Fetus</td>
<td>1:4,500</td>
</tr>
<tr>
<td></td>
<td>Cre;CKI/CKI</td>
<td>-</td>
<td>Fetus</td>
<td>1:14,750</td>
</tr>
</tbody>
</table>

Table 6.1. Frequency of contaminating erythroid/myeloid progenitors in sorted endothelium
Figure 6.3. Postnatal vascular beds are not competent to respecification into hemogenic endothelium by Runx1
Figure 6.3. Postnatal vascular beds are not competent to respecification into hemogenic endothelium by Runx1. A) Confocal Z-projection of livers and lungs from CKI/CKI and Cre;CKI/CKI 1-month-old mice. Samples were immunostained for CD31 (i), Runx1 (i,ii) and Kit (i,iii). Boxed region on the Cre;CKI/CKI liver Z-projection indicates the magnified region in the row below. Arrowheads indicate ectopic phenotypic hematopoietic cluster cells (CD31+ Runx1+ Kit+) at the periphery of the Cre;CKI/CKI liver. Scheme demonstrates experimental outline; 1-month-old mice were given 2mg of tamoxifen via oral gavage and their livers and lungs were removed 24 hours later for analysis. All scale bars = 100µm. B) Confocal Z-projections of retinas from CKI/CKI and Cre;CKI/CKI postnatal day (P)5 mice immunostained for CD31 (i), Runx1 (i,ii) and Kit (i,iii). Z-projection in second row is a magnified image of a blood vessel in the retina. Scheme demonstrates experimental outline; P2 mice were injected with 0.5mg of tamoxifen, 72 hours later their retinas were removed for analysis. All scale bars = 100µm.
Figure 6.4. Respecified embryonic and fetal endothelium give rise to phenotypic multilineage hematopoietic cells
Figure 6.4. Respecified embryonic and fetal endothelium give rise to phenotypic multilineage hematopoietic cells A) Scheme illustrating the plating of embryonic and fetal endothelium for the derivation of erythroid, myeloid, B, and T cells. B) Scatter plots showing phenotypic erythroid (Ter119⁺) and myeloid (Mac1⁺ and Gr1⁺) cells derived from E9.5 Cre;CKI/+ and E13.5 Cre;CKI/CKI endothelium. C) Scatter plots showing phenotypic T (CD45⁺ CD90⁺ CD25⁺) and B (CD45⁺ B220⁺) cells. D) Scatter plots indicating the percentage of Ter119⁺, Mac1⁺, Gr1⁺, T cells and B cells derived from respecified embryonic and fetal endothelium as well as control endothelium. Bar indicates the mean.
Figure 6.5. Respecified embryonic and fetal endothelium generates phenotypic hematopoietic stem cells.
Figure 6.5. Respecified embryonic and fetal endothelium generates phenotypic hematopoietic stem cells. (A) Scheme illustrating the plating of embryonic and fetal endothelium derived from the region superior to the fetal liver on AKT-EC for the derivation of hematopoietic cells. (B) Light microscopy image of round semi-adherent cells that form in co-cultures of AKT-ECs and E9.5 Cre;CKI/+ or E13.5 Cre;CKI/CKI endothelium. Image taken 10 days after plating. C) Representative scatter plots showing phenotypic HSCs (Lin⁻ Kit⁺ Sca1⁺ Mac1⁺⁻) derived from E9.5 Cre;CKI/+ and E13.5 Cre;CKI/CKI endothelium.
Figure 6.6. Conditional endothelial-specific expression of Runx1 initiated before E9.5 increases the percentage of phenotypic hematopoietic cells in the yolk sac by E10.5
Figure 6.6. Conditional endothelial-specific expression of Runx1 initiated before E9.5 increases the percentage of phenotypic hematopoietic cells in the yolk sac by E10.5. A) Scheme for the initiation of ectopic endothelial-specific expression of Runx1 in utero at embryonic day (E)7.5, E8.5 or E9.5 by the intraperitoneal (IP) injection of tamoxifen into the pregnant mouse and the subsequent collection of embryos for analysis at E10.5. B) Percentage of endothelium (VEC⁺ CD31⁺ CD41⁻ CD45⁻ Ter119⁻) expressing Runx1 in the yolk sac and embryo proper of E10.5 CKI/+ and Cre;CKI/+ littermates after the initiation of Runx1 at E7.5, E8.5 or E9.5 (mean ± SD). Unpaired 2-tailed Student’s t-test was applied to determine significance. *** indicates that P ≤ 0.001. Data are from two litters per time point. C) Gross images and confocal Z-projection of CKI/CKI and Cre;CKI/CKI embryos and yolk sacs. Samples were immunostained for CD31 (i), Runx1 (i,ii) and Kit (i,iii). Arrowheads indicate hemorrhaging and arrows indicate the vitelline artery (VA) in the yolk sac. All scale bars = 500µm. D) Percentage of VEC⁺ Kit⁺ cells in the embryo proper (left) or yolk sac (right) of E10.5 CKI (CKI/+ and CKI/CKI), Cre;CKI/+ and Cre;CKI/CKI after initiation of ectopic Runx1 expression at E7.5, E8.5 or E9.5. Data are from four to six litters per time point (mean ± SD). 1-way ANOVA and Tukey’s multiple comparison test applied to determine significance. *** indicates that P ≤ 0.001, ** indicates that P ≤ 0.01 and * indicates that P ≤ 0.05.
Figure 6.7. Kit⁺ cells in the yolk sacs and embryos of E10.5 Cre;CKI/+ and Cre;CKI/CKI mice express CD41 and CD45 and therefore likely to be phenotypic hematopoietic cells rather than hemogenic endothelium.
Figure 6.7. Kit$^+$ cells in the yolk sacs and embryos of E10.5 Cre;CKI/+ and Cre;CKI/CKI mice express CD41 and CD45 and therefore likely to be phenotypic hematopoietic cells rather than hemogenic endothelium. A) Representative scatter plots of CD41 and CD45 expression in the E10.5 embryo or yolk sac after the initiation of ectopic Runx1 expression at E7.5, E8.5 or E9.5. Populations are gated through single, live and represent the Kit$^+$ population. B) Stacked bar graphs representing the percent of Kit$^+$ cells in CKI, Cre;CKI/+ and Cre;CKI/CKI embryos that are CD41$^-$CD45$^-$, CD41$^+$CD45$^-$, CD41$^+$CD45$^+$ or CD41$^-$CD45$^+$. 
Figure 6.8. Conditional endothelial-specific expression of Runx1 initiated at E7.5 increases hematopoietic cells in the heart but not the dorsal aorta (DA) by E10.5
Figure 6.8. Conditional endothelial-specific expression of Runx1 initiated at E7.5 increases hematopoietic cells in the heart but not the dorsal aorta (DA) by E10.5.

A) Confocal Z-projections of DAs with z-intervals of 2 µm from E10.5 Cre, CKI, Cre;CKI/+ and Cre;CKI/CKI embryos immunostained for CD31(i), Runx1 (i,ii) and Kit (i,iii). The dorsal (D) side of the embryo is on the left and the ventral (V) side is on the right. Dotted lines demarcate the ventral and dorsal sides of the DA used for cluster counts in B. All scale bars = 100µm. B) Quantification of hematopoietic cluster cells (CD31⁺ Runx1⁺ Kit⁺) in the dorsal aortas of Cre (n= 3), CKI (n= 8), Cre;CKI/+ (n= 4) and Cre;CKI/CKI (n= 5) embryos. Cluster cells on the dorsal side of the DA indicated by black bar and cluster cells on the ventral side indicated by white bar (mean ± SD). 1-way ANOVA applied to determine significance (P ≤ 0.143). C) Confocal Z-projections of hearts E10.5 CKI, Cre;CKI/+ and Cre;CKI/CKI embryos immunostained for CD31(i), Runx1 (i,ii) and Kit (i,iii). Arrows indicate ectopic angiogenic sprouts, arrowheads indicate ectopic phenotypic hematopoietic cells in the atrium (A) and ventricle (V). All scale bars = 100µm. D) Quantification of erythroid myeloid progenitors (EMP) in the hearts (including atrium, ventricles and outflow tract) of E10.5 CKI, Cre;CKI/+ and Cre;CKI/CKI embryos (mean ± SD). Data are from 6 litters. 1-way ANOVA and Tukey’s multiple comparison test applied to determine significance. *** indicates that P ≤ 0.001.
Figure 6.9. Endogenous Runx1 expression is not required for hematopoietic cluster formation in E10.5 Cre;CKI/CKI embryos and yolk sacs when ectopic Runx1 expression is initiated at E7.5.
Figure 6.9. Endogenous Runx1 expression is not required for hematopoietic cluster formation in E10.5 Cre;CKI/CKI embryos and yolk sacs when ectopic Runx1 expression is initiated at E7.5. (A) Confocal Z-projections of DAs with z-intervals of 2 µm from an E10.5 CKI/CKI;Runx1Δ/Δ and Cre;CKI/CKI; Runx1Δ/Δ embryo. The dorsal side of the embryo is on the left and the ventral side is on the right. Scale bar = 100µm. B) Confocal Z-projection of E10.5 CKI/CKI;Runx1Δ/Δ and Cre;CKI/CKI; Runx1Δ/Δ hearts. Scale bar = 100µm. C) Confocal Z-projection of E10.5 CKI/CKI;Runx1Δ/Δ and Cre;CKI/CKI; Runx1Δ/Δ yolk sacs. Scale bar = 500µm. All samples immunostained for CD31(i), Runx1 (i,ii) and Kit (i,iii).
Figure 6.10 Conditional endothelial-specific expression of Runx1 initiated at E7.5 but no later increases the total number of erythroid myeloid progenitors (EMPs) but not lymphoid progenitors (LP)
Figure 6.10. Conditional endothelial-specific expression of Runx1 initiated at E7.5 but no later increases the total number of erythroid myeloid progenitors (EMPs) but not lymphoid progenitors (LP) A) Scheme of experimental outline for the quantification of EMPs in the yolk sac and embryo proper after the initiation of ectopic Runx1 expression at E7.5, E8.5 or E9.5. B) Total number of EMPs in the yolk sac and embryo proper of Cre, CKI, Cre;CKI/+ and Cre;CKI/CKI E10.5 conceptuses (mean ± SD). Data are from ten IP E7.5 litters, six IP E8.5 litters and seven IP E9.5 litters. 1-way ANOVA and Tukey’s multiple comparison test applied to determine significance. *** indicates that $P \leq 0.001$ and ** indicates that $P \leq 0.01$. C) Scheme of experimental outline for the quantification of lymphoid progenitors in the yolk sac and embryos of CKI/+ and Cre;CKI/+ littermates after an intraperitoneal (IP) injection of tamoxifen at E7.5. D) Representative scatter plots of Kit$^+$ cells sorted for analysis. E) Total number of lymphoid progenitors within the Kit$^+$ population sorted from the embryo proper and yolk sacs of CKI/+ and Cre;CKI/+ conceptuses after the initiation of ectopic Runx1 expression at E7.5 (mean ± SD). Data are from two independent experiments of pooled E10.5 litters and each tested dilution had 10 replicates per experiment. Unpaired 2-tailed Student’s $t$-test was applied to determine significance. For B cells $P \leq 0.1482$ and for T cells $P \leq 0.6632$. 
Figure 6.11. Prolonging ectopic endothelial-specific expression of Runx1 in vivo does not increase phenotypic or functional hematopoietic cells
Figure 6.11. Prolonging ectopic endothelial-specific expression of Runx1 in vivo does not increase phenotypic or functional hematopoietic cells. A) Scheme demonstrating the experimental outline for the initiation of ectopic Runx1 expression in utero at E9.5 and analysis of hematopoietic cells at E12.5. B) Gross images of E12.5 CKI/+ and Cre;CKI/+ littermates after ectopic expression of Runx1 initiated at E9.5. C) Representative scatter plots of VEC+ Kit+ cells in the yolk sac and embryo proper of E12.5 CKI/+ and Cre;CKI/+ conceptuses. D) Percentage of VEC+ Kit+ cells in the embryo proper and yolk. Data are from two independent experiments and two litters (mean ± SD). Unpaired 2-tailed Student’s t-test was applied to determine significance, P ≤ 0.44 for embryos and P ≤ 0.21 for yolk sacs. E) Total number of EMPs in the yolk sac and embryo proper of CKI/+ and Cre;CKI/+ E12.5 conceptuses (mean ± SD). Data are from three independent experiments. Unpaired 2-tailed Student’s t-test was applied to determine significance, *** indicates that P ≤ 0.001.
Movie 6.1. 3-dimensional reconstruction of the dorsal aorta of an E10.5 CKI;CKI mouse. Sample is immunostained for CD31 (red), Runx1 (green) and Kit (cyan).

Movie 6.2. 3-dimensional reconstruction of the dorsal aorta of an E10.5 Cre:CKI;CKI mouse after initiation of ectopic Runx1 expression at E7.5. Sample is immunostained for CD31 (red), Runx1 (green) and Kit (cyan). Arrow points to an ectopic angiogenic sprout associated with hematopoietic cells.

Movie 6.3. 3-dimensional reconstruction of the dorsal aorta of an E10.5 Cre:CKI;CKI mouse after initiation of ectopic Runx1 expression at E7.5. Sample is immunostained for CD31 (red), Runx1 (green) and Kit (cyan). Arrow points to an ectopic extravascular island near the dorsal aorta.
CHAPTER 7: Conclusions and future directions
Confocal analysis of embryonic hematopoiesis

Scientists have observed hematopoiesis in developing embryos through the lenses of microscopes for more than one hundred years. In 1916, microscopic observations of chick, turtle, pig and mongoose embryos revealed the close association of hematopoietic cells and endothelial cells (Emmel, 1916, Jordan, 1916). In describing his observation, H.E. Jordan wrote, "my attention was arrested by the presence of peculiar cell clusters in the aorta...they were intimately associated with the endothelium; in some clusters true endothelium appears to be entirely lacking beneath the mass of primitive blood cells" (Jordan, 1916). This observation along with similar observations made by Drs. Emmel and Sabin lead to the hypothesis that the precursor of hematopoietic cells lies within the endothelium; a hypothesis that was not experimentally supported until the 1990s-2010s with the advent of lineage tracing and live-imaging experiments (Jaffredo et al., 1998, Jaffredo et al., 2000, Zovein et al., 2008, Chen et al., 2009, Eilken et al., 2009, Kissa and Herbomel, 2010, Bertrand et al., 2010, Boisset et al., 2010, Lam et al., 2010, Emmel, 1916, Sabin, 1920). My observations of embryonic hematopoiesis via confocal microscopy will build upon the knowledge garnered by the scientists who came before me and hopefully bring the field closer to understanding the de novo generation of hematopoietic cells.

During my confocal studies, I found that embryonic hematopoiesis is not as spatially restricted as previously thought. I identified hematopoietic clusters and Runx1+ hemogenic endothelial (HE) cells within the somitic vessels and the venous plexus of the yolk sac; two sites not previously associated with hematopoiesis. I also described three waves of hematopoiesis that occur from the endocardium between E9.5 and E12.5.
Furthermore, I determined that the nuclear shape of hematopoietic cluster cells is heterogeneous. Specifically, nuclei are round, ring or bean-shaped. Ring-shaped nuclei are indicative of the myeloid lineage and therefore the identification of cells with ring-shaped nuclei suggests that hematopoietic cells begin to mature and acquire cell identity before entering circulation. In congruence, I found that Ly6a-GFP\textsuperscript{+} cluster cells, which are enriched for lymphoid and HSC-potential, have round or bean-shaped nuclei. My observations, though descriptive in nature, provide insight into the de novo generation of hematopoietic cells.

Perhaps, my most interesting confocal microscopic observation was that of extravascular islands. Extravascular islands are aggregates of endothelial and hematopoietic cells that are independent of the cardiovascular system. Extravascular islands were first observed in the mesentery of chick embryos in 1913 (Miller, 1913). At the time, it was hypothesized that they developed in situ independent of the cardiovascular system. It wasn’t until 2010 that Ann Zovein and colleagues revealed the origin of extravascular islands when they demonstrated that the remodeling of the vitelline artery resulted in the extravascular emergence of hematopoietic clusters (Zovein et al., 2010). However, a subsequent study cast doubt on the existence of extravascular islands by attributing them to artifacts of sample sectioning and concluded that they did not exist within whole embryos (Yokomizo et al., 2011). Using confocal microscopy to analyze unsectioned embryos, I provided clear evidence that extravascular islands are present not only in the mesentery of midgestation mouse embryos but also in the extravascular yolk sac and placenta. Furthermore, I demonstrated that they arise from both the vitelline artery (as described by Zovein et al.) and the umbilical artery via at
least two mechanisms: vascular remodeling and extrusion. I also observed the elongation of extravascular islands and the merging of extravascular islands to developing blood vessels. My data suggested that extravascular islands function in a novel mechanism of vasculogenesis whereby established vessels contribute endothelial and hematopoietic cells to nearby developing vascular beds.

My results shed some light on the development and function of extravascular islands but much remains to be discovered. To definitively demonstrate that extravascular islands are in fact derived from the umbilical and vitelline arteries, live imaging of the vasculature of midgestation embryos will be necessary. Although live-imaging of embryos has been performed (Boisset et al., 2010), the resolution is likely too low to detect the emergence of extravascular islands in whole embryos, therefore direct evidence of their emergence may have to await the development of higher resolution live-imaging techniques. Furthermore, my results demonstrated that extravascular islands contribute to the vasculature but the molecular mechanisms underlying their development are a mystery. Laser capture microdissection of emerging and sprouting extravascular islands and subsequent transcriptional analysis may lend some clues to the factors regulating their emergence. Lastly, it is unknown if extravascular islands form during other vasculogeneic/angiogeneic events such as wound healing and tumorgenesis therefore microscopic analysis of the vasculature during these events would be informative.

During embryogenesis, the formation of extravascular islands is not restricted to the arteries they also form on the heart. Cardiac blood island (CBI) formation constitutes the third wave of hematopoiesis in the heart and is marked by protrusions of blood-filled
endocardial sacs on the ventricle. During mid to late gestation CBIs pinch off from the ventricles and contribute to the coronary vasculature. A previous study found that CBIs are associated with Ter119+ erythrocytes, CD41+ platelets and CD45+ leukocytes and contribute only minimally to the coronary plexus with a bulk of the coronary plexus thought to be derived from the sinus venosus (Red-Horse et al., 2010). Since CBIs are only a minute component of the coronary plexus it is not clear if they are necessary for vascular development or a remanent of evolution. Before this question can be addressed a better understanding of the molecular development of CBIs will have to be determined. Towards understanding the development of CBIs, I found that the total number of CBIs on the ventricles of mouse fetuses significantly increases when the Ras-GAP, NF1 is knocked out in the germ line. Therefore, activation of the Ras pathway increases CBI formation. Interestingly, I did not see an increase in extravascular islands from the umbilical and vitelline arteries of Nf1 deficient embryos suggesting that the formation of CBIs and extravascular islands may be differentially regulated.

My confocal studies demonstrated that there is still a lot to learn about the embryo from simple observation. Descriptive research, while not necessarily the most popular, brings us back to the roots of research and allows us to make observations and to discover previously unknown developmental processes that can then be further probed to determine the why and how.

**The specification of hemogenic endothelial cells**
Each year, more than 8,000 people in the United States are diagnosed with hematological malignancies in which the best course of treatment is transplantation of HSCs from a donor (allogeneic transplant) (Health Resources and Service Administration, 2016). However, obtaining a human leukocyte antigen (HLA)-matched donor is a challenge faced by many patients, especially minorities (Health Resources and Service Administration, 2016). To overcome this challenge research has focused on the derivation of HSCs in vitro either through the directed differentiation of induced pluripotent stem cells or reprogrammed primary cells. The most successfully technique for the derivation of HSCs has been established at Cornell University in Dr. Shahin Rafii’s lab. The Rafii lab demonstrated that human adult dermal cells could be reprogrammed into transplantable HSCs by ectopic expression of Fosb, Gfi1, Runx1 and Spi1 in a permissive vascular niche platform (Sandler et al., 2014). However, before in vitro derived HSCs can be used in the clinic it will be necessary to increase the efficiency of reprogramming, gain a better understanding of how reprogramming occurs on a genomic level so we can generate more streamlined methods and minimize manipulation of the cells in order to increase safety.

My results suggest that embryonic and fetal endothelial cells are competent to respecification into HE by ectopic expression of Runx1 alone but postnatal endothelium is not. Comparing cell populations competent to respecification (embryonic and fetal endothelium) to non-competent cells (postnatal endothelium) may provide valuable insight into what defines a competent cell state and ultimately allow for more efficient reprogramming. Comparing the populations via ATAC-seq will provide a map of the open chromatin regions in competent and non-competent populations. Chromatin
condensation is a barrier to cell reprogramming and if competent cells have a more open chromatin structure compared to non-competent cells, reducing chromatin condensation via chromatin remodeling inhibitors may enhance reprogramming. Furthermore, two of the reprogramming factors (Spi1 and Gfi1) used by the Raffi lab to reprogram cells are direct downstream targets of Runx1, therefore it would be interesting to determine if Runx1 can bind these targets in competent versus non-competent cell populations via Runx1 ChIP-seq analysis. Furthermore, if we can determine the hallmarks of a competent cell we can screen multiple postnatal populations to determine which are the best candidates for reprogramming. If postnatal cells that fit the competent cell signature can be reprogrammed by Runx1 alone it will reduce the amount of genetic manipulation necessary and therefore be a safer method of cellular reprogramming.
CHAPTER 8: Bibliography


KUMANO, K., CHIBA, S., KUNISATO, A., SATA, M., SAITO, T., NAKAGAMI-YAMAGUCHI, E., YAMAGUCHI, T., MASUDA, S., SHIMIZU, K., TAKAHASHI, T., OGAWA, S., HAMADA, Y. &...


LEVANON, D., BERNSTEIN, Y., NEGREANU, V., GHOZI, M. C., BAR-AM, I., ALOYA, R., GOLDENBERG, D., LOTEM, J. & GRONER, Y. 1996. A large variety of alternatively spliced
and differentially expressed mRNAs are encoded by the human acute myeloid leukemia gene AML1. DNA Cell Biol, 15, 175-85.


MILLER, A. 1913. Histogenesis and morphogenesis of the thoracic duct in the chick; development of blood cells and their passage to the blood stream via the thoracic duct. The American Journal of Anatomy, 15, 131-197.


NORTH, T. E., STACY, T., MATHENY, C. J., SPECK, N. A. & DE BRUIJN, M. F. 2004. Runx1 is expressed in adult mouse hematopoietic stem cells and differentiating myeloid and lymphoid cells, but not in maturing erythroid cells. Stem Cells, 22, 158-68.


SABIN, F. R. 1920. Studies on the origin of blood-vessels and of red blood-corpuscles as seen in the living blastoderm of chicks during the second day of incubation. Contributions to Embryology.


The differential activities of Runx1 promoters define milestones during embryonic hematopoiesis. *Blood*, 114, 5279-89.


Fate tracing reveals the endothelial origin of hematopoietic stem cells. *Cell Stem Cell*, 3, 625-36.