The Role of *Gata2* in Hematopoietic and Vascular Development

by

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Dedication

To my family, without whom this PhD would never have been possible.

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Abstract

Transcription factors play demonstrably critical roles in development. The transcription factor *Gata2* is required for the proliferation of hematopoietic progenitors and proper urogenital development. *Gata2* null mutant mice die at embryonic day 10.5 from a complete failure of primitive and definitive hematopoiesis; hematopoietic-rescued compound mutant animals succumb to hydroureteronephrosis shortly after birth. The *Gata2* null mutant background deficiencies were successfully rescued using a 413-kilobase pair (kbp) linked-Bacterial Artificial Transgene (BAC) containing both the 5' hematopoietic and 3' urogenital enhancers. Rescued transgenic mice are both viable and fertile, indicating that the endpoints of this linked-BAC define the functional boundaries of the *Gata2* locus. A 290-bp endothelial enhancer of *Gata2*, termed VE (vascular enhancer), was localized within *Gata2* intron 4 and characterized in detail, revealing a requirement for an E box binding site for proper expression from this element. To determine whether GATA-2 is required for the differentiation of definitive hemangioblasts, presumptive common progenitors of both the endothelial and hematopoietic lineages, mice carrying the *Gata2* VE element driving cre expression ($Tg^{VE\text{-}Cre}$) were generated in order to conditionally ablate *Gata2* in this precursor cell type. Through lineage tracing studies in $Tg^{VE\text{-}Cre}$:$Rosa26R^{lacZ}$ mice, we provide evidence that precursor cells from the mesoderm give rise to at least a subset of definitive, lineage-restricted hematopoietic cells and endothelial cells. Through understanding of

the *cis* regulatory elements and *trans*-acting factors that govern *Gata2* expression and its role in these developmental processes, our comprehension of the functional networks that are employed during embryonic development has been expanded.

Chapter 1

Introduction

The development of a living organism is an intricate and complex process. The complete blueprint resides within every individual cell, and yet throughout development each cell becomes ever more highly restricted to carrying out specific tasks. This is made possible through the process of differentiation. While each cell contains every gene necessary for life, a specialized group of proteins called transcription factors decide which genes are activated or repressed in any given cell.

Purified eukaryotic RNA polymerase II is incapable of initiating transcription *in vitro,* unlike bacterial RNA polymerase. This finding led to the discovery of supplementary factors that are necessary for initiating transcription. These general transcription factors bind to DNA and allow RNA polymerase to function. While these factors are necessary for initiation of all gene transcription, other transcription factors have evolved more specific functions.

Eukaryotic DNA contains regulatory sequences with the ability to promote, enhance, or silence gene transcription. Transcription factors have evolved the ability to bind to these regulatory elements (Mitchell and Tjian, 1989), allowing for the unique expression of a subset of genes in any given cell. With multiple transcription factors working in concert to regulate which genes are activated or repressed in a given cell, the complex pattern of differentiation is unveiled throughout development.

Regulatory transcription factors can be divided into two major categories: developmental transcription factors and signal-dependent transcription factors. Signal-dependent transcription factors function precisely as their name implies, through activation by one of three main extrinsic stimuli: steroid hormones, small intracellular signaling molecules, or cell-surface receptors (Brivanlou and Darnell, 2002). Developmental transcription factors enter the nucleus directly without requiring

posttranslational modifications. However, their production is regulated, often depending upon specific intracellular or extracellular signals. Various developmental transcription factors work in concert to influence cell determination and differentiation (Brivanlou and Darnell, 2002). Understanding how these factors are regulated, and what in turn they regulate, will help elucidate the control mechanisms underlying development. This information becomes exceptionally useful when the transcriptional machinery becomes corrupted and leads to disease, such as in the setting of human congenital or acquired deficiencies (*e.g*. cancer).

This dissertation focuses on the developmental transcription factor GATA-2, which has been found to regulate a number of independent developmental pathways. It is expressed in discrete tissue types, and displays distinct functions in these various physiological domains. Understanding the mechanism(s) that regulate these processes will help to elucidate one or more of the pathways by which developmental progression proceeds.

The GATA Family of Transcription Factors

The first GATA factor to be discovered, originally named Eryf1, was found in chicken erythroid cells while studying the regulation of the β-globin gene (Evans et al., 1988). Eryf1, also called GF-1 in the mouse, was found to contain two zinc fingers (Evans and Felsenfeld, 1989; Tsai et al., 1989) and bind to a consensus (A/T)GATA(A/G) sequence (Ko and Engel, 1993; Merika and Orkin, 1993), from which the factor obtained its modern namesake. Using a murine GATA-1 probe, two additional GATA transcription factors (GATA-2 and GATA-3) were discovered in chickens (Yamamoto et al., 1990), and then in all other vertebrates. The discovery of these two additional family members was followed some years later by the cloning of GATA-4, which was identified in an embryonic day 6.5 (e6.5) mouse embryo library (Arceci et al., 1993). This was followed soon thereafter by the cloning of GATA-5 (Kelley et al., 1993; Laverriere et al., 1994; Morrisey et al., 1997) and GATA-6 (Laverriere et al., 1994; Morrisey et al., 1996; Suzuki et al., 1996). All six of these vertebrate family members bind to the same consensus GATA sequence and contain two zinc fingers.

By the beginning of the third millennium AD, ninety-three proteins, ranging from cellular slime mold to plants to humans, have been discovered that contain at least one GATA-type zinc finger (Lowry and Atchley, 2000). Five GATA factors have been discovered in *Drosophila* (Gillis et al., 2008), some of which retain more similar functions to their vertebrate counterparts (Brown and Castelli-Gair Hombría, 2000; Fossett and Schulz, 2001; Lebestky et al., 2000) than others (Gillis et al., 2008). Eleven GATA factors have been discovered in *Caenorhabditis elegans,* which also appear to have overlapping as well as unique activities (Patient and McGhee, 2002). Fungal GATA factors regulate distinctly invertebrate processes including nitrogen metabolism, mating-type switching, and chromatin rearrangement (Scazzocchio, 2000). Clearly, while GATA factors have taken on unique roles in different phyla, their function is evolutionarily critical.

Focusing on the vertebrate GATA factors, the amino-terminus functions as a transcriptional activator (Martin and Orkin, 1990; Yang and Evans, 1992). The GATA factors contain two zinc fingers, which contain four cysteine residues that chelate a single zinc ion (Teakle and Gilmartin, 1998), but are functionally distinct. The amino finger can specify and stabilize DNA binding (Trainor et al., 1996), can bind weakly to a GATC consensus sequence (Newton et al., 2001), and also contains key residues that allow specific binding to cofactor proteins such as FOG1 (Fox et al., 1998; Tsang et al., 1997). The carboxyl finger directly binds the consensus GATA recognition sequence on DNA (Martin and Orkin, 1990; Yang and Evans, 1992), but also is capable of physically interacting with other transcription factors, including Sp1 and PU.1 (Merika and Orkin, 1995; Rekhtman et al., 1999). The C-terminus of the GATA factors contain basic motifs and augment DNA binding (Yang and Evans, 1992).

In keeping with the strong conservation of the GATA factor family, all but one of the GATA genes has been found to contain two distinct promoters and alternate first exons capable of independently regulating gene expression in distinct tissues or cell lineages. The alternative promoter for GATA-1 directs expression in Sertoli cells of the testis (Ito et al., 1993; Yomogida et al., 1994). GATA-2's alternative promoter directs expression to hematopoietic progenitor cells and the midbrain (Minegishi et al., 1998; Pan et al., 2000). An alternative GATA-3 promoter directs expression in the brain and in

T lymphocytes (Asnagli et al., 2002). The alternative promoter for GATA-5 creates an isoform that splices to exon three, creating a protein that contains only a single zinc finger (MacNeill et al., 1997), though its function remains to be determined. Finally, the alternative promoter of GATA-6 creates an isoform that is expressed in the same tissue-specific and developmental stage-specific pattern as the more abundantly transcribed promoter, although the longer protein appears to have a more robust transactivation activity (Brewer et al., 1999).

The GATA family can be functionally divided into two subgroups. GATA-1/2/3 are primarily involved in hematopoietic lineage development (Ness and Engel, 1994), while GATA-4/5/6 are primarily involved in mesoderm- and endoderm-derived tissue development including the gonad, gut, heart, liver, and lung (Molkentin, 2000).

GATA-1

The founding and best characterized member of the GATA family of transcription factors (likely because its expression pattern is far simpler than the other factors), GATA-1, was discovered in erythroid cells bound to two sites in the chicken β-globin enhancer (Evans et al., 1988), and was discovered soon thereafter in mice (Tsai et al., 1989). GATA-1 can activate erythroid-specific genes (Evans and Felsenfeld, 1989; Mignotte et al., 1989), including β-globin (Evans et al., 1988; Talbot and Grosveld, 1991), and is induced by erythropoietin in response to tissue hypoxia (Krantz, 1991). GATA-1 is important for the characteristic chromatin structure of the β-globin locus by creating a hypersensitive site in the locus control region (Stamatoyannopoulos et al., 1995), but requires other erythroid factors to function in an additive fashion (Boyes and Felsenfeld, 1996). Recently, Ikaros has been found to interact with GATA-1 to impair the β-globin locus control region from being in close proximity to the human γ-globin genes, potentially augmenting the switch from γ to β-globin (Bottardi et al., 2009).

By disrupting GATA-1 in embryonic stem cells, it was shown that erythroid differentiation is blocked in the absence of this factor in chimeric mice (Pevny et al., 1991) at the proerythroblast stage (Pevny et al., 1995; Weiss et al., 1994). This was also found to be true in conditional knockout mice in which the erythroid promoter of GATA-1 was ablated (Takahashi et al., 1997). These mice could be fully rescued by transgenic

GATA-1 cDNA expression, but only partially by transgenes expressing GATA-2 or GATA-3, indicating that the hematopoietic GATA factor proteins are not functionally equivalent (Takahashi et al., 2000).

GATA-1 was also found to suppress apoptosis in maturing erythroid cells (Weiss and Orkin, 1995), a process that can be regulated by steroid hormones (Blobel et al., 1998). Caspase-mediated cleavage of GATA-1 functions as a negative feedback loop of erythropoiesis (De Maria et al., 1999).

GATA-1 is also expressed in other myeloid lineages, including megakaryocytes (Martin et al., 1990; Romeo et al., 1990), mast cells (Martin et al., 1990), and eosinophils (Hirasawa et al., 2002; Zon et al., 1993). GATA-1 was shown to induce megakaryocyte differentiation when forcibly expressed in an early mouse myeloid cell line (Visvader et al., 1992). In chicken myeloblasts, GATA-1 induces the generation of erythroblasts, eosinophils, and megakaryocytes (thromboblasts) (Kulessa et al., 1995). Megakaryocyte lineage-specific knockout of *Gata1* in mice resulted in impaired maturation of megakaryocytes and quantifiably fewer platelets (Shivdasani et al., 1997), though complete maturation was possible (Pevny et al., 1995). Mutations of *GATA1* resulting in premature stop codons preceed the generation of acute megakaryoblastic leukemia in Downs syndrome children (Wechsler et al., 2002). GATA-1 has recently been shown to interact with the positive transcriptional elongation factor P-TEFb for megakaryocyte differentiation (Elagib et al., 2008). Inhibition of cyclin-dependent kinase 9, part of the P-TEFb complex, produces a megakaryoblastic disorder similar to Downs syndrome (Elagib et al., 2008).

Eosinophil differentiation relies on the carboxy-terminal finger of GATA-1 (Hirasawa et al., 2002), as well as a double GATA motif in the erythroid promoter of GATA-1 (Yu et al., 2002). Interestingly, the double GATA motif appears to be dispensable for erythroid, megakaryocyte, mast cell, or other early hematopoietic precursors (Yu et al., 2002).

Gata1 has a separate promoter upstream from the erythroid promoter (Ito et al., 1993). This was discovered through expression of GATA-1 in the seminiferous tubules of the testes (Ito et al., 1993). GATA-1 expression initiates concurrently in Sertoli cells

with the first wave of spermatogenesis, but is turned off in maturing germ cells (Yomogida et al., 1994).

The zinc fingers of GATA-1 can bind the histone acetyltransferase CBP (Ogryzko et al., 1996) to augment gene expression and differentiation (Blobel et al., 1998). Acetylation of GATA-1 allows for a more open chromatin structure enhancing the ability of GATA-1 to function as a transcription factor (Boyes et al., 1998), though the presence of GATA-1 alone is inadequate to create an open chromatin state (Yamamoto et al., 1997). Interestingly, the N-finger of GATA-1 is critical for erythroid differentiation *in vitro*, even though it is dispensable for binding most GATA motifs (Weiss et al., 1997). When tested *in vivo,* the N-finger was found to be critical for definitive erythropoiesis, but dispensable for primitive erythropoiesis (Shimizu et al., 2001). The N-terminal transactivation domain was found to be important for definitive erythropoiesis, but not absolutely required (Shimizu et al., 2001). Furthermore, primitive erythroid cells only require a 5' enhancer of *Gata1* for expression, while definitive erythroid cells require both the 5' enhancer as well as an element in the first intron (Nishimura et al., 2000; Onodera et al., 1997).

Using yeast two-hybrid assays, the zinc finger protein Friend of GATA-1 (FOG1), was found to bind to the N-finger of *Gata1* and synergistically activate transcription during erythroid and megakaryocyte differentiation (Tsang et al., 1997). FOG1 knockout mice demonstrated that GATA-1 requires FOG1 for erythroid maturation, and that FOG1 appears to have a GATA-independent role in the earliest stages of megakaryocyte differentiation (Tsang et al., 1998). Mutation of the binding site for FOG1 on the amino-finger of *Gata1* results in x-linked dyserythropoietic anaemia and thrombocytopenia (Nichols et al., 2000). However, GATA-1 appears to regulate mast cell differentiation in a FOG1-independent matter by binding to PU.1; in fact, if FOG1 is forcibly expressed, PU.1 binding to GATA-1 is disrupted and converts earlier progenitors into neutrophils, erythroid, megakaryocytic and granulocytic lineages instead (Cantor et al., 2008; Sugiyama et al., 2008).

Another FOG protein, FOG2, also binds to the N-finger of *Gata1* (Tevosian et al., 1999). FOG2 functionally complements FOG1 in at least some aspects (even though the

primary sequence is quite divergent) as FOG2 can rescue terminal erythroid differentiation in *Fog1*$^{-/-}$ hematopoietic cells (Tevosian et al., 1999).

Similar to GATA-1, the basic helix-loop-helix protein Stem Cell Leukemia (SCL) is necessary for proper maturation of erythroid and megakaryocytic precursors (Porcher et al., 1999). SCL also binds as a complex with LMO2 and its interacting protein GATA-1 on composite E-box-GATA DNA binding sites (Wadman et al., 1997). SCL, LMO2, and GATA-1 together induce the erythroid program in naïve ectoderm of *Xenopus* embryos (Mead et al., 2001). This was also shown in hematopoietic cells where SCL acts to assemble this complex and GATA-1 functions as a DNA tether to activate the transcription of the erythroid marker *glycophorin A* (Lahlil et al., 2004). Interestingly, a recent study has shown that SCL is bound to GATA-1 when it functions to activate transcription, yet is depleted when GATA-1 functions as a transcriptional repressor (Tripic et al., 2009).

GATA-3

Using a mouse GATA-1 cDNA as a probe, GATA-2 and GATA-3 cDNAs were discovered as homologues in the chicken (Yamamoto et al., 1990), mouse and human (Ko et al. 1991) genomes. GATA-3 is highly conserved across species and functions most clearly to regulate T-cell differentiation (Ko et al., 1991). However, GATA-3 expression is also observed in the placenta, definitive erythrocytes, kidney, adrenal gland, central and peripheral nervous systems, the embryonic liver, the skin, in hair follicles and in breast epithelial cells as well as in T-lymphocytes, and in several cases has been shown to be required for specific aspects of their organogenesis (George et al., 1994; Kornhauser et al., 1994; Yamamoto et al., 1990). GATA-3 is capable of binding to GATA sites in an enhancer of *CD8*, potentially augmenting the formation of DNase I hypersensitive sites (Landry et al., 1993). GATA-3 also has broader DNA recognition properties than GATA-1, due in part to its ability to bind GATC sequences as well as GATA (Ko and Engel, 1993). Distinct regulatory elements of *Gata3* have been discovered for early muscle mass, a subset of PNS neurons, the genital tubercle, branchial arches, and endocardium, which helps to explain the ability of GATA factors to

contribute to distinct temporal and spatial patterns of cellular differentiation (Lakshmanan et al., 1999; Lieuw et al., 1997).

Gata3 heterozygous mutant mice are normal, but null mutants die between e11 and e12 from hemorrhage, growth retardation, brain and spinal cord deformities, and irregularities in fetal liver hematopoiesis (Pandolfi et al., 1995). The specific cause of mortality was found to be a neuroendocrine deficiency, with reduced levels of tyrosine hydroxylase and dopamine β-hydroxylase in the sympathetic nervous system. Feeding catechol intermediates to pregnant dams was able to significantly prolong the life of *Gata3* null animals, revealing new deficits including renal hypoplasia and defects in cephalic neural crest cell contributions (Lim et al., 2000). However, the pharmacologically rescued mutants still succumbed to embyronic lethality, most likely from cardiac developmental abnormalities (Lim et al., 2000). Specific transgenic GATA-3 expression in sympathoadrenal (SA) lineages was also capable of rescuing SA cells, with induced expression of Mash1, Hand2, and Phox2b, which are believed to create a mutually reinforcing transcriptional network (Moriguchi et al., 2006). GATA-3 haplo-insufficiency has also been found to be the causal mutation in human HDR syndrome (Hypoparathyroidism, sensorineural Deafness, and Renal dysplasia). Both nonsense mutations and intragenic deletions of GATA-3 have resulted in this DiGeorge-like syndrome, resulting in hypoparathyroidism, deafness, renal and craniofacial abnormalities (Van Esch et al., 2000).

Within the kidney, the loss of GATA-3 results in a failure of the nephric duct to extend into or augment the differentiation of the metanephric mesenchyme (Lim et al., 2000). An enhancer directing GATA-3 in the kidney was isolated and used to create transgenic mice, which were then used to complement the *Gata3* null mutation. From these studies, it was found that at least 50% of endogenous GATA-3 protein abundance is required for normal nephrogenesis. Less than 50% results in localized kidney defects similar to those encountered in HDR syndrome (Hasegawa et al., 2007). Recently, a piece of the genetic cascade regulating these events has been resolved. β-catenin appears to regulate GATA-3 to prevent premature cell differentiation in the nephric duct. Independent of this activity, GATA-3 also positively regulates Ret expression.

Conditional knockout of GATA-3 specifically in nephric duct cells reduces Ret expression and results in ectopic ureter budding (Grote et al., 2008).

Within hematopoietic lineages, GATA-3 is primarily restricted to thymocytes and T cells. GATA-3 binding sites in multiple T cell specific genes have been functionally identified (Hambor et al., 1993; Ho et al., 1991; Ko et al., 1991; Marine and Winoto, 1991). Mutation of *Gata3* in embryonic stem cells has revealed that T cells are blocked at or before the formation of double negative (CD4-/CD8-) thymocytes, indicating that GATA-3 is required for early thymocyte development (Ting et al., 1996), including the expansion of T cell progenitors (Hendriks et al., 1999). Further characterization of GATA-3's role in the thymus has found that it is necessary for β selection and single CD4-positive thymocyte development (Pai et al., 2003). Finally, GATA-3 is required for the development and maintenance of T helper type 2 (Th2) cells, but not Th1 cells (Pai et al., 2004; Zhang et al., 1997). This occurs through two mechanisms: reduced Th1 development through IL-12 receptor repression and positive GATA-3 feedback autoregulation (Zhou and Ouyang, 2003). A signal transducer of the transforming growth factor β (TGFβ) family, phosphorylated Smad3 can bind GATA-3 to cooperatively activate both *IL-5* and *IL-10* (Blokzijl et al., 2002). This is one of the first studies to demonstrate the process by which extracellular signals can affect nuclear programming. Acetylation of GATA-3 is also critical for its function, as a hypo-acetylated mutant results in prolonged survival after activation and defective T cell homing to systemic lymph nodes (Yamagata et al., 2000).

Similar to GATA-1 expression in the testis, GATA-3 contains an alternative promoter that regulates expression specifically in the brain (Asnagli et al., 2002). These two GATA-3 isoforms have also been shown to have differential activity through different stages of Th2 development, indicating a potentially unique role for each isoform in this process (Asnagli et al., 2002). In the developing brain, GATA-3 is critical for the development and locomotor function of serotonergic neurons (van Doorninck et al., 1999). GATA-3 is also downstream of GATA-2 and Hoxb1 in ventral rhombomere 4 supporting the migration of facial branchiomotor neurons and projection of contralateral vestibuloacoustic (CVA) efferent neurons (Pata et al., 1999).

Recently, GATA-3 has also been implicated in the maintenance of luminal epithelial differentiation in the adult mammary gland. *Gata3*, when conditionally deleted specifically from mammary cells in mice, fails to properly form terminal end buds resulting in mammary developmental defects (Kouros-Mehr et al., 2006). Loss of *Gata3* results in early breast cancer carcinoma progression along with tumor propagation (Kouros-Mehr et al., 2008).

GATA-4

Discovered through an embryonic mouse library screen using a probe to the highly conserved *Gata1* zinc finger domain, GATA-4 is expressed in the heart, intestinal epithelium, primitive endoderm, and gonads (Arceci et al., 1993; Kelley et al., 1993). GATA-4's role in cardiac development was enhanced through regulation of *cardiac muscle-specific troponin C* (cTnC) (Ip et al., 1994), *cardiac actin* (Jiang and Evans, 1996)*, atrial natriuretic factor* (Grépin et al., 1994; Morin et al., 2001), *brain natriuretic factor* (Grépin et al., 1994; Thuerauf et al., 1994), *myosin heavy chain* α (Jiang and Evans, 1996; Molkentin et al., 1994), and *Nkx2.5* (Lien et al., 1999). A role for GATA-4 in heart development was further verified through a chromosomal deletion containing the *Gata4* gene that results in congenital heart disease (Bhatia et al., 1999; Pehlivan et al., 1999). A missense mutation in the *Gata4* gene, preempting GATA-4 binding with the T-box protein TBX5, implicated GATA-4 in cardiac septal defects (Garg et al., 2003).

Gata4 null mutant mice die between e7.0 and e10.5 (Kuo et al., 1997; Molkentin et al., 1997). These mice develop partially outside of the yolk sac, suffer from cardia bifida, and contain disorganized foregut and anterior intestinal portal. These deformities indicate a role for GATA-4 in the caudal and ventral folding of the embryo (Kuo et al., 1997; Molkentin et al., 1997). In embryonic stem cells, forced expression of GATA-4 can induce differentiation of extraembryonic endoderm (Fujikura et al., 2002).

The zinc finger protein FOG-2 has been found to interact with GATA-4 to cause both synergistic activation and repression of GATA-dependent promoters depending on the target and cell type involved (Lu et al., 1999). *Fog2* null mice lack coronary vasculature, though cross regulation is not expected, as GATA-4 expression is normal in these mice (Tevosian et al., 2000). FOG-2 and GATA-4 are also required for normal

gonadal differentiation. Without the physical interaction between FOG-2 and GATA-4, testis cords fail to develop and genes required for Sertoli cell function, *Sox 9, Mis* and *Dhh,* are not expressed, indicating a role for both FOG-2 and GATA-4 in sex determination (Tevosian et al., 2002). Recently, developmental defects in ovarian development were also found to rely on the interaction of GATA-4 to FOG-2 (Manuylov et al., 2008).

GATA-4 also interacts with the GTPase RhoA to induce sarcomere reorganization (Charron et al., 2001). During myocardial cell hypertrophy, the kinase ROCK-1 is involved in GATA-4-dependent *ET-1* transcription, which is abrogated in the absence of ROCK-1 (Yanazume et al., 2002). Recently, a nonclassical nuclear localization signal of GATA-4 has been discovered that can actively transport GATA-4 to the nucleus of cardiac myocytes. Importin β interacts with four arginine residues in this region to mediate transport. These residues were also found to be important for DNA binding and cardiac-specific gene activation of GATA-4 (Philips et al., 2007).

Bone Morphogenetic Proteins (BMPs) regulate the septum transversum mesenchyme to induce liver genes and exclude a pancreatic fate in the endoderm, at least partially through elevating the protein levels of GATA-4 (Nemer and Nemer, 2003; Rossi et al., 2001). GATA-4 binds an enhancer of the albumin gene, seemingly even within compacted chromatin, indicating that early transcription factors are capable of accessing and opening local chromatin structures (Cirillo et al., 2002). GATA-4 also binds to the erythropoietin promoter in hepatocytes, with RNAi of GATA-4 resulting in reduced Epo transcription in Hep3B cells (Dame et al., 2004). Epo expression transitions from the fetal liver to the adult kidney during development. While GATA-4 is highly expressed in hepatocytes of the fetal liver, it is low in the adult liver, indicating a potential role for GATA-4 in the transition of Epo from the fetal liver to the adult kidney (Dame et al., 2004).

GATA-4 expression has also recently been reported in the developing adrenal gland, more specifically in the less differentiated proliferating cells of the fetal cortex (Parviainen et al., 2007). GATA-4 has also been shown to regulate the expression of genes associated with normal adrenal function, namely *inhibin-α* and *steroidogenic factor-1* (Tremblay and Viger, 2003). Gonadotropin dependent mouse adrenocortical

tumors also contain higher levels of GATA-4 than does wild type cortex. It has been shown that gonadotropins augment the expression of GATA-4 (Looyenga and Hammer, 2006), which in turn transactivates luteinizing hormone receptor (Rahman et al., 2004).

GATA-5

GATA-5 was originally discovered in *Xenopus* (Kelley et al., 1993). It is expressed in the cardiac crescent before the heart tube is formed (Laverriere et al., 1994). GATA-5 is then expressed in lateral plate derivatives, including the endocardium and myocardium. It is also found in the primitive embryonic gut, and at later stages in specific regions of gastrointestinal epithelia, along with the spleen (Laverriere et al., 1994). Murine GATA-5 is expressed in the precardiac mesoderm of the primitive streak, and is found early in heart development. GATA-5 is expressed in both atrial and ventricular chambers at e9.5, just the atrial endocardium by e12.5, and not at all at later stages (Morrisey et al., 1997). GATA-5 is also uniquely expressed in the pulmonary mesenchyme and restricted subsets of smooth muscle cells in the bladder (Morrisey et al., 1997).

GATA-5 is required for myocardial precursors to expand properly (Reiter et al., 1999). Cardiac primordial migration to the embryonic midline and morphogenesis of the endoderm also requires proper GATA-5 expression (Reiter et al., 1999). This occurs at least in part through GATA-5 regulation of the cardiac-specific factor *Nkx2.5* (Reiter et al., 1999). Endodermal patterning has been found to require nodal signaling, which induces GATA-5 expression along with the homeobox transcription factor Mixer, in turn regulating a Sox-like factor to stimulate endodermal genes (Patient and McGhee, 2002).

GATA-5 is the only GATA family transcription factor not absolutely required for embryonic development, as null mutations in all other family members incorporated into the germ line of mice result in embryonic lethality. *Gata5* null mutant mice are viable and fertile. Female *Gata5* null mice contained vaginal and uterine defects, along with hypospadias, whereas males showed no genitourinary abnormalities. The female urogenital developmental deficiency was determined to be a defect in partitioning of the urogenital sinus (Molkentin et al., 2000).

GATA-6

A cDNA clone originally identified in chickens denoted GATA-6 was identified and found to be distinct from other GATA factor members (Laverriere et al., 1994). This protein is highly expressed in liver, lung, stomach, and ovary, and to a lesser extent in the intestines and heart (Laverriere et al., 1994). The murine homologue binds to the *cardiac-specific troponin C* gene (*cTnC*). It is expressed in the precardiac mesoderm, the embryonic heart tube, the primitive gut, the developing bronchi, arterial smooth muscle cells, and the urogenital ridge and bladder (Morrisey et al., 1996). The human homologue has a similar expression profile (Suzuki et al., 1996).

GATA-6 has a broader DNA binding sequence specificity than other GATA family members (except GATA-3), with the consensus GATA sequence still being the highest affinity, followed by GATT and then GATC; the core motif is (A/T)GATA(A/G), but GATA-6 binds the tightest when flanked on both sides by adenines (Sakai et al., 1998).

GATA-6 is the GATA family member that is required first in embryonic development, as *Gata6* null mutant mice die between e6.5 and e7.5 from defects in the differentiation of visceral endoderm; expression of a number of endodermal markers, including α-fetoprotein, GATA-4, HNF3β, and HNF4 were all greatly reduced or altogether absent in *Gata6* null mutants (Koutsourakis et al., 1999; Morrisey et al., 1998). Analysis of chimeras revealed that GATA-6 is only required for the branching and differentiation of epithelial cells, not the process of endoderm specification itself (Keijzer et al., 2001).

GATA-6 is upregulated by tumor necrosis factor-α, which enhances binding and transcriptional activation of vascular cell adhesion molecule-1, which functions in inflammatory response (Umetani et al., 2001). Furthermore, NKX3.2, serum response factor, and GATA-6 form a complex to transactivate the smooth muscle genes α_1 *integrin, SM22α,* and *caldesmon* (Nishida et al., 2002); this process appears to be at least partially activated by statins (Wada et al., 2008). GATA-6 also plays a role in maintaining vascular smooth muscle quiescence (Perlman et al., 1998); it is down regulated upon arterial injury to promote vessel lesion formation (Mano et al., 1999).

Further clarification of a role for GATA-6 in vascular smooth muscle differentiation showed that it could displace myocardin from the serum-response factor to repress telokin promoter activity; at the same time, GATA-6 activates *smooth muscle myosin heavy chain* and *smooth muscle actin alpha-actin* promoters (Yin and Herring, 2005).

The adrenal gland also appears to require the expression of GATA-6 (Kiiveri et al., 2002), particularly for developmental changes and corticosteroid production in the zona fasciculata (Nakamura et al., 2007). Interestingly, *Gata6* appears to be dispensable for early stages of megakaryocyte differentiation, but is specifically up regulated in late stage megakaryocytes, potentially cooperating with Elf-1 to induce expression of megakaryocyte-specific genes (Dumon et al., 2006).

Given its early role in extraembryonic endoderm development, it has been difficult to study later roles of GATA-6 *in vivo;* to circumvent this problem, a conditional knockout of *Gata6* has been created with *LoxP* sites flanking exon 2 of the *Gata6* gene (Sodhi et al., 2006). The efficacy of these conditional mice was tested through a cross to mice expressing Cre from the *Villin* promoter, which is specifically expressed in the intestine. The homozygous conditional mice had significantly reduced levels of GATA-6, indicating that this conditional knock-out mouse should prove to be a valuable tool in addressing the role of GATA-6 during later developmental stages (Sodhi et al., 2006).

GATA-2

Gata2, the principal focus of these studies, was originally isolated as a chicken cDNA clone by virtue of its homology to *Gata1* (Yamamoto et al., 1990). GATA-2 expression was found in all developmental stage erythroid cells, along with embryonic brain, liver, cardiac muscle, adult kidney and fibroblasts (Yamamoto et al., 1990). The human homologue was discovered soon thereafter and found to regulate endothelin-1 (Kawana et al., 1995; Lee et al., 2001). Other early studies found GATA-2 to be important for regulation of endothelial cells (Dorfman et al., 1992; Lee et al., 2001), in early – but not mature – erythroid cells (Leonard et al., 1993), mast cells (Zon et al., 1991), megakaryocytes (Visvader and Adams, 1993), eosinophils (Zon et al., 1993), basophils (Zon et al., 1993), neutrophils (Zon et al., 1993), hematopoietic stem cells (Orlic et al., 1995) in the AGM region of the developing embryo (Minegishi et al., 1999),

placental trophoblasts (Ma et al., 1997; Ng et al., 1994), gonadotropes and thyrotropes of the pituitary gland (Charles et al., 2006; Dasen et al., 1999), urogenital development (Zhou et al., 1998), adipocyte differentiation (Okitsu et al., 2007; Tong et al., 2005), V2 interneurons (Zhou et al., 2000), the central nervous system (Nardelli et al., 1999), vascular endothelium (Wozniak et al., 2007), and serotonergic neurons of the hindbrain (Craven et al., 2004; Krueger and Deneris, 2008).

Gata2 null mutant embryos die at e10.5 from a failure of primitive and definitive hematopoiesis (Tsai et al., 1994). *In vitro* studies have shown that GATA-2 is required for survival and proliferation of hematopoietic progenitors (Briegel et al., 1993), possibly through regulation by Evi-1 and repression of TGF-β signaling (Sato et al., 2008). GATA-2 is also required for the production of mast cells; however, it is not required for the terminal differentiation of macrophages or erythroid cells (Tsai and Orkin, 1997). *Gata2* haploinsufficiency studies reveal that GATA-2 is required for the production and proliferation of hematopoietic stem cells (HSC) in the aorta-gonad-mesonephros region of the embryo, as well as propagation of HSCs in adult bone marrow (Ling et al., 2004). BMP-4 stimulates GATA-2 expression in the ventral mesoderm (Maeno et al., 1996). The threshold of GATA-2 expression plays a role in self-renewal *versus* differentiation of hematopoietic progenitors (Heyworth et al., 1999; Persons et al., 1999). This threshold appears to be at least partially regulated by Notch1, which maintains GATA-2 expression allowing it to sustain the undifferentiated state of hematopoietic progenitors (Kumano et al., 2001; Minegishi et al., 2003). GATA-2 can maintain quiescence by inhibiting the cell cycle of these progenitors (Tipping et al., 2009).

The ability of GATA-2 to undergo a broader array of regulatory functions than GATA-1 may stem at least partially from its ability to bind to a consensus GATC sequence (Ko and Engel, 1993). This binding may occur through the N-terminal finger, as opposed to the traditional binding by the C-terminal finger; this binding is stabilized by two basic regions on either side of the amino finger, one of which is lacking in GATA-1 (Pedone et al., 1997).

GATA-2 transcription, like almost all GATA factor family members, is regulated by alternate promoters (Minegishi et al., 1998). The downstream 1G (general) promoter sequence is similar to the promoters described for *Xenopus* and human, but the upstream

1S (specific) promoter shows unique expression in hematopoietic progenitor cells and neural tissues (Minegishi et al., 1998; Pan et al., 2000). A knock-in reporter driving GFP expression from the *Gata2* 1S promoter was used to evaluate hematopoietic stem cell function in the bone marrow (Suzuki et al., 2006). From these studies HSCs appear to be solitary and maintain intimate contact with osteoblasts that characterize the HSC niche (Suzuki et al., 2006). Another interesting observation is that, even though GATA-2 expression driven from the 1S promoter marks putative hematopoietic/endothelial precursor cells (Kobayashi-Osaki et al., 2005), no hematopoietic deficiency was observed when 1S promoter activity was abrogated, indicating that transcription from the 1G promoter can fully complement deficient 1S activity (Suzuki et al., 2006).

GATA-2 is required in neurogenesis for the generation of V2 interneurons (Zhou et al., 2000). This expression was localized to an enhancer in intron 5 of the *Gata2* locus (Zhou et al., 2000). GATA-2 is necessary for the proper expression of GATA-3 in the central nervous system (Nardelli et al., 1999; Pata et al., 1999). A developmental cascade involving GATA-2 has also recently been deduced for the differentiation of serotonergic neurons in the hindbrain. Sonic hedgehog activates the homeodomain proteins Nkx2.2 and Nkx6.1. These proteins then cooperate to activate GATA-2, which goes on to stimulate the expression of the transcription factors *Lmx1b*, *Pet1*, and *Gata3*. This process induces serotonergic neuron production within rhombomere 1 of the hindbrain (Craven et al., 2004). GATA-2 also regulates the ETS transcription factor *fifth Ewing variant* (FEV) in serotonergic neurons (Krueger and Deneris, 2008).

In attempting to rescue *Gata2* null embryonic lethality, a YAC transgene was found that encompasses two hematopoietic enhancers located 100 to 150 kilobase pairs upstream of the *Gata2* structural gene. Expression of the YAC in a *Gata2* null mutant background was able to rescue hematopoiesis; however, these mice then die perinatally from complications in urogenital development including hydroureternephrosis (Zhou et al., 1998). Following the development of a novel BAC-mapping strategy, two distinct urogenital enhancers were identified (Khandekar et al., 2004). One enhancer is expressed specifically in the mesenchyme surrounding the nephric duct along with the mesonephric mesenchyme; the second regulates *Gata2* expression in the mesenchyme around the ureteric bud along with the urogenital sinus (Khandekar et al., 2004). A third urogenital

enhancer was deduced to exist, but not specifically localized, as the *Gata2-GFP* reporter mice showed expression in the ureteric epithelium, but neither of the isolated urogenital enhancers conveyed expression in this tissue (Khandekar et al., 2004). A piece of this transcriptional pathway has recently been resolved: a GATA-2 hypomorph reduces the expression level of BMP-4 resulting in abnormalities resembling human congenital anomalies of the kidney and urinary tract (CAKUT) (Hoshino et al., 2008).

GATA-2 only binds to a small subset of the composite GATA sequence elements that are available in the genome. Recently, a set of unique molecular hallmarks demarcating these sites from unoccupied sites has been elucidated, including a distinct epigenetic signature, specific neighboring *cis* elements, and binding by the transcription factor SCL (Wozniak et al., 2008).

Deregulation of GATA-2 has been linked to acute myeloid leukemia through analysis of 3q21 aberrations (Wieser et al., 2000). Concurrently, GATA-2 has been shown to complex with promyelocytic leukemia protein to modify the transactivation capacity of GATA-2 (Tsuzuki and Enver, 2002; Tsuzuki et al., 2000). Recently, GATA-2 has also been implicated in atherosclerosis (Seo et al., 2004), early-onset coronary artery disease (Connelly 2006), chronic myelogenous leukemia (Zhang et al., 2008), and Parkinson's disease (Scherzer et al., 2008).

Interplay between GATA Factors

While each of the GATA factor family members play distinct roles in embryonic development, they also interact and functionally complement each other in multiple developmental processes. GATA-1 was proposed to repress GATA-2 in erythroid maturation soon after their discovery (Weiss et al., 1994). Given their role in hematopoiesis, one of the first combinatorial studies focused on elucidating the phenotype of GATA-1/2 double knockouts. Double heterozygote mice die at mid-gestation from anemia. In the double knockout, the yolk sac vascular network appeared to be intact, but no circulating cells were observed. This severe phenotype indicates that GATA-1 and GATA-2 functionally overlap at the earliest stages of primitive hematopoiesis; the *Gata2* null phenotype predominates in definitive hematopoiesis, as the

phenotype of the double knockout resembles that of the *Gata2* null (Fujiwara et al., 2004).

GATA-2 appears to positively autoregulate the *Gata2* gene during hematopoietic development specifically through an enhancer located 2.8 kbp upstream of the *Gata2* 1S promoter, even though there are dozens of other GATA consensus sites in the region (Grass et al., 2003). GATA-1 then displaces GATA-2 specifically at this enhancer, resulting in reduced acetylation to generate a repressive chromatin state (Grass et al., 2003). This process has been termed a "GATA switch" (Grass et al., 2003), and appears to require the coregulator Friend of GATA-1 (FOG-1) for the GATA switch to occur by facilitating chromatin occupancy with GATA-1 (Pal et al., 2004a). A similar GATA switch occurs at other locations in the *Gata2* locus (Grass et al., 2006; Martowicz et al., 2005), the *α-globin* promoter (Anguita et al., 2004), and *neurokinin-B* promoter (Pal et al., 2004b).

Interestingly, GATA-1 requires FOG-1 to displace GATA-2 in the *Gata2* -2.8 kbp enhancer to promote erythroid differentiation, but GATA-1 must act independently of FOG-1 to promote mast cell maturation (Cantor et al., 2008). Forced expression of FOG-1 in mast cell progenitors actually redirects them to other hematopoietic lineages, implicating FOG-1 as a negative regulator of the mast cell fate (Cantor et al., 2008). GATA-1 has also been shown to enhance histone acetylation in other settings, together with CBP (Letting et al., 2003), indicating that GATA factor function relies on specific cofactor binding to elicit the desired chromatin state.

Recently, a similar GATA switch has been reported between GATA-2 and GATA-3. GATA-3, in association with FOG-1, directly represses *Gata2* in undifferentiated trophoblast cells. GATA-2 then displaces GATA-3 and FOG-1 at two separate regions of the mouse *Gata2* locus (-3.9 and +9.5 kbp) to initiate trophoblast giant-cell specific differentiation (Ray et al., 2009). It is also interesting that regulatory elements at -1.8, -2.8, and -77 kbp, known to be important for hematopoietic regulation, are not occupied by GATA factors in trophoblast cells, presumably through sequestration in repressed chromatin structure (Ray et al., 2009).

GATA-4 and GATA-6 have a similar relationship to GATA-1 and GATA-2. While they overlap or synergize expression in some tissues, such as cardiac myocytes

(Charron et al., 1999; Kuo et al., 1997; Molkentin et al., 1997), it has been proposed that GATA-6 confers positive regulation on GATA-4, while GATA-4 acts in a negative feedback manner (Morrisey et al., 1998). The abundance of each transcription factor has also been shown to be important for multiple processes. An interesting study recently found that while mice heterozygous for a *Gata4* or *Gata6* null allele are phenotypically normal, compound heterozygotes are embryonic lethal from an array of cardiovascular defects (Xin et al., 2006). In contrast, in the mouse pancreas GATA-4 expression seems to be restricted to exocrine pancreatic differentiation, while GATA-6 is directed to the endocrine pancreas (Ketola et al., 2004). It will be interesting to uncover the mechanism behind their restricted expression domains, and if it mimics the GATA switch of the hematopoietic factors.

Hemangioblasts, Hematopoiesis, and Vasculogenesis

A hypothesis put forth at the beginning of the 20th century was that since endothelial and hematopoietic lineages arise from the same location at the same time, they might arise from a common progenitor -- a cell termed the "hemangioblast" (Murray, 1932; Sabin, 1920). More recent evidence to support this idea is the identification of proteins that are expressed in both of these cell types, including CD34 (Young et al., 1995), Flk-1 (Kabrun et al., 1997; Millauer et al., 1993; Yamaguchi et al., 1993), Flt-1 (Fong et al., 1996), TIE-2 (Takakura et al., 1998), SCL (Kallianpur et al., 1994), GATA-2 (Orkin, 1992), and PECAM-1 (Watt et al., 1995). Since then, a few of these proteins have been shown to play physiologically important roles in both hematopoietic and endothelial cell development, including cloche (Stainier et al., 1995), Flk-1 (Eichmann et al., 1997; Shalaby et al., 1995), and SCL (Visvader et al., 1998).

Embryonic stem cell derived embryoid bodies have the capacity to produce both hematopoietic as well as endothelial cells. Mixing studies demonstrate that both of these cell types arise from the same cell – termed the blast colony-forming cell (BL-CFC) (Choi et al., 1998). This BL-CFC is believed to be the *in vitro* equivalent of the hemangioblast. A cell with similar properties has been isolated from the primitive streak of e7.0 mouse embryos (Huber et al., 2004).

The site of origin for the primitive hemangioblast was found to be the embryonic blood islands of the yolk sac (Sabin, 1920). Peripheral endothelial precursors (ie – angioblasts) surround hematopoietic precursors (Risau and Flamme, 1995). For some time, hematopoietic stem cells arising from these yolk sac blood islands were thought to colonize hematopoietic organ rudiments including the fetal liver, spleen, and bone marrow (Moore and Owen, 1965). Quail/chick grafting experiments showed that while the yolk sac was capable of generating a transient wave of hematopoiesis, the hematopoietic organs were seeded principally by embryo-derived hematopoietic stem cells (Beaupain et al., 1979; Dieterlen-Lievre, 1975; Martin et al., 1978). However, recent lineage tracing studies have found that yolk sac hematopoiesis may still contribute a small percentage of hematopoietic stem cells to hematopoietic organ rudiments (Samokhvalov et al., 2007).

Vasculogenesis is defined as the differentiation of angioblasts from the mesoderm and the subsequent amassing of these angioblasts into blood vessels (Risau and Flamme, 1995). The first intraembryonic angioblasts arise adjacent to the endoderm (Coffin and Poole, 1988). Some angioblasts then fuse with other angioblasts *in situ* to form vessels; others migrate as single cells or clusters and incorporate into developing vessels or form new vessels at remote locations (Coffin and Poole, 1988; Poole and Coffin, 1989). Capillaries then sprout off of these primary vessels in a process termed angiogenesis to form the mature vasculature (Folkman and Shing, 1992). The first known molecular marker expressed on angioblasts is Flk-1 (Millauer et al., 1993). *Flk1* null mutant mice can form some endothelial precursors cells, but they never aggregate into mature vessels (Shalaby et al., 1995). Intraembryonic vasculogenesis originates in the embryo proper at e7.3, where Flk1 is already strongly expressed (Drake and Fleming, 2000). By e9.0, the dorsal aorta, intersomitic vessels and the intervertebral arteries are already formed (Drake and Fleming, 2000).

The origin of definitive hematopoietic stem cells must reside within the embryo proper, possibly arising from a definitive form of the hemangioblast. Early studies noted that putative hematopoietic cells have been seen lining the ventral wall of the dorsal aorta endothelium (Jordan, 1917). These cells take up basophilic stain and express hematopoietic markers (Dieterlen-Lièvre and Martin, 1981; Jaffredo et al., 1998;

Pardanaud et al., 1987). Further studies demonstrated that the aorta-gonad-mesonephros (AGM) region of the developing mouse embryo is the site of definitive hematopoietic progenitor generation (Cormier, 1993; Godin et al., 1993; Medvinsky et al., 1993). Hematopoietic stem cells (HSC) first emerge from the AGM at e10.5 of development (Medvinsky and Dzierzak, 1996) along with contributions from the vitelline and umbilical arteries (de Bruijn et al., 2000). Definitive HSCs can be found in the yolk sac one day later at e11.5 (Medvinsky and Dzierzak, 1996; Müller et al., 1994).

Since HSCs are first generated in the AGM, the next question is whether they are derived directly from hemangioblasts, from "hemogenic endothelium", or from some other undefined mesodermal progenitor (Dzierzak et al., 1998; Orkin, 2000). Hemogenic endothelium is defined as specified endothelial cells that can give rise to hematopoietic cells (Jaffredo et al., 2005). Based on the expression of a *Sca1*-directed GFP reporter gene in mice, HSCs do not appear in the mesenchyme, but rather localize specifically to the endothelium lining the floor of the dorsal aorta (de Bruijn et al., 2002). Accordingly, DiI-conjugated acetylated low-density lipoprotein (LDL) tagged endothelial cells were able to give rise to DiI labeled circulating erythroid cells (Sugiyama et al., 2003). Mice haploinsufficient for *Runx1*, a transcription factor that is required for definitive HSC generation, labeled HSCs in the hematopoietic clusters, the endothelium, and in the underlying mesenchymal cell population, indicating that HSC precursors may reside in the mesenchyme in an undifferentiated state (North et al., 2002). Recent studies may have reconciled these events, as the primary support for a direct hemangioblast model came from BL-CFC's *in vitro* directly giving rise to hematopoietic and endothelial cells. Lancrin *et al* have recently shown that BL-CFC's actually give rise to a transiently adherent cell population, the hemogenic endothelium equivalent, before quickly differentiating into distinct hematopoietic and endothelial populations (Lancrin et al., 2009). Also, separate labelling of the endothelium versus its underlying mesenchyme has shown that hematopoietic cells specifically and uniquely arise from the endothelial population (Zovein et al., 2008).

Hematopoietic clusters are generally localized to the ventral floor of the dorsal aorta (Jaffredo, 1999). A potential explanation for this has been elucidated through grafting experiments in avian embryos. The roof and walls of the dorsal aorta are derived

from paraxial mesoderm, while the floor is derived from the splanchnopleural mesoderm (Pardanaud et al., 1996). These splanchnopleural cells are capable of giving rise to hematopoietic cells, but only in the proper microenvironment (Pardanaud et al., 1996). While one factor, Glypican-1, has recently been shown to support hematopoietic development (Nagao et al., 2008), three hematopoietic transcription factors in particular have been shown to play crucial roles in hematopoietic stem cell formation – SCL, GATA-2, and RUNX1 (Jaffredo et al., 2005). GATA-2's role in hematopoietic development has been described above.

SCL (also known as TAL-1) is a helix-loop-helix protein that was originally identified through its participation in a chromosomal translocation that results in stem cell leukemia, from which the protein garners its name (Begley et al., 1989). *Scl* null mutant mice suffer from hematopoietic failure and die around e9.5 (Robb et al., 1995). Studies of *Scl* mutant embryonic stem cells in chimeric mice show that SCL is required for the development of all hematopoietic lineages (Porcher et al., 1996). An enhancer of *Scl* was uncovered that was found to bind GATA-2, Fli-1, and Elf-1 *in vivo*, uncovering part of the regulatory network in hematopoietic development (Göttgens et al., 2002). Expression of Flk-1 and SCL enrich the hemangioblast population in cell culture studies (Chung et al., 2002). GATA-2 appears to stimulate the regulation of SCL, inducing Flk-1 positive mesoderm to become $Flk1^{+}SCL^{+}$ hemangioblasts, and then rapidly differentiate into hematopoietic or endothelial cells (Lugus et al., 2007). SCL has been found to be necessary for angiogenic remodeling of the capillary network of the yolk sac (Visvader et al., 1998), and is expressed at sites of neovascularization and lymphangiogenesis (Tang et al., 2006).

Runx1 (also known as *Cbfa2* and *Aml1*), a member of the core binding factor family, was originally discovered from a translocation resulting in acute myeloid leukemia (Miyoshi et al., 1991). *Runx1* null mutant animals die around e12.5 from hemorrhaging in the central nervous system (Wang et al., 1996) and a failure to initiate definitive hematopoiesis (Okuda et al., 1996). While definitive hematopoiesis is abrogated, primitive hematopoiesis is not affected (Lacaud et al., 2002; Okuda et al., 1996). RUNX1 protein expression is restricted to the ventral aspect of the dorsal aorta and intra-aortic hematopoietic clusters are not formed in its absence (North et al., 1999).

Haploinsufficiency of *Runx1* results in alteration of the temporal and spatial emergence of hematopoietic stem cells (Cai et al., 2000; Lacaud et al., 2004). Therefore, it is believed that RUNX1 functions during the transition from hemogenic endothelium to hematopoietic stem cells (North et al., 1999; Yokomizo et al., 2001). Part of the mechanism behind this transition may involve the downregulation of *Flk1* through direct repression by Runx1, as demonstrated in embryonic stem cell differentiation cultures (Hirai et al., 2005). *Runx1* contains an enhancer that binds GATA-2, Ets proteins, and SCL to initiate the generation of hematopoietic stem cells (Nottingham et al., 2007). Recent conditional deletion studies have shown that RUNX1 is required for the differentiation of hematopoietic cells from the endothelium, but is not required once these cells express Vav1 – an early HSC specific pan-hematopoietic gene (Chen et al., 2009). Another study has proposed that the hemogenic endothelium is $Tie2^{hi}$c-$kit^{+}CD41^{-}$, and cannot reach this stage without functional SCL; differentiation of hemogenic endothelium to HSCs then requires RUNX1 repression of Tie2, allowing CD41, another early hematopoietic marker, expression to increase (Lancrin et al., 2009).

While a distinct process from the epithelial-to-mesenchymal cell transition (EMT), the budding of hematopoietic cells from the dorsal aorta is a similar progression, which may implicate a number of other factors in this development process as well. Key inducers of EMT include the bHLH transcription factor *Twist* along with members of the Snail superfamily, which function as transcriptional repressors of *E-cadherin* and components of tight junctions (Moreno-Bueno et al., 2008).

The Role of GATA-2 in Development

The developmental role and patterning of the GATA family of transcription factors are quite diverse. Numerous individual enhancer elements have been localized for *Gata2* alone, including two hematopoietic (Zhou et al., 1998), one endothelial (Chapter 2; (Wozniak et al., 2007), two central nervous system (Zhou et al., 2000), and three urogenital enhancers (Khandekar et al., 2004), as well as the two *Gata2* promoters (Minegishi et al., 1998). This is without the other regulatory elements that deductively must exist given the involvement of GATA-2 in many other developmental events, such

as the generation of placental trophoblasts, adipose tissue, and pituitary specification (Dasen et al., 1999; Ma et al., 1997; Tong et al., 2000).

In this work, I have sought to elucidate mechanisms that regulate *Gata2* and to determine the role that GATA-2 plays in specific aspects of mammalian development. Chapter 2 describes the isolation and characterization of an endothelial enhancer that was first localized within *Gata2* intron 4. In Chapter 3, we intended to generate and then test whether a linked BAC would encompass all of the necessary regulatory elements required for embryonic viability in the *Gata2* locus, or to hypothetically reveal new essential roles for GATA-2 in development. In Chapter 4, I describe my most recent studies examining the role of GATA-2 in the differentiation of the definitive hemangioblast and hemogenic endothelium. Through understanding the *cis* regulatory elements and *trans* acting factors governing *Gata2* expression and its role in various developmental processes, we can begin to understand the functional networks governing development, and thereby provide clues as to how we might repair them when they go awry.

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Chapter 2

A *Gata2* Intronic Enhancer Confers its Pan-Endothelia-Specific Regulation

Introduction

The early murine blood vasculature develops from angioblasts associated with blood islands of the yolk sac. This process, known as vasculogenesis, results in the formation of the initial vascular network (Pardanaud et al., 1987), which consists of paired dorsal aortae, the cardinal veins, the vitelline artery and vein, and the endocardial tubes. The primordial differentiation of this network then proceeds based on the mesodermal germ layer involved. Paraxial mesoderm gives rise to angioblasts capable of colonizing the endothelial lining of vessels, including the roof and sides of the dorsal aorta. Splanchnopleural mesoderm, while also contributing to the endothelial lining of vessels, can invade visceral organs and contribute to the floor of the dorsal aorta (Pardanaud et al., 1996), where definitive hematopoietic precursors first become evident (de Bruijn et al., 2002).

New endothelial cells and vessels are generated later via a process called angiogenesis, which proceeds via two distinct mechanisms: sprouting and non-sprouting angiogenesis (Risau, 1997). Sprouting angiogenesis involves the extension of pre-existing vessels. The extracellular matrix is removed via proteolytic degradation. Endothelial cells then proliferate, form a new lumen and mature into a functional endothelium. This is the primary method of vascularization of the brain. Non-sprouting angiogenesis, or intussusception, is when pre-existing vessels split via transcapillary pillars or posts of extracellular matrix. This is the primary method of vascularization in the embryonic lung. Multiple organs develop through concurrent utilization of both methods of angiogenesis. Superfluous branches are then pruned or remodeled, resulting in the formation of the mature vascular network (Risau, 1997).

Throughout development, hematopoietic and endothelial progenitors originate in the same locales. In the yolk sac, the blood islands contain primitive erythrocytes surrounded by a thin layer of angioblasts. In the aorta-gonad-mesonephros region of the embryo proper, which is the initial site of definitive hematopoiesis, hematopoietic stem cells can be detected budding from the endothelium of the dorsal aorta (de Bruijn et al., 2002). Given the close physical proximity of the very earliest hematopoietic and endothelial cells, it has been speculated that they originate from a common progenitor cell, which has been termed the hemangioblast (Murray, 1932). A number of transcription factors have been shown to play a role in the development of both cell lineages: for example, *cloche* is required for the formation of endothelial and hematopoietic progenitors in zebrafish (Stainier et al., 1995) and *Scl* (also known as *Tal1* – Mouse Genome Informatics), which encodes a basic helix-loop-helix transcription factor, was initially shown to be required for hematopoietic development in mice (Robb et al., 1995; Shivdasani et al., 1995). Subsequent transgenic rescue of the hematopoietic defect in *Scl*-null embryos revealed a requirement for SCL in the remodeling of the yolk sac vasculature (Visvader et al., 1998), and it has since been shown to play a role in vasculogenesis (Patterson et al., 2005), as well as in the migration and morphogenesis of endothelial cells (Lazrak et al., 2004). Transgenic expression of SCL is able to rescue the phenotypic consequences of *cloche* mutation in the zebrafish, suggesting that *Scl* functions downstream of *cloche* (Liao et al., 1998). LMO2, a member of the LIM domain family, is required for primitive erythropoiesis in the embryo; *Lmo2* ablation results in death at embryonic day (E) 9.75 secondary to hematopoietic failure (Warren et al., 1994). Analysis of chimeric mice bearing contributions from $Lmo2^{-/-}$ embryonic stem (ES) cells revealed that angiogenic remodeling of blood vessels requires *Lmo2* (Yamada et al., 2000). Similarly, targeted disruption of the receptor tyrosine kinase *Flk1* results in embryonic lethality around 8.5 days post-coitum (dpc) from a lack of organized blood vessels and a severely reduced number of hematopoietic progenitors (Shalaby et al., 1995).

Embryonic stem cell-derived embryoid bodies, in response to vascular endothelial growth factor (the ligand of Flk1), give rise to blast colony-forming cells, which in turn can directly differentiate to hematopoietic and endothelial precursors *in vitro* (Choi et al.,

1998). Moreover, single-cell-resolution fate maps in zebrafish have now shown *in vivo* that individual cells along the ventral mesoderm can contribute to both lineages (Vogeli et al., 2006).

Given the dual requirement for many hematopoietic transcription factors in angiogenesis, and the fact that expression of GATA factors had been reported in several endothelial cell lines (Dorfman et al., 1992; Umetani et al., 2001), it seemed reasonable that GATA family members might share a similar role in development. GATA factors belong to an evolutionarily conserved family of C_4 zinc-finger transcription factors that play demonstrably crucial roles in development. There are six GATA family members in vertebrates, which have historically been subdivided into two subfamilies. GATA-1, GATA-2 and GATA-3 are important for hematopoietic determination and differentiation, participating respectively in erythroid, hematopoietic progenitor and T-lymphoid development, among other activities (Pandolfi et al., 1995; Pevny et al., 1995; Tsai et al., 1994). Similarly, GATA-4, GATA-5 and GATA-6 have been shown to be involved in cardiac, genitourinary and multiple endodermal developmental events (Molkentin, 2000; Molkentin et al., 1997; Molkentin et al., 2000; Morrisey et al., 1998).

GATA-2 was originally cloned from a chicken reticulocyte cDNA library (Yamamoto et al., 1990). It is expressed in a wide variety of tissues, including hematopoietic, neuronal and endothelial cells. *Gata2*-null mutant embryos die at mid-gestation due to a block in primitive hematopoiesis (Tsai et al., 1994). Further examination of *Gata2* gain-of-function and in vitro differentiation of *Gata2* null ES cells showed that GATA-2 plays a key role in the proliferation of very early hematopoietic progenitors (Briegel et al., 1993; Kitajima et al., 2002; Tsai and Orkin, 1997), accentuating the conclusions from the initial loss-of-function experiments.

Given that many genes involved in hematopoiesis also participate in vascular development and that GATA-2 is strongly expressed in endothelial cell lines, it was originally believed that loss of GATA-2 function would result in vascular defects. Adding further to this expectation was early evidence that many genes that appeared to be crucial for endothelial development and function are regulated via GATA-binding sites (Dorfman et al., 1992). For example, GATA sites have been implicated in the regulation of the endothelium-specific genes *preproendothelin* (immature form of EDN1) (Dorfman

et al., 1992; Yamashita et al., 2001), *Pecam1* (Gumina et al., 1997), *Flk1* (Kappel et al., 2000; Minami et al., 2004), *eNOS* (also known as *Nos3* – Mouse Genome Informatics) (German et al., 2000) and *Icam2* (Cowan et al., 1998). Mutation of a GATA-binding site in the *Flk1* endothelium-specific enhancer completely abolished its activity in transgenic reporter assays, indicating that *Flk1* expression is dependent on GATA activity *in vivo* (Kappel et al., 2000). Surprisingly, however, the analysis of *Gata2*-null embryos failed to reveal any obvious defects in the vasculature at the time of their early embryonic demise (~e10) (Tsai et al., 1994), leaving the role for GATA-2 in endothelial function undefined.

To begin to investigate the role of GATA-2 in endothelial function, GFP expression in the developing vasculature of *Gata2*-GFP knock-in embryos was examined systematically during embryogenesis (Khandekar et al., 2007). It was found that GFP was expressed in cells lining arterial and venous vessels formed during vasculogenesis and angiogenesis, and that its expression continued postnatally. A 1.2-kbp region of *Gata2* was then functionally identified in intron 4 that could regulate the expression of a cis-linked reporter transgene in cardiovascular and lymphatic endothelial cells. Additionally, using site-specific mutagenesis, it was found that the potency of the minimal endothelium-specific enhancer is crucially dependent on an E-box (CANNTG) motif. By contrast, disruption of three ETS-binding sites quantitatively reduced, but did not abolish, enhancer activity (Khandekar et al., 2007). These studies also demonstrated that the vascular enhancer can be dissected into a core 167 base pair (bp) element capable of recapitulating vascular expression autonomously everywhere except in the endocardium; a 155-bp element, which is dependent on the 167-bp element, resides immediately upstream of the core enhancer and confers expression in the endocardium. We also showed that SCL is able to bind the critical E-box motif in the core enhancer *in vitro*.

Materials and Methods

Transgenic mice

Wild-type CD1 mice were mated with *Gata2*-GFP knock-in heterozygous mice, which had GFP inserted (in frame) at the translation initiation codon in *Gata2* exon 2 (Suzuki et

al., 2006). Embryos were harvested at the times indicated in the text and figure legends as previously described (Khandekar et al., 2004). Digital images were recorded as previously described (Khandekar et al., 2004).

For founder transgenic analyses, expression constructs were purified for microinjection into fertilized ova as previously described (Khandekar et al., 2004). At the indicated times, embryos from foster mothers or a *Gata2* YAC d16Z transgenic line were harvested for X-gal staining and PCR genotyped as previously described (Zhou et al., 1998). Transgenic embryos were photographed as whole-mount or cryosectioned specimens as described previously (Zhou et al., 1998).

Expression-plasmid construction

Gata2 fragments examined here were cloned 3' to the herpes simplex virus (HSV) thymidine kinase (TK) gene promoter in TKβ (Clontech) to mimic their natural position in the *Gata2* locus. To generate TKBXβ, a 2.9-kbp *Bam*HI-*Sal*I fragment from plasmid GR22 was first subcloned into pBluescript II (Stratagene) and then excised with *Xba*I before re-cloning into *Xba*I-digested TKβ. To generate TKSXβ, TKBXβ was treated with *Spe*I-*Sfi*I and T4 DNA polymerase before self-religation. To construct TKAAβ, a 460-bp *Alw*NI-*Apa*I fragment was excised from plasmid GR22 and treated with T4 DNA polymerase before being cloned into TKβ, which had been treated sequentially with *Xba*I and with Klenow polymerase. For microinjection, TKANβ was generated from TKAAβ by *Nco*I restriction-enzyme digestion. To delete the internal *Alw*NI-*Apa*I fragment, a plasmid subclone containing the 1.2-kbp *Sfi*I-*Xba*I *Gata2* intron 4 was treated with *Alw*NI-*Apa*I and T4 polymerase before self-religation. The resultant 0.8-kbp *Sfi*I-*Xba*I fragment was cloned into TKβ to generate TKSXΔAAβ. To clone the vascular endothelium-specific (VE) enhancer into TKβ (thereby generating TKVEβ, Fig. 2.2*B*), primers Endocons(f) and Endocons(r) containing an engineered *Xba*I site (5'-ggtctagaCCATGGAGTCACCTATACTGTG-3' and 5'-ggtctagaACTGAGTCGAGGTGGCTCTG-3', respectively) were used to generate a 167-bp amplicon (defined by the arrows in Fig. 2.1*A*, Fig. 2.2*A*), which was verified by sequencing.

To mutate the E-box in the VE enhancer, oligonucleotide-based PCR mutagenesis

was performed to introduce mutations (from 5'-CATCTG-3' to 5'-CAcccG-3'; mutations are lowercase) that had been shown to eliminate SCL binding in gel shift assays (Kappel et al., 2000). Primers EcSCLmut(f) (5'-CGGACAcccGCAGCCG-3') and Endocons(r) (shown above) were used to generate a 3' fragment using GR22 plasmid as template in a PCR reaction. Similarly, EcSCLmut(r) (5'-CGGCTGCgggTGTCCG-3') and Endocons(f) were used to generate a 5' fragment. The resultant amplicons were gel-purified and pooled as templates in a PCR reaction using Endocons(f) and Endocons(r) as primers. The gel-purified PCR products were sequenced to verify incorporation of the mutation and were then digested with *Xba*I prior to cloning into the *Xba*I site of TKβ to generate TKVEβmScl (Fig. 2.2*D*).

A similar strategy was used to introduce a mutation (5'-CGGA-3' to 5'- CGcg-3') that had been shown to eliminate ETS-factor binding (O'Reilly et al., 2003) into all three ETS-binding sites in the *Gata2* VE enhancer. The primers Ecmets1(f) (5'-CTCCTGCCGcgGTTTCCTAT-3'), Ecmets1(r) (5'-ATAGGAAACcgCGGCAGGAG-3'), Ecmets2(f) (5'-TTCCTATCCGcgCATCTGCAG- 3'), Ecmets2(r) (5'-CTGCAGATGcgCGGATAGGAA- 3'), Ecmets3(f) (5'-TGTTTCCGcgCCGGCAA-3') and Ecmets3(r) (5'-TTGCCGGcgCGGAAACA-3') were used to mutagenize the VE enhancer, as described above, and the enhancer was then sequenced to verify incorporation of the desired mutations. Fragments containing mutations in either the first two or all three ETS-binding motifs were digested with *Xba*I and then cloned into the *Xba*I site of TKβ to generate TKVEβmEts1,2 (data not shown) or TKVEβmEts1,2,3 (Fig. 2.2*C*), respectively.

Electrophoretic mobility shift assay

Nuclear extracts were prepared as described previously (Tanimoto et al., 2000) from 293T human embryonic kidney cells that were either mock transfected or transfected with EF-1α promoter-directed SCL cDNA alone or with a CMV promoter-directed E12 expression plasmid. Either no extract or nuclear extract (35 μg) was added to binding buffer containing 20 mM HEPES (pH 7.9), 1 mM $MgCl_2$, 0.5 mM DTT and 37.5 ng/μl poly(dI-dC) at 4°C. Unlabeled oligonucleotides [20- or 200-fold molar excess; wild type (5'-TCCGGACATCTGCAGCCGGT-3'; E-box underlined) or mutant (5'-

TCCGGACAcccGCAGCCGGT-3'; E-box underlined, mutated nucleotides lowercase)] or antibodies (Rodriguez et al., 2005) were added as indicated in the legend to Fig. 2.3. After 1 hour of pre-incubation, 2 μl ($2x10^5$ cpm) of radiolabeled wild-type oligonucleotide probe was added to each sample and incubated for an additional 30 minutes. All samples were fractionated by electrophoresis on neutral 6% TBE/polyacrylamide gels. After electrophoresis, the gels were dried and recorded using a PhosphorImager (Molecular Dynamics).

Results

Localization of a *Gata2* endothelium-specific enhancer

Melin Khandekar, a former graduate student in the lab, was able to localize the endothelium-specific enhancer of *Gata2* to a 1.2 kilobase pair (kbp) region in intron 4 (Khandekar et al., 2007). He next tested a series of smaller constructs (Fig. 2.1*A*) to establish the boundaries of a minimum enhancer element required to achieve the *Gata2* endothelium-specific expression pattern. Deletion of the 1.2-kbp SX fragment from only the 3' end (in TKSRβ) or from both termini (in TKAAβ) did not alter the cardiovascular endothelium-specific expression in E10.5 transgenic embryos (Fig. 2.1*B,C*). By contrast, deletion of the internal *Alw*NI-*Apa*I (AA) 460-bp fragment from the SX enhancer fragment eliminated all endothelial enhancer activity (0/15; Fig. 2.1*A,D*). Thus, the minimal endothelium-specific enhancer as defined by the AA restriction fragment is sufficient for endothelium-specific *Gata2* enhancer activity.

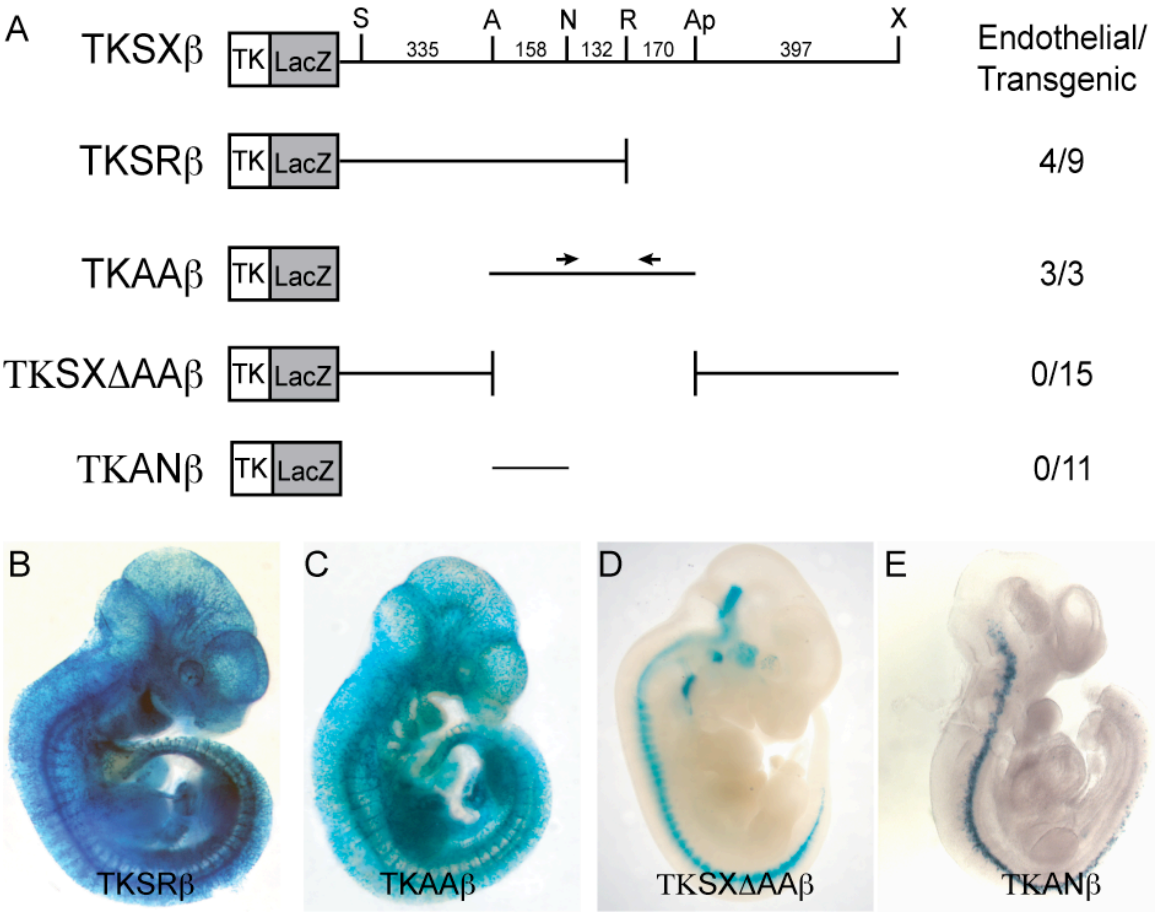

Figure 2.1 - Fine localization of the *Gata2* endothelium-specific enhancer. (A) Schematic illustrations of transgenic constructs (TKSXβ, TKSRβ, TKAAβ, TKSXΔAAβ and TKANβ) used to functionally localize the *Gata2* endothelium-specific enhancer element. Sub-fragments of *Gata2* intron 4 were individually cis-linked to a TK promoter-*lacZ* reporter gene. The positions of relevant restriction enzyme sites (*Alw*NI, A; *Apa*I, Ap; *Nco*I, N; *Rsr*II, R, *Sfi*I, S; *Xba*I, X) and the restriction fragment lengths (in bp) are indicated. The numbers on the right indicate the number of embryos with cardiovascular β-gal staining/total number of transgene-positive embryos. The arrows represent the positions of the primer pairs used to amplify the 167-bp VE enhancer (see Fig. 2.2). (**B-E**) E10.5 embryos bearing TKSRβ (B) or TKAAβ (C) transgenes showed widespread endothelial β-gal staining, whereas the TKSXΔAAβ (D) and TKANβ (E) transgenic embryos were devoid of endothelial X-gal accumulation. In the latter embryos, only ectopic β-gal activity was observed.

Identification of key regulatory motifs within the *Gata2* minimal endothelium-specific enhancer

Regulatory elements are thought to diverge more slowly than sequences that surround them (Loots et al., 2000). Comparison of the mouse *Gata2* 460-bp endothelium-specific sequence to that of the human sequence demonstrated that a 355-bp region within the AA fragment displayed 92% sequence identity, as well as a nearby 58-bp region that harbored 96% identity (data not shown). This extreme degree of evolutionary sequence

conservation strongly implies an associated functional significance. Analysis of the 460-bp element using Matinspector 2.2 (Quandt et al., 1995), which uses the consensus transcription factor-binding motifs from the TRANSFAC database, identified a number of candidate regulatory molecules that might bind to this enhancer (Fig. 2.2*A*). A closer examination of this restriction fragment showed an unusual clustering of binding sites within a central 167-bp core region (as delineated by the two convergent arrows shown in Fig. 2.1*A* and Fig. 2.2*A*). Interestingly, the 3' terminus of this region corresponded closely to the *Rsr*II site that was identified previously as defining the 3' functional boundary of endothelium-specific activity (Fig. 2.1*A,B*).

The mouse 167-bp endothelium-specific fragment (called VE) bearing the highest human-mouse identity was cloned into TKβ to test its ability to recapitulate endothelial expression. Analysis of transgenic founders showed that TKVEβ was able to confer endothelial expression in a range of vascular tissue (14/17; Fig. 2.2*B*) that did not differ from the TKAAβ construct. Strikingly, however, none of these embryos (0/17) exhibited endocardium-specific β-gal staining (Fig. 2.2*B*, arrow). We surmise from these data, in conjunction with data presented earlier (Fig. 2.1), that the endothelium-specific activity is largely contained within the 290-bp *Alw*NI-*Rsr*II fragment of *Gata2* intron 4 and that the 5'-most 155-bp of the *Alw*NI-*Rsr*II fragment are required for *Gata*2 expression in the endocardium while the adjoining 3' 167-bp can autonomously direct transgene expression in blood endothelia (Fig. 2.1*A* and Fig. 2.2*A*). Examination of the former sequences revealed the presence of consensus binding sites for the NFAT, Nkx2.5 (also known as Nkx2-5), SMAD, AP2 (also known as Tcfap2a) and MEF2C transcription factors, some of which have been shown previously to directly regulate endocardial enhancers and to be involved in regulating endocardial differentiation (Nemer and Nemer, 2002; Zhou et al., 2005). Whether or not these serve as bona fide binding motifs for any of these factors, and determination of the underlying mechanism via which they contribute to endocardial endothelial development, will require further investigation.

To ascertain whether the 5'-most 155-bp fragment was sufficient for endocardium-specific reporter gene activation, we tested the *Alw*NI-*Nco*I fragment in the context of TKβ (Fig. 2.1*A*), and found that, of 11 recovered transgenic embryos, none displayed endocardial X-gal staining despite exhibiting variably ectopic X-gal staining

(Fig. 2.1*A,E*). We conclude that the 5'-most 155-bp of the endothelium-specific enhancer alone is incapable of independently directing *Gata2* endocardium expression.

The Ets family of transcription factors have been shown to play crucial roles in vascular development (Ayadi et al., 2001; Wang et al., 1997), and have also been reported to play prominent roles in endothelium-specific enhancer activity (Göttgens et al., 2002; Kappel et al., 2000). The existence of three ETS-binding sites within the 167-bp *Gata2* VE enhancer suggested that one or multiple Ets family members might modulate its activity. To directly address this hypothesis, the three ETS-binding sites were individually mutated from CGGA to CGcg, a mutation that was previously shown to eliminate ETS-factor binding (O'Reilly et al., 2003). Mutation of either two or all three ETS-binding sites reduced the overall number of embryos displaying weak, albeit endothelium-specific, staining (1/12 and 3/25, respectively; data not shown and Fig. 2.2*C*).

Next we determined whether the single E-box motif present in the 167-bp VE enhancer is important for its overall activity. A mutation (5'-CATCTG-3' to 5'-CAcccG-3') that was previously shown to eliminate SCL binding (Kappel et al., 2000) was incorporated into the TKVEβ plasmid. Among the 24 recovered TKVEβmE-box transgenic embryos, none displayed endothelial β-gal staining (e.g. Fig. 2.2*D*). This lack of staining was presumably not due to the effect of the transgene integration site, because some of the transgenic embryos (9/24) exhibited ectopic staining in the spinal cord or head. These data demonstrate that the single *Gata2* intron 4 E-box exerts a profound effect on the activity of the VE enhancer and, by extension, in the regulation of *Gata2* expression throughout the blood endothelium.

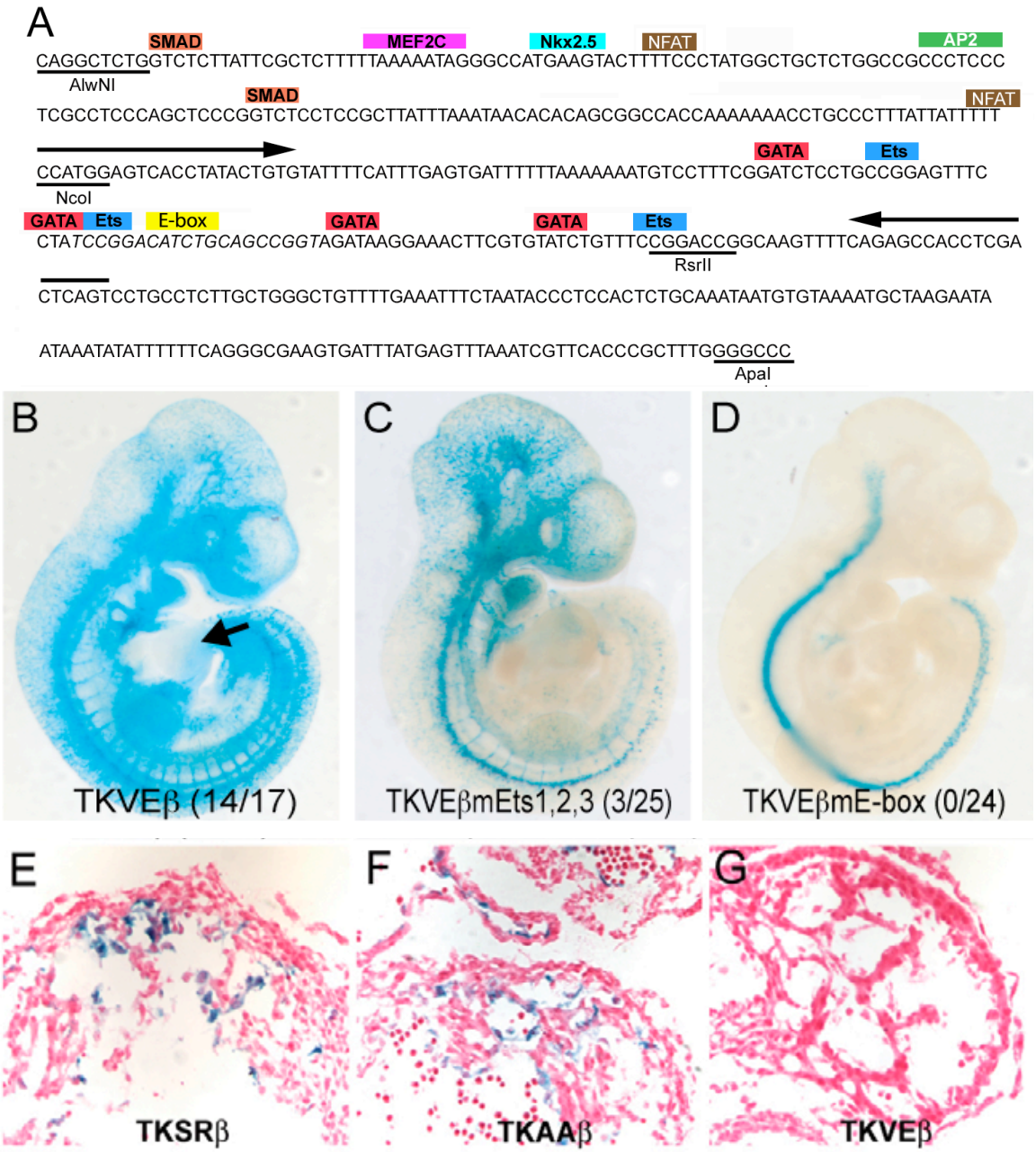

Figure 2.2 - Identification of a crucial E-box for *Gata2* vascular endothelium enhancer activity. (**A**) Consensus binding motifs for candidate regulatory effectors within the evolutionarily conserved 460-bp *Alw*NI-*Apa*I endothelium-specific enhancer sequence are highlighted. The 167-bp minimal vascular endothelium-specific (VE) enhancer in TKVEβ (B) was generated using the PCR primer pairs indicated by the two convergent arrows. The italicized sequences correspond to the radiolabeled probe used for EMSA studies (see Fig. 2.3). (**B**) TKVEβ recapitulates widespread vascular (14/17), but not endocardial (0/17, arrow), endothelial *lacZ* expression in e10.5 transgenic embryos. (**C**) Simultaneous mutation of all three ETS1-binding consensus sites (A) in TKVEβmEts1,2,3 resulted in far fewer (3/25) transgenic embryos that displayed vascular endothelium-specific *lacZ* expression. (**D**) Disruption of the single SCL-binding site (A) in TKVEβmScl completely abrogated vascular endothelium-specific X-gal accumulation (0/24). (**E-G**) Transverse sections through the hearts of e10.5 embryos bearing TKSRβ (E; Fig. 2.1*B*), TKAAβ (F; Fig. 2.1*C*) or TKVEβ (G; Fig. 2.2*B*) transgenes. Notice the conspicuous absence of X-gal staining in the endocardium of the ventricular chamber of the TKVEβ embryo.

An SCL-E12 heterodimer avidly binds to the E-box motif in the *Gata2* VE enhancer

Because SCL had been shown previously to be essential for endothelial differentiation (Visvader et al., 1998), we wished to determine whether SCL could bind to the E-box in the VE fragment. To do so, we performed electrophoretic mobility shift assays (EMSAs) using nuclear extracts from 293T cells transfected with either SCL, or SCL plus E12, expression vectors. Incubation of radiolabeled E-box oligonucleotide probes with nuclear extracts containing SCL-E12, but not SCL alone, resulted in a low-mobility complex (Fig. 2.3, lane 4), which could be specifically competed by the addition of an excess of unlabeled E-box oligonucleotide (Fig. 2.3, lanes 5,6), but not by mutant E-box oligonucleotide (Fig. 2.3, lanes 7,8). The binding specificity and protein identity were confirmed by showing that addition of an anti-SCL antibody (Fig. 2.3, lanes 9,10), but not control IgG (Fig. 2.3, lane 11), significantly reduced EMSA complex formation. Thus, the crucial E-box motif in the *Gata2* VE enhancer can be strongly bound by the basic helix-loop-helix transcription factor SCL.

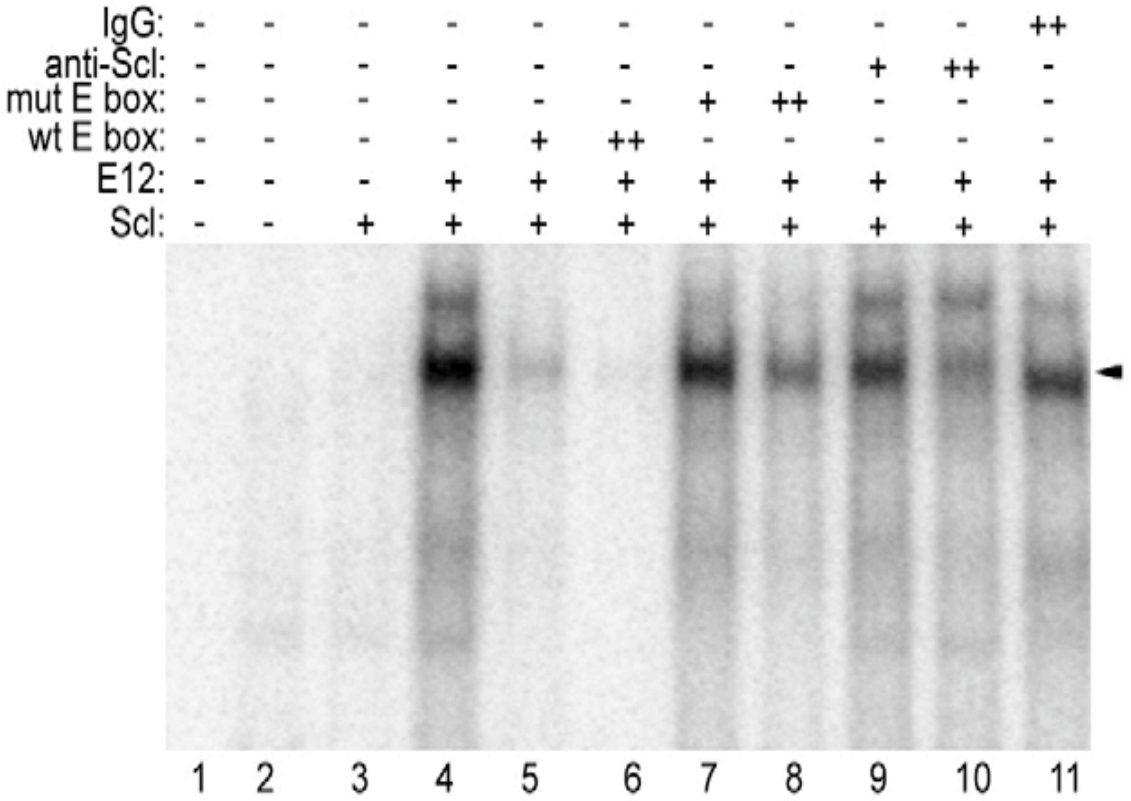

Figure 2.3 - An SCL-E12 complex binds specifically to the *Gata2* VE-enhancer E-box. No extract (lane 1) or nuclear extracts from 293T cells, which were mock transfected (lane 2), transfected with SCL alone (lane 3) or with SCL plus E12 expression plasmids (lanes 4-11), were incubated with radiolabeled E-box oligonucleotide probe. To demonstrate binding specificity, unlabeled competitors (20- or 200-fold excess) containing wild-type (wt; lanes 5,6) or mutant (mut; lanes 7,8) E-box, anti-SCL antibody (1 or 3 μl; lanes 9,10) or control mouse IgG (lane 11) were added to separate binding reactions. Formation of a lower-mobility complex was observed only when both SCL and E12 were present in the extract and was specifically disrupted by wild-type, but not mutant, competitors as well as by the addition of an anti-SCL antibody.

Discussion

In summary, the data presented here delimit the boundaries of a functionally defined 460-bp *Gata2* fourth intron endothelium-specific enhancer element that is capable of autonomously directing reporter gene expression *in vivo* in vascular and endocardial endothelial cells, thus precisely mimicking endogenous GATA-2 expression. Both ETS and E-box binding sites contribute to the potency of the VE enhancer, thus implicating Ets family member(s) and SCL as candidate regulatory effectors of *Gata2* vascular endothelial expression.

Identification of *Gata2* pan-endothelium-specific enhancer

The existence of an endothelial enhancer for *Gata2* raises several intriguing questions about its function. Earlier genetic data indicated that GATA-2 plays a crucial role in early hematopoietic development (Tsai et al., 1994) and is indeed capable of specifically marking hematopoietic stem cells (Suzuki et al., 2006). Given that the earliest hemangiogenic cells are closely related to the endothelial lineage (Choi et al., 1998), one might speculate that this endothelial enhancer could also target a subset of hematopoietic cells that are generated from the aortic endothelium. Suggestively, we identified *lacZ*-positive cells that appear to be 'budding' from the aortic wall (data not shown). Whether these cells are bona fide hematopoietic cells or simply endothelial cells being sloughed into the aorta is yet to be determined (Khandekar et al., 2007). However, the existence of an endothelium-specific enhancer of *Scl*, which also marks hematopoietic progenitors (Göttgens et al., 2004), suggests that the *Gata2* intron 4 enhancer identified here may play some role in the appropriate regulation of *Gata2* in hematopoietic progenitors as well, particularly in light of the recent observation that this enhancer appears to be active in definitive erythroid cells (Grass et al., 2006) (see below).

In the blood vasculature, the lack of any reported phenotype in *Gata2*$^{-/-}$ embryos led to the initial conclusion that functional redundancy of other GATA family members, including GATA-4 and GATA-6, may compensate for any lack of GATA-2 in the endothelium. Another equally plausible possibility is that *Gata2* mutant embryos simply die too early (~e10) (Tsai et al., 1994) to generate a robust vascular phenotype. Based on the analysis of *Gata3-lacZ* knock-in mice, *Gata3* expression in the endothelium does not appear to be widespread, but this closely related GATA family member does seem to be sporadically active (our unpublished data). *Gata4*, which plays a crucial role in heart development, does not appear to be expressed in mature endothelial cells (Umetani et al., 2001), although it is expressed in endothelial progenitors (Hatzopoulos et al., 1998). *Gata6* is expressed in both endothelial precursors as well as in mature endothelial cells (Hatzopoulos et al., 1998; Umetani et al., 2001), making GATA-6 a prime candidate for a possible *Gata2*-complementing endothelium activity. Whereas *Gata4* and *Gata6* heterozygotes are normal, compound heterozygotes display cardiovascular defects (Xin et al., 2006). Notably, these embryos displayed a less intricate patterning, which was

disorganized, of the cranial and intersomitic vasculature, as well as hemorrhaging. The recent generation of a *Gata6* conditional loss-of-function allele (Sodhi et al., 2006) should now permit exploration of cell autonomous GATA-6 involvement, if any, in endothelial development.

Role of Ets family transcription factors in *Gata2* endothelium-specific enhancer activity

Within an initial functionally defined restriction fragment describing the *Gata2* endothelial enhancer, we subsequently identified a 167-bp core enhancer that was sufficient to recapitulate vascular endothelial expression. The existence of three putative Ets family-member binding sites implicated a role for these factors in the control of this VE enhancer. The Ets family of transcription factors have been shown to play an important role in vascular development *in vivo* (Sumanas and Lin, 2006) and have been shown to be functionally important in the activation of a number of endothelial-specific enhancers, including *Scl* (Göttgens et al., 2004), *Tie2* (also known as *Tek*) (Minami et al., 2003) and *Flk1* (also known as *Kdr*) (Elvert et al., 2003). Furthermore, disruption of *Tel* (also known as *Etv6*), an Ets family member, results in defective yolk sac angiogenesis (Wang et al., 1997), suggesting that TEL plays an important role in vascular remodeling. However, targeted mutation of other ETS factors has not revealed vascular deficiencies, suggesting that these factors may either play no role or may also be functionally redundant in endothelium development.

In the *Gata2* VE enhancer defined here, mutations predicted to disrupt ETS-binding sites significantly attenuated enhancer activity, as indicated by the number of, and X-gal-staining intensity in, transgenic embryos displaying endothelial β-gal staining. However, the weak staining pattern detected in these embryos appeared to remain endothelium-specific, indicating that the ETS-binding sites are not essential for the tissue specificity of the enhancer, but rather may serve to augment its overall potency. Because this cis mutation has been shown in a similar assay to eliminate DNA binding for some members of the family (O'Reilly et al., 2003), we cannot rule out the possibility that the mutation does not abolish the binding of the multiple Ets family members that are expressed within the endothelium (Lelièvre et al., 2001). Additionally, the heterogeneity

of ETS-binding sites suggests that some family members may be able to bind to other sequences within the enhancer, enabling endothelial activation despite mutations within canonical high affinity binding sites.

An E-box binding factor is required for *Gata2* endothelial enhancer activity

The transcription factor SCL has been shown to play crucial roles in both hematopoiesis (Shivdasani et al., 1995) and vascular development (Patterson et al., 2005; Visvader et al., 1998), leading to the speculation that SCL may be important for the ontogeny of the hemangioblast. A comprehensive analysis of the transcriptional regulation of *Scl* has identified several tissue specific enhancers that are required for its appropriate expression (Barton et al., 2001; Göttgens et al., 2002; Sinclair et al., 1999). Interestingly, the enhancer specific for hematopoietic progenitors has GATA sites that are crucial for *Scl* enhancer activity *in vivo*. The factor responsible for binding to these sites in hematopoietic cell lines appears to be GATA-2, suggesting that GATA-2 is responsible for activating *Scl* in early hematopoiesis. However, there is no GATA-binding site in the endothelial enhancer of human *SCL*, although it remains possible that a GATA factor is acting without directly binding to DNA (Göttgens et al., 2004). The data presented here are consistent with the possibility that *Gata2* and *Scl* encode reciprocally reinforcing activators in these developmentally related tissues, although other interpretations are clearly not excluded from the data presented.

To assess the relationship between *Scl* and *Gata2* in the endothelium, we mutated the single E-box present within the 167-bp *Gata2* VE enhancer. *Scl* has previously been shown to be regulated by GATA factors in both the CNS (Sinclair et al., 1999) and hematopoietic progenitors (Göttgens et al., 2002), suggesting that the nature of the epistatic relationship between these two factors may be dependent on the specific tissue in question. However, these data also underscore the point that the functions of these two factors are often intimately intertwined during development. Lending further credence to this point is the evidence that *Scl* expression in the endothelium is crucially dependent on Ets-family activity (Göttgens et al., 2004). The endothelial enhancer of *Scl* contains five ETS-binding sites that are required for the activity of the enhancer in trans-activation assays. Given that both *Scl* and *Gata2* appear to be regulated by ETS factors in the

endothelium, we surmise that ETS, SCL and GATA-2 together constitute a regulatory circuitry wherein, in the simplest scenario envisaged, ETS factors activate *Scl*, and ETS and SCL then cooperate to activate *Gata2* in endothelial cells. Similarly, the data are also consistent with the possibility that Ets family members collaborate with GATA-2 to reinforce *Scl* expression in a positive feedback loop.

The similarities between the regulation of *Scl* and *Gata2* are also underscored by the similarity of their functions in the hematopoietic system. Targeted mutation of both genes results in defects in both primitive and definitive hematopoiesis, resulting in mid-gestational lethality (Shivdasani et al., 1995; Tsai et al., 1994). This phenotypic similarity has not been demonstrated in the vascular system, where SCL has been shown to play a prominent role in vascular remodeling, whereas GATA-2 has not. However, because the vascular defects in *Scl*-null mutants were only revealed after selective rescue of hematopoiesis, it seems likely that the early lethality of *Gata2*-null mice precludes a more precise analysis of the function of GATA-2 in the vascular system. Experiments are underway to circumvent the embryonic hematopoietic lethality and explore possible functions of GATA-2 in the vasculature (Chapter 4).

The presence of multiple GATA-binding motifs within the VE enhancer also raises the issue of whether this enhancer might be auto-regulated by GATA-2, or even by another GATA factor. Of specific interest here, Grass et al. recently identified this same element through its evolutionary sequence conservation during an analysis of GATA-1 regulation of the *Gata2* gene in erythroid cells (Grass *et al*., 2006). In that study, the authors demonstrated that sequences overlapping the VE element exhibited robust activity in transfected erythroid cells and that elimination of the GATA sites abrogated the erythroid enhancer activity. Here, we show that the VE element is at least equally as active and as specific for endothelial cells in a rigorous *in vivo* assay. Whether the activity identified by Grass *et al*. and the activity we defined here represents an endothelial-enhancer activity that can simply be surreptitiously activated in erythroid cells or whether the element represents one that can be a *bona fide* target for GATA-factor activation in both hematopoietic and endothelial cells (as one might imagine for a hemangioblast-responsive element) awaits resolution following further investigation (Chapter 4).

Acknowledgments

Melin Khandekar, a former graduate student in the lab, initiated this work. He examined expression of *Gata2* in the endothelium, and he refined the position of the element and analyzed its expression, including all of the founder analysis except for TKANβ, which was performed by Kim Chew-Lim. Melin also performed all of the analysis of the conserved regions and putative regulatory elements. I analyzed the endocardial sequence for potential binding partners, and analyzed the transverse sections through the endocardium of the founders. I also performed the EMSA study, and helped write and prepare the manuscript and figures for publication. The whole article can be found in (Khandekar et al., 2007).

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Chapter 3

Defining the Functional Boundaries of the *Gata2* Locus by Rescue with a Linked Bacterial Artificial Chromosome Transgene

Introduction

The transcription factor GATA-2 is exemplary of a vital developmental factor whose regulation reveals many of the intricacies of the controlling apparatus that is required for proper temporal and tissue-specific expression of a gene in many different tissues and organs that are critical for mammalian development. The evolutionarily conserved C_4 zinc finger GATA transcription factors play demonstrably crucial roles in embryogenesis. GATA-2, originally cloned from a chicken cDNA library (Yamamoto et al., 1990), was shown to be essential for the proliferation and/or differentiation of early hematopoietic progenitors, as *Gata2* null mutant mice die around mid-gestation from a block in primitive hematopoiesis (Tsai et al., 1994). Further examination of *Gata2* gain-of-function mice and *in vitro* differentiation of *Gata2* null mutant ES cells underscored the fundamental conclusions from the initial loss of function experiments, and showed that GATA-2 plays a pivotal role in the proliferation of very early hematopoietic progenitors (Briegel et al., 1993; Kitajima et al., 2002; Tsai and Orkin, 1997).

We showed several years ago that *Gata2* null mutant embryonic lethality could be rescued by complementation with a 247 kilobase pair (kbp) transgenic yeast artificial chromosome (YAC) bearing sequences from -174 to +73 kbp (revised endpoints relative to the *Gata2* translational initiation site); the YAC contains all of the regulatory information required to rescue *Gata2* function in primitive and definitive hematopoiesis (Zhou et al., 1998). We subsequently found that *Gata2* hematopoietic regulatory activity is conferred by at least two distinct cooperative *cis* elements, one lying about 170 kbp 5' to the gene and another lying somewhere between 40 and 100 kbp 5' (Suwabe and Engel, unpublished observations). However, compound mutant mice bearing this YAC, but

lacking endogenous *Gata2*, expired perinatally of kidney failure and uretero-vesicular obstruction, because the YAC-rescued *Gata2* null mutant mice failed to develop a patent connection between the ureters and the bladder, causing the mice to develop cystic, non-functional kidneys and megaureters (Zhou et al., 1998). Because the rescuing YAC was not expressed in the urogenital system, while endogenous GATA-2 is prominently expressed there, we speculated that the development of hydroureters and cystic kidneys was due to the absence of tissue-specific enhancers that were responsible for directing proper *Gata2*-mediated patterning of the developing urogenital system in the YAC transgene.

Several years ago, we devised a BAC transgenic founder mapping strategy (BAC-traps) that ultimately revealed the positions of the regulatory elements that control *Gata2* expression in the developing urogenital system (Khandekar et al., 2004). One *cis* element, responsible for *Gata2* activation in the ureteric epithelium, was not specifically localized, but was predicted to lie very far, either upstream or downstream, from the structural gene. However, two enhancers that were finely localized are responsible for mesenchymal expression: one (UG2, located +113 kbp 3' to the translational initiation site) controls *Gata2* activation in the rostral metanephric mesenchyme, whereas a second (UG4, centered at +75 kbp) is responsible for *Gata2* activation in the caudal periureteric mesenchyme and urogenital sinus.

We have now regionally or precisely localized about a dozen individual *cis* elements that are required for *Gata2* expression, including two hematopoietic (Zhou et al., 1998), two central nervous system (Zhou et al., 2000), one endothelial (Khandekar et al., 2007), and three urogenital enhancers (Khandekar et al., 2004), as well as the two *Gata2* promoters (Minegishi et al., 1998); these individual elements are scattered over 400 kbp 5' and 3' to (as well as within) the *Gata2* structural gene. However, dozens of other elements must deductively exist, because *Gata2* is involved in many other quite diverse organotypic developmental events (*e.g.* for the generation of placental trophoblasts, fat, and pituitary subtype specification (Dasen et al., 1999; Ma et al., 1997; Tong et al., 2000)), and indeed, our initial BAC-trap surveys demonstrated that other *Gata2* enhancers lie even further away (*e.g.* a whisker follicle enhancer that maps to between +560 and +787 kbp 3' to *Gata2* (Khandekar et al., 2004)).

To determine whether the *Gata2* urogenital enhancers that were identified in the initial BAC-trap survey defined the required functional domains of gene activity that are responsible for proper ureter/bladder morphogenesis, in this study we created a transgene that would recapitulate the expression of *Gata2* in both the hematopoietic as well as the urogenital compartments. Although YACs have been generated that contain genomic inserts of the size required, numerous technical problems with YACs have also come to light, including chimerism (Larionov et al., 1994; Zhou et al., 1998) and innate replicative instability (Kouprina et al., 1999). BACs, while surmounting many of the problems encountered with YACs, posed a different problem, because the average genomic DNA insert size in BACs is only ~200 kbp (Osoegawa et al., 2000), which is insufficient in this case to span the interval between the most distant *Gata2* hematopoietic (-170 kbp) and urogenital (+113 kbp) enhancers.

To circumvent the size limitation of BACs, we devised a new general strategy to precisely link two BACs together to generate a single recombinant that contained a very large but stable genomic DNA fragment. Generation of mice bearing this BAC were predicted to create a transgene spanning the entire interval between the distant enhancer elements that were either known (hematopoietic) or suspected (urogenital) of being required for *Gata2* function. Following breeding between *Gata2* BAC transgenic mice and *Gata2* germ line null mutant mice, we were able to formally test whether complementation of both the hematopoietic and urogenital functions would fully rescue *Gata2* null mutants. Alternatively, complementation with the linked BAC might only partially rescue, and thereby reveal novel *Gata2* mutant phenotypes due to as yet undiscovered enhancers that lie beyond the boundaries of the linked BAC.

The present experiment revealed that rescue of the *Gata2* null mutant phenotype with a 413-kbp linked BAC was complete using either of two independent rescuing BAC transgenic lines. Both lines allowed animals to survive to adulthood, and the rescued mice exhibited no obvious behavioral or reproductive anomalies. Therefore, we can place outer boundaries on the size of the *Gata2* locus, including all sequences that are required to fulfill its most vital developmental functions. These data suggest that documented enhancers that lie outside these boundaries are either unnecessary for vital GATA-2-mediated physiological functions in some tissues where that factor is expressed, or that

those functions are fully complemented by other molecules.

Materials and Methods

Bacterial Artificial Chromosomes

BACs spanning the mouse *Gata2* locus were identified (Osoegawa et al., 2000), purified (Marra et al., 1997), and modified (Khandekar et al., 2004) as previously described.

Construction of Specialized Recombineering Plasmids

plox514Neo: Two loxP514 sites (Siegel et al., 2001) with convenient cloning sites were created. lox514nx oligonucleotides (Table 3.1) were annealed and digested with NotI and XbaI and then cloned into the EagI and SpeI sites of the pFrtNeo plasmid. The resulting plasmid was digested with PstI and EcoRI and ligated to annealed lox514pm oligonucleotides (Table 3.1), which had been digested with NsiI and MfeI. The resulting plasmid, plox514Neo, was verified by sequencing and then used as a template for the generation of the BAC targeting constructs. pFrtAmp: to generate a plasmid containing the Frt-flanked ampicillin gene, we used Famp oligonucleotides (Table 3.1) containing Frt sites, sequences complementary to the pUC19*Amp*^{R} gene and convenient cloning sites. Using these primers, the *Amp*^{R} gene was amplified from pUC19 by PCR, gel-purified, and digested with BamH1 and EcoR1. This fragment was cloned into the BamH1/EcoR1 sites of pIRES2-eGFP vector (Clontech). The fragment was sequenced to verify correct generation of the flanking Frt sites. Tn5FlpeTet: a plasmid that constitutively expresses Flp recombinase was generated in a manner similar to that described by Liu *et al.* (Liu et al., 2003).

Generation of Targeting Constructs by PCR

To generate the bacterial targeting constructs, synthetic oligonucleotides were used as outlined in previous experiments (Lee et al., 2001). Targeting fragments were generated by PCR with ExTaq (Takara), purified from agarose using GenecleanII (Q-biogene), and digested with DpnI to remove (methylated) template DNA. 100–200 ng of

targeting fragment was used in each experiment. To generate single lox sites, the same primers (Table 3.1) were used to amplify a targeting fragment containing tandem lox sites. After insertion of this fragment into the BAC by homologous recombination, induction of cre recombinase resulted in deletion of the intervening selection marker, leaving a single loxP site incorporated at the point of selectable marker insertion.

cre Recombinase Induction

The EL350 strain was used for cre recombinase induction, as previously described (Khandekar et al., 2004). Because the frequency of recombination between loxP and loxP511 or loxP514 sites is finite, varying the duration of cre induction was used to optimize recovery of the desired homologous recombination product.

Modification of Parental BACs 115E9 and 81F7

To generate the 5' BAC, homologous recombination was used to insert a loxP514 site ~13 kbp upstream of the *Gata2* translational initiation site. Simultaneously, this insertion was designed to delete a loxP site in the pBACe3.6 vector backbone by directing the 3' homologous recombination to position 4862 of the vector sequence (Frengen et al., 1999). The primers (-13lox514(f) and bacdellox(r); Table 3.1) and the template plasmid plox514Neo were used for PCR to generate a targeting fragment, which was then used to modify the 115E9 BAC by homologous recombination. The neomycin gene that was used as the positive selection marker was removed by cre induction, leaving a single loxP514 site at -13 kbp, and at the same time deleting the loxP site in the vector backbone. The resulting BAC was further modified to include an Frt-flanked PGK-directed neomycin resistance gene for positive selection. BacNeo primers (Table 3.1) were used to generate a targeting fragment by PCR using the pFrtNeo plasmid as template. The resulting fragment was then inserted into the 115E9lox BAC by homologous recombination.

To generate the 3' BAC, a loxP514 site was again inserted 13 kbp upstream of the *Gata2* translational start site, at the same position that was modified in BAC 115E9. The -13lox514 forward and reverse primers (Table 3.1) and the ploxP514 plasmid were used to generate a fragment that was subsequently inserted into the 81F7 BAC by homologous

recombination. The neomycin gene was again removed by cre induction, leaving a single loxP514 site at the -13 kbp position. This BAC was further modified to insert an Frt-flanked ampicillin resistance gene to allow for selection of products that had undergone intermolecular recombination. This was achieved by using Famp2977 and Famp4233 primers (Table 3.1) together with the FrtAmp plasmid to generate a targeting fragment that was then inserted into 81F7lox BAC by homologous recombination, and in doing so, inserted the FrtAmp cassette between positions 2977 and 4233 of the pBACe3.6 vector backbone (Frengen et al., 1999).

cre-mediated BAC Recombination

To join the two modified BACs, *Escherichia coli* EL350 bearing the 115E9loxNeo BAC was grown in overnight culture. After diluting the culture 1:10 and allowing it to grow for 10 min, arabinose was added to 1 mg/ml before growing the culture for an additional 2 h. The cells were made competent for transformation (Khandekar et al., 2004) and then electroporated with the 81F7loxAmp BAC; recombinants were selected on Cam/Kan/Amp plates. Homologous recombination was verified by HindIII fingerprinting, as well as by P1-SceI digestion and pulsed field gel electrophoresis under the conditions: 6 V/cm, linear ramp switch time (10–50 s) 24 h at 14 °C (Fig. 3.1*B*).

Verification of Homologous Recombination

To verify recovery of the correct recombination products by Southern blot analyses, three probes from different regions of the recombined BAC were employed. A 5’ Nco/Spe probe fragment corresponding to -173.9 kbp of the *Gata2* locus, which was predicted to yield a restriction fragment of 3.9-kbp only from the parental 115E9 BAC and the recombinants, was hybridized to HindIII-digested BAC DNAs (Fig. 3.1*D*). To generate a -13 kbp probe, a 600-bp fragment was generated by PCR from the 81F7 BAC using the G2–13 primers (Table 3.1). Using this probe and digesting the BACs with PmeI and PvuI was predicted to yield a 5-kbp restriction fragment in the case of the 115E9 parental BAC, or a 10-kbp fragment after appropriate recombination at the loxP514 site (Fig. 3.1*E*). For the 3’ probe, a 900-bp fragment corresponding to a position +200 kbp

(relative to the *Gata2* translational initiation site) was generated by PCR from the 81F7 BAC using G2 +200 primers (Table 3.1). Digestion with HindIII followed by hybridization with this probe was predicted to yield a 7-kbp band from the 81F7 parental BAC as well as the homologous recombinants but not hybridize to the 115E9 BAC (Fig. 3.1*F*).

Flp-mediated Removal of the Neomycin Selection Cassette

Because the neomycin resistance gene carries a eukaryotic promoter, it was removed from the linked BAC to avoid possible promoter interference effects. To accomplish this, the Tn5FlpeTet plasmid was transformed into bacteria carrying the linked BAC (Fig. 3.1*A, R*), which were then grown on plates containing Cam/Kan/Amp/Tet. The Tn5FlpeTet plasmid constitutively expresses Flp recombinase, which catalyzed excision of the Neo gene. To subsequently eliminate this plasmid from the bacteria bearing the linked BAC, we used nickel ion-based counterselection, because bacteria that are tetracycline-resistant are sensitive to nickel death (Podolsky 1996). We reasoned that growth in L-broth containing $NiSO_4$ would select for the rare bacteria that had lost the Tn5FlpeTet plasmid during cell division. Cam/Kan/Amp/Tet colonies were grown in 5 ml of L-broth plus Cam for 3 h, at which time $NiSO_4$ was added to 5 mM. The cultures were grown until turbid and then streaked onto Tet/Cam, Kan/Cam/Amp, and Cam plates. Cultures that showed slower growth on Tet/Cam and Kan/Cam/Amp plates *versus* Cam plates were replica-spotted onto Cam and Kan/Cam plates to verify loss of neomycin resistance. Miniprep DNA from the resulting Cam^R/Kan^S clones was verified by HindIII restriction digest, pulsed field gel electrophoresis, and Southern blot analyses as described above.

Transgenic and Knock-out Mice

BACs were purified by double banding on CsCl/EtBr gradients and then the purified supercoiled DNA was microinjected into oocytes as previously described (Khandekar et al., 2004). The *Gata2* EII-KI mice have been previously described (Suzuki et al., 2006).

Immunohistochemistry

Embryos were harvested and prepared as previously described (Khandekar et al., 2004). Purified rat anti-chicken GATA-2 monoclonal antibody, RC1.1 (1:10) (Zhou et al., 1998), which is cross-reactive with murine GATA-2, was added along with an anti-green fluorescent protein (anti-GFP) antibody (Molecular Probes, A11122, 1:2000) to the embryo sections, which were then incubated overnight at 4°C. The next day the RC1.1 antibody was detected using a Cy3-conjugated goat anti-rat secondary antibody (Zymed Laboratories Inc.), whereas the anti-GFP antibody was detected using Alexa488-conjugated goat anti-rabbit secondary antibody (Molecular Probes). Both secondary antibodies were incubated at 1:200 for 1 h. Finally the sections were treated with 4',6-diamidino-2-phenylindole (DAPI) and ProLong Gold antifade reagent (Invitrogen). Images were recorded electronically and merged as previously described (Khandekar et al., 2004).

Results

Linking BACs

In this strategy, we first identified two overlapping BACs, which together encompass the complete structural gene as well as the regulatory domains required for *Gata2* hematopoietic and urogenital expression (Khandekar et al., 2004; Zhou et al., 1998), from BAC library RPMI-23. The selected parental BACs, 115E9 (P1) and 81F7 (P2), bear genomic sequences from -187 to -10 kbp and from -34 to +226 kbp of the *Gata2* locus, respectively (Fig. 3.1*A*). To fuse the two BACs precisely in register, we employed cre-mediated homologous recombination to exploit the documented differential recombination efficiency between homologous *versus* heterologous loxP sequences (Hoess et al., 1986). A single loxP514 site (Fig. 3.1*A, yellow arrow*), which is distinct from the loxP511 sites (Fig. 3.1*A, green arrows*) in the pBACe3.6 vector backbone (Frengen et al., 1999), was inserted at -13 kbp (relative to the *Gata2* start codon) of the *Gata2* locus in both BACs, P1 and P2. Next, Frt-flanked antibiotic resistance markers (neomycin or ampicillin) were introduced at the 5' end of P1 or at the 3' end of P2, respectively, to facilitate drug selection of intermolecular recombinants. The

electroporation of P1 into P2-harboring bacteria, in conjunction with cre recombinase induction in the *E. coli* EL350 strain, led to simultaneous loxP514/514 and loxP511/511 homologous recombination in *trans* (Fig. 3.1*A*, *gray bars*) and the insertion of *Gata2* genomic DNA from P1 into P2, thus generating the 413-kbp (from -187 to +226 kbp of the *Gata2* locus) recombinant BAC (Fig. 3.1*A, R*). After Flp-mediated deletion of the Frt-flanked positive selection markers from this recombinant, the structural integrity of the resultant linked BAC (referred to hereafter as *G2BAC*) was confirmed by pulsed field gel electrophoresis (Fig. 3.1*B*), restriction digest fingerprinting (Fig. 3.1*C*), and Southern blotting analyses using three radiolabeled probes dispersed throughout the *Gata2* locus (Fig. 3.1*A*, the positions of the probes are indicated as *D*, *E*, and *F*). Hence, we successfully generated a 413-kbp *Gata2*-linked BAC using cre-mediated intermolecular recombination.

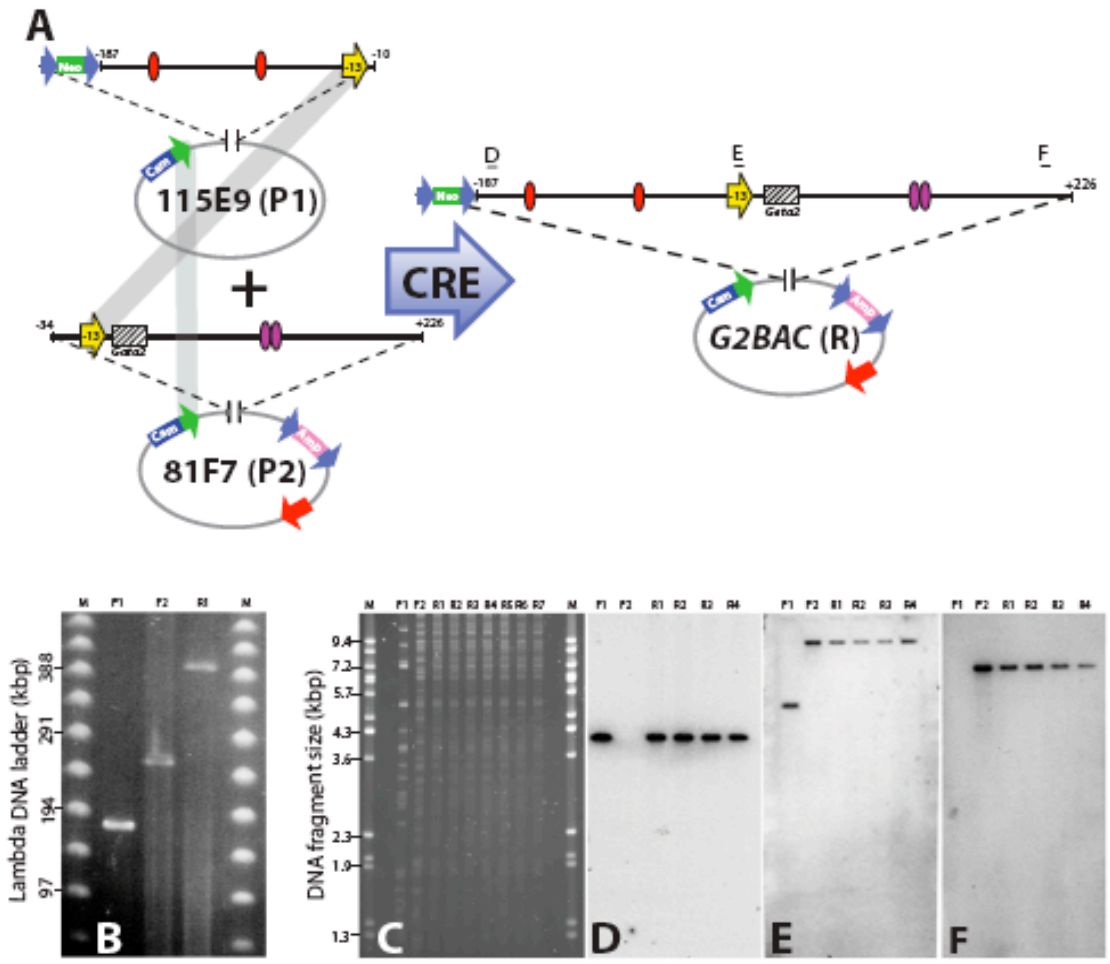

Figure 3.1 - Cre-mediated generation of a 413-kbp linked BAC. (A) A strategy was devised for recombining two overlapping parental BACs so that the combination of urogenital and hematopoietic enhancers could be examined as a single transgene. The relative positions of the *Gata2* structural gene (*hatched box*) and the enhancers known to be critical for hematopoietic (*red ovals*) or urogenital (*purple ovals*) development are indicated. Site-specific cre-mediated recombination was employed to fuse the two parental BACs, 115E9 (P1) and 81F7 (P2), by simultaneous intermolecular homologous recombination between loxP514 (*yellow arrowheads*) and loxP511 (*green arrowheads*) sites. The strategy was predicted to generate a 413-kbp BAC bearing contiguous sequence from -187 to +226 kbp surrounding the *Gata2* gene (*G2BAC*, *right*). The neomycin-positive selection marker was subsequently removed by Flp-mediated recombination between Frt sites (*blue arrowheads*). **(B)** Pulsed-field gel electrophoresis of the two parental (*P1* and *P2*) BACs as well as a recombinant-linked BAC (*R*). The BACs were digested with PI-SceI; molecular weight markers (*M*) are λ DNA concatemers. **(C)** HindIII restriction digest DNA fingerprinting shows that all of the recombinants (*R1–R7*) contain all of the bands within both parental BACs (*P1* and *P2*). Molecular weight markers (*M*) for panels *D-F* are a mixture of HindIII- or BstEII-digested λ DNA samples. **(D-F)** Southern blot analysis of the parental (*P1* and *P2*) and linked recombinant (*R1–R4*) BACs display the anticipated sizes. (D) Probe D, located at -174 kbp (see panel *A*), hybridizes to a 3.9-kbp HindIII fragment from P1 (that is not detected in P2) and all recombinant BACs. (E) Hybridization to probe E, located at -13 kbp, generates a 5-kbp band in P1 as well as a 10-kbp band in P2 and all of the recombinant BACs after digestion with PmeI+PvuII. (F) Probe F, located at approximately +200 kbp, hybridizes to a 7-kbp HindIII fragment from P2 and the recombinant BACs, while no hybridization is detected in P1 DNA.

Linked BAC Transgenic Mice

Due to fragmentation problems encountered when very large linear or nicked circular BAC DNAs are used for microinjection (Khandekar and Engel, unpublished observations), we injected supercoiled DNA into fertilized mouse ova. Founder animals were genotyped using PCR primers (Table 3.1) located at the junction between the BAC vector and the most 5' end of the *Gata2* genomic DNA insert at -187 kbp, at the loxP514 insertion site situated at -13 kbp of the *Gata2* locus, and in the chloramphenicol acetyltransferase gene. Fourteen founders were verified to be transgenic by Southern blot analyses of tail DNA (data not shown). Of these, at least two appeared to contain complete transgenes that were transmitted stably to progeny for multiple generations (below), so these two lines were investigated further.

To simultaneously quantify the BAC copy number and roughly assess the integrity of the transgenes, we employed a quantitative PCR (Q-PCR) strategy. After breeding the lines for several generations to ensure that no further segregation of unlinked transgenes occurred, DNA was prepared from the tails of several transgenic littermates of each line. Q-PCR was performed on these DNA samples in triplicate using Sybr green (Applied Biosystems). PCR primer pairs were designed (Table 3.1) to amplify *Gata2* genomic sequences at ~50-kbp intervals throughout the length of the linked *G2BAC*. *Gata2* genomic sequences lying immediately beyond the boundaries of the *G2BAC* (at approximate positions corresponding to -230 and +270 kbp) as well as the β-actin gene were used as diploid copy number controls to ensure the validity of the assay. As shown in Fig. 3.2, transgenic line 2.2 appears to have incorporated three stably integrated transgene copies (three transgenic plus two endogenous = 5 copies), whereas line 2.12 appears to bear two. Given the similarity of the quantified results reflected by PCR primer pairs spread across the entire locus, we assume that at least one of the integrated BAC transgenes is intact in each line, but we performed no additional experiments (*e.g.* pulsed field Southern mapping) to test this hypothesis.

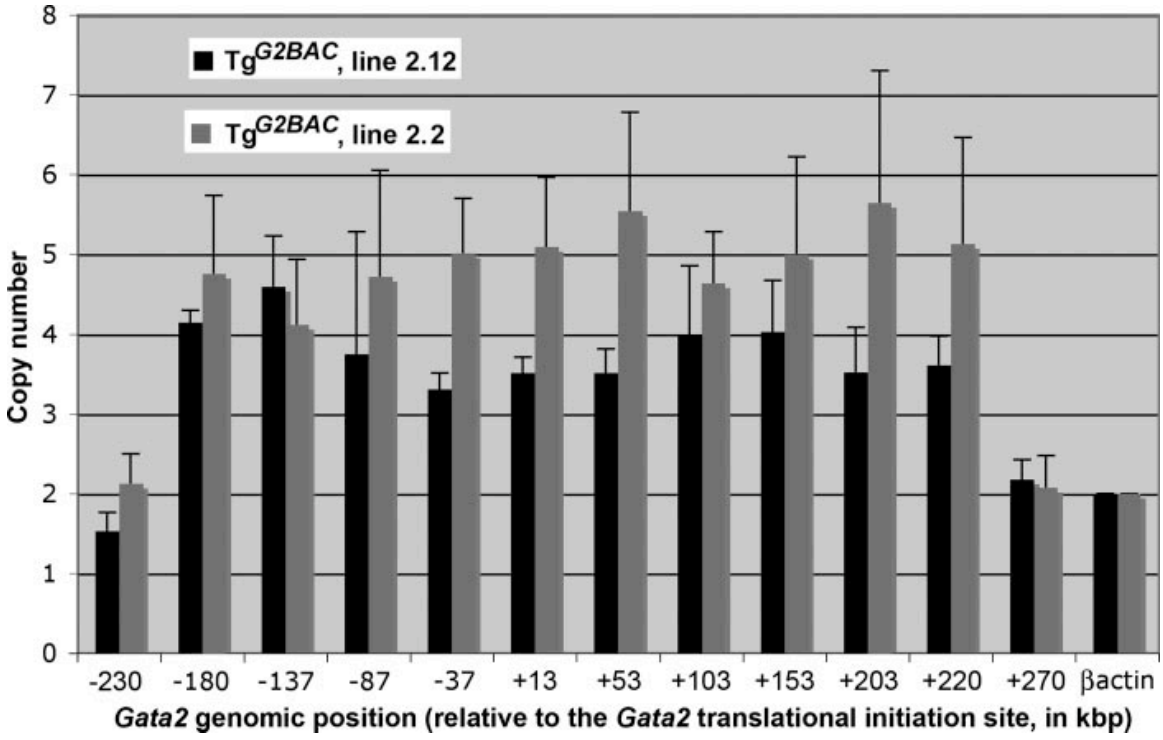

Figure 3.2 - *Gata2*-linked BAC copy number as determined by Q-PCR. Tail DNA was recovered from animals in which the transgenes in two different established lines (called 2.2 and 2.12) were stably transmitted to offspring for more than four generations. PCR primers were developed using Primer Express, which would detect unique sequences approximately every 50 kbp along the length of the linked BAC, as well as at positions outside the boundaries of the BAC (as a diploid copy number control; Table 3.1). The normalization strategy for each genomic DNA sample involved first standardizing the quantified PCR product from *Gata2*-specific primer pairs to that of the β-*actin* gene, and then normalizing the values of transgenic mice to those of wild-type controls.

Rescue of the Gata2 Null Mutation with a Linked BAC Transgene

A *Gata2* null mutant (EII-KI) mouse in which the green fluorescent protein (GFP) was integrated at the translational start site in *Gata2* exon II has been previously characterized (Suzuki et al., 2006). GFP expression was detected in all of the anticipated organs and tissues (Suzuki et al., 2006). We mated the two independent linked BAC transgenic (Tg^{G2BAC}) lines (2.2 and 2.12) to *Gata2* germ line mutant heterozygotes to generate $Gata2^{+/-}$:Tg^{G2BAC} compound mutant animals; none of the compound mutant offspring exhibited abnormal behavior. The transgenic heterozygotes were backcrossed again to *Gata2* heterozygotes to generate *Gata2* homozygous null mutant transgenic mice, which were genotyped by Q-PCR (Fig. 3.3*A*). The quantitative methodology was validated by breeding an F_3 $Gata2^{-/-}$:Tg^{G2BAC} (presumptive rescued null mutant) parent to wild-type females: all progeny contained one germ line mutant *Gata2* allele (Fig. 3.3*C*), thus demonstrating unambiguously that this parent was indeed of $Gata2^{-/-}$:Tg^{G2BAC} genotype.

Because a *Gata2* ureteric epithelial enhancer had not been previously identified (Khandekar et al., 2004), we speculated that rescue of the *Gata2* null mutants with the linked BAC might lead to full complementation of the hematopoietic phenotype, as we previously observed with a smaller transgenic YAC (Zhou et al., 1998). However, an additional expectation was that the BAC-rescued mice might also exhibit perinatal or postnatal urogenital deficiencies, given that a ureteric epithelial enhancer was not detected among the BACs surveyed in the original BAC-trap screen, and was therefore expected to lie somewhere outside the boundaries described by the linked BAC transgene (Khandekar et al., 2004).

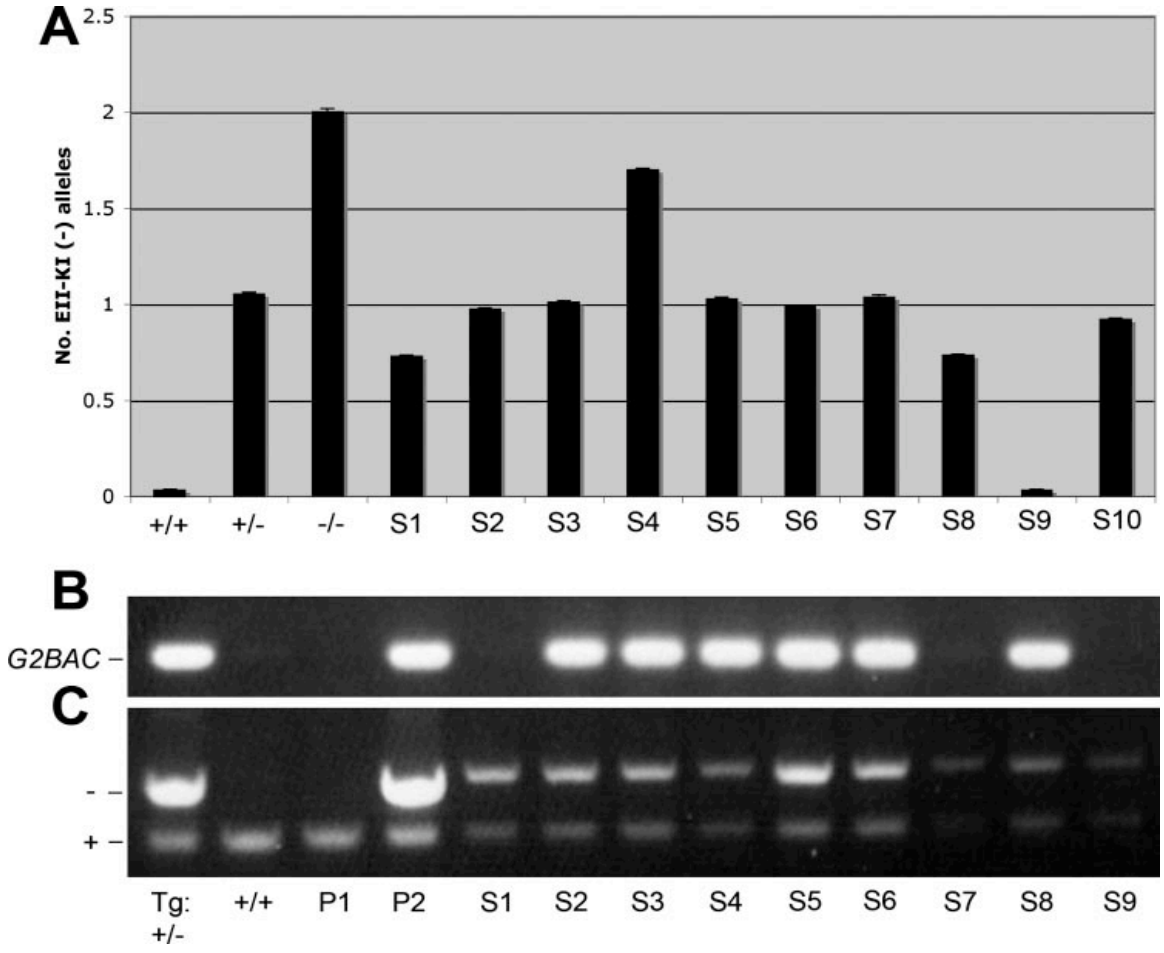

Figure 3.3 - BAC-complemented *Gata2*$^{-/-}$ mice are born in a Mendelian distribution. (A) Q-PCR was performed on genomic DNA samples prepared from the tails of sibling pups (*S1–S10*) from a presumptive *Gata2*$^{+/-}$:Tg^{G2BAC} *x Gata2*$^{+/-}$ intercross (primer sequences for the EII-KI allele are shown in Table 3.1, *Q-eGFP*); β-*actin* Q-PCR was used as a diploid copy number control. The strategy was designed to detect the EII-KI (*Gata2*$^{-}$) allele in all GFP^{+} mice (Table 3.1), and therefore wild-type mice have a ratio (*Gata2*$^{-}$/β-*actin*) of 0, EII-KI heterozygotes (*e.g.* both parents) have a ratio of 1, and EII-KI homozygotes (*Gata2* null mutant mice) have a ratio of 2. **(B and C)** Representative semi-quantitative PCR results depicting detection of the linked BAC transgene allele (*B*, using BAC5'jxn primers, Table 3.1) or the EII-KI (-) or wild-type (+) alleles (*C*, Table 3.1) from a presumptive *Gata2*$^{-/-}$:Tg^{G2BAC} X *Gata2*$^{+/+}$ intercross. Note that every sibling (*S1–S9*) from this mating carries a (-) germ line allele, whereas only six of nine sibs harbor a BAC allele, proving that the genotype of the original transgenic parent (*P2*) is *Gata2*$^{-/-}$:Tg^{G2BAC}. Note that the + allele can be contributed either through the germ line or from a BAC-derived *Gata2* gene.

After crossing the two independent Tg^{G2BAC} lines into the *Gata2* null mutant background, we were able to draw several conclusions. First, all genotypes were recovered at the anticipated Mendelian frequency from *Gata2*$^{+/-}$ x *Gata2*$^{+/-}$:Tg^{G2BAC} intercrosses (actual: 60:127:23; expected: 52:105:26, for *Gata2*$^{+/+}$ (both with and without the *G2BAC*), *Gata2*$^{+/-}$ (again, both with and without the *G2BAC*), and *Gata2*$^{-/-}$ (with the *G2BAC* only)) genotypes (Table 3.2), respectively, when performed with either of the

G2BAC transgenic lines. Second, BAC-complemented null mutant mice suffer from neither hematopoietic nor urogenital defects; neither do they exhibit any adult urological deficiency. Furthermore, *Gata2*$^{-/-}$:Tg^{G2BAC} mice exhibit normal life expectancy, body weights, and litter sizes (of expected Mendelian frequencies) compared with wild-type controls.

Given the lack of expression of any of these BAC and YAC transgenes in the ureteric epithelium (Khandekar et al., 2004), the observation that adult animals were generally normal was somewhat surprising, and therefore we collected, sectioned, and stained e14.5 embryos to specifically assess the early development of the ureter and the expression of the linked BAC transgenes. Hematoxylin and eosin staining showed that the overall morphology of the genitourinary system is unaltered in the *Gata2*$^{-/-}$:Tg^{G2BAC} embryos (Fig. 3.4, *A* and *E*). GFP expression (from the *Gata2* knock-in allele) localized to the same cells in BAC-complemented *Gata2*$^{-/-}$:Tg^{G2BAC} compound mutant animals as in heterozygous mutant (*Gata2*$^{+/-}$) controls (Fig. 3.4, *B* and *F*). However, much to our surprise, GATA-2 protein was expressed in both the urogenital epithelial and mesenchymal compartments in the *G2BAC*-rescued transgenic mice (Fig. 3.4,*C* and *G*). Although we anticipated that these compound mutant mice might be rescued by the linked BAC possibly via non-cell autonomous inductive effects (perhaps as a consequence of the mesenchyme inducing ureteric epithelial differentiation), on the basis of previous data we did not expect to observe GATA-2 expression in the epithelium (Khandekar et al., 2004). The previously characterized *lacZ*-tagged YAC transgene (called d16; Zhou et al., 1998) has boundaries that map to positions -174 to +73 kbp according to the revised genome sequence, and an overlapping BAC (333I12, from +60 to +268 kbp of the *Gata2* locus) was also previously examined in the "BAC-trap" *lacZ* reporter assay. These two sequences together encompass sequences represented by both the 5'BAC (115E9, -187/-10 kbp of the *Gata2* locus) and the 3'BAC (81F7, -34/+226 kbp) that we examined in this study, and in neither of the former reporter transgenic studies were we able to detect GATA-2 activity reflected as reporter staining in the ureteric epithelium. Thus the present data indicating that the linked BAC is able to confer tissue specificity for epithelial expression that is not observed in the BACs or YAC when they are analyzed individually (Khandekar et al., 2004).

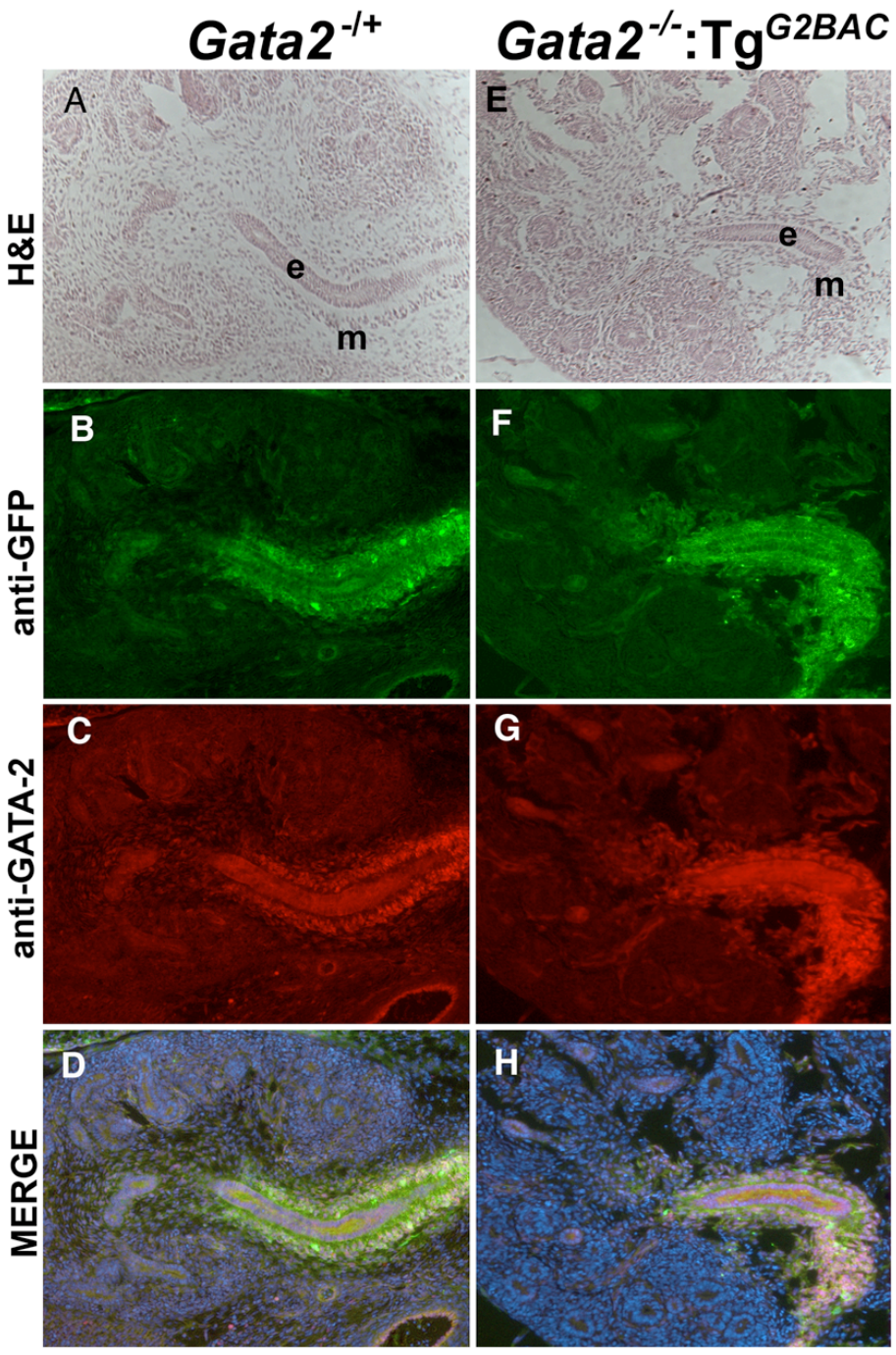

Figure 3.4 - The *G2BAC* transgene recapitulates mesenchymal and epithelial *Gata2* urogenital expression. Transverse cryosections of e14.5 *Gata2*$^{+/-}$ **(A–D)** and *Gata2*$^{-/-}$:Tg^{G2BAC} **(E–H)** embryos were subjected to co-immunostaining using anti-GFP (*B* and *F*) and anti-GATA-2 (*C* and *G*) antibodies, prior to hematoxylin and eosin histological staining (*A* and *E*). **(B and F)** GFP immunofluorescence reflects the endogenous GATA-2 expression pattern in the urogenital mesenchymal and epithelial cells of *Gata2*$^{+/-}$ and *Gata2*$^{-/-}$:Tg^{G2BAC} embryos. **(C and G)** GATA-2 protein was immunologically detected in the urogenital mesenchymal and epithelial compartments of the *Gata2*$^{+/-}$ embryo (*C*). In the *Gata2*$^{-/-}$:Tg^{G2BAC} embryo, anti-GATA-2 immunoreactivity was present not only in the urogenital mesenchyme, but also in the ureteric epithelium (*G*). **(D and H)** Merged images of anti-GFP and anti-GATA-2 immunofluorescence. *e*, ureteric epithelium; *m*, urogenital mesenchyme.

Discussion

GATA-2 is expressed in multiple tissues during vertebrate development and has been shown in several instances to be required for their differentiation. However, there are at least an equal number of examples of tissues in which this transcription factor is robustly expressed but also for which its *in vivo* requirement has not yet been completely elucidated. Because it is well established that *Gata2* null mutants expire from hematopoietic failure (Tsai et al., 1994) and that *Gata2* YAC-rescued null mutants, after surmounting the lethal hematopoietic block, expire from urogenital patterning failures (Zhou et al., 1998), it seemed reasonable to ask: if both of these embryonic deficiencies were complemented by a transgene bearing activities that would correct both deficiencies, would the animals survive? The alternative, which might have been expected in this case, is that the animals would probably not survive to reproductive age, but expire of a second perinatal lethal urogenital failure (due to the anticipated absence from the transgene of any urogenital epithelia-complementing activity), or even from a deficiency in some new tissue that was regulated by an enhancer located outside the boundaries circumscribed by the linked BAC.

Through the analyses of two independent transgenic lines, complementation of the germ line *Gata2* null mutation by the 413-kbp linked BAC appears to be complete: $Gata2^{-/-}$:Tg^{G2BAC} mice are born in the anticipated Mendelian ratio, they display no obvious physiological deficiencies, and they parent offspring without apparent difficulty. The size and general health of all viable progeny derived from $Gata2^{+/-}$ x $Gata2^{+/-}$:Tg^{G2BAC} intercross is indistinguishable from that of wild-type mice (data not shown, Table 3.2).

One major unexpected consequence resulting from the BAC linking was the observation that fusion of the two parental BACs led to *Gata2* expression in the urogenital epithelium, whereas all of our previous studies examining the expression of *lacZ*-tagged BACs and YACs that fully overlapped this domain gave no indication that a reporter gene could be expressed in that specific tissue (Khandekar et al., 2004; Zhou et al., 1998; Zhou et al., 2000). The current genetic and histological data support the notion that urogenital epithelial expression is conferred by two distinct, delocalized sequences,

one contained within each of the two parental BACs (Fig. 3.1), which become a functional enhancer element only when the two BACs are joined in *cis*. This novel concept of a delocalized synthetic enhancer has no precedent insofar as we are aware. Recent studies have also described the existence of domain boundaries, which is established by an array of *cis* elements (including insulators, boundary elements) that serve to delimit an active transcriptional state in a given region of the genome (Blanton et al., 2003; Cléard et al., 2006; Saitoh et al., 2000; West et al., 2004). Thus a possible scenario is that there is a single *Gata2* ureteric epithelial enhancer, which requires the full complement of domain boundaries present only in the linked BAC to activate GATA-2 expression in the ureteric epithelium. Further investigation into the mechanism by which the ureteric epithelial enhancer becomes functional promises to reveal novel and fundamental insights into transcriptional regulation.

We conclude from the data shown here that the urogenital deficiency detected in the YAC-rescued *Gata2*$^{-/-}$ mice is indeed the cause of their perinatal lethality (Zhou et al., 1998). We also conclude that *Gata2* enhancers that have been identified lying outside the -187/+226 kbp boundaries (Khandekar et al., 2004) encompassed by the *G2BAC* are unnecessary for viability or healthy reproductive status. The possibility that these even more distant enhancers control functions for which GATA-2 activity is redundant seems likely, but this hypothesis remains to be tested.

Finally, recombineering-based mutagenesis strategies to link overlapping BACs via homologous recombination have been previously described (Kotzamanis and Huxley, 2004; Sopher and La Spada, 2006; Zhang and Huang, 2003), although none of the earlier reports attempted transgenic rescue with the manipulated DNA. Here, we employed a somewhat different approach by utilizing cre-mediated homologous recombination to exploit the documented differential recombination efficiency between homologous *versus* heterologous loxP sequences to create a fully rescuing BAC transgene. This could provide the scientific community with a powerful genetic tool that could be used to quickly and efficiently mutate specific sequences *in vivo* in any rescuing transgene. Interrogation of genomic locus activity using these straightforward recombineering strategies could provide a much more rapid way of assessing the effects of any mutation when compared with the lengthy process involved in the generation of germ line mutants

by ES cell targeting, especially if one wishes to examine (*e.g.*) dozens of different mutations in the same genetic locus. Thus this BAC complementation strategy may offer several advantages over the creation of conditional germ line mutants for animals in which stable null mutations already exist.

Acknowledgements

Melin Khandekar, a former graduate student in the lab, initiated this work. He linked the BACs and created founders with the assistance of Xia Jiang. He did the structural analyses including pulsed field gel electrophoresis, restriction digest fingerprinting, and southern blotting. I performed the Q-PCR experiment, crossed the Tg^{G2BAC} mice in to the *Gata2* null mutant background, and performed all of the subsequent experiments.

Table 3.1: Oligonucleotides and primer sequences

Lox514pm	5' tataacttcgtataatgtacgctatacgaagaagt tatac 3'	5' aattgtataacttcttcgtatagcgtacattatacgaag ttatatgca 3'
Lox514nx	5' ggccgtaacttcgtataatgtacgctatacgaag ttatat 3'	5' ctagatataacttcgtatagcgtacattatacgaagt tac 3'
Famp	5' cgggatcctgaagttcctatactttctagagaata ggaacttcactaccgttaagggatt 3'	5' cgaattcgaagttcctattctctagaaagtataggaac ttcatggttttagacgtcag 3'
-13Lox514	5' cactaggccttggatgtttgtttgtcttcatcttagg tctcaccgcggtggcggccgtaac 3'	5' aaggcacaggcaacttgcagctggtgaccagaaca gagagtttaacctcgaatgtataa 3'
Bacdellox		5' gtcgctgtcgacggtgaccctatagtcgagggacct aatagcttgatctcgaatgtataa 3'
BacNeo	5' caagcactactgtcttaggctgtatgtatgtgcac tctttgctaccatggagaagttcctattc 3'	5' gcatcggtcgagcttgacattgtaggactatattgctc taagataactgatcagcttgaagttctt 3'
Famp2977	5' ttacgactgcacttctggcaggaggagcgactc aagccttgctcaagcttcgaa 3'	
Famp4233		5' catgtagcttgtgataaccacattgttgcctttggcttg caggggcggatcctgaagtt 3'
G2-13	5' ggcacgttcagttgtaaaca 3'	5' cccatatccactcagtacagag 3'
G2+200	5' accacaggcacttacacctcaa 3'	5' gcttctcacatgctagtcagagac 3'
BAC5'jxn	5' atcggtcgagcttgacattgta 3'	5' cctttgaggattacagacctcactc 3'
BAC-13	5' atggcaccagcagctctaggt 3'	5' cagagaagcttgatctcgaatgtataa 3'
CAT	5' cagtcagttgctcaatgtacc 3'	5' actggtgaaactcaccca 3'
Q-230 kb	5' tgttcactgctaaaggaggcc 3'	5' tggcaaggtctgtgagtcaca 3'
Q-180 kb	5' ggcagcctattagccacattg 3'	5' tagccacagcatttcccagc 3'
Q-137 kb	5' ccattctttctttccaccgtg 3'	5' ttggtggcgacctcacaac 3'
Q-87 kb	5' ctctcacccttcgtccctgtt 3'	5' cgctggatctcaggatgagc 3'
Q-37 kb	5' ttctgcatggccttctcctt 3'	5' ccattcccacagacctgctac 3'
Q+13 kb	5' tctccagagccaagggttca 3'	5' tctccaccaaagcttgccc 3'
Q+53 kb	5' ttttctcgacccctagctgg 3'	5' ttctggctctcgtcccaact 3'
Q+103 kb	5' tgtaggctccttccttctccc 3'	5' ggtaaggctatgctgtgccc 3'
Q+153 kb	5' acaaggatggcactgtgcatt 3'	5' ctgcacaagctgtgattggc 3'
Q+203 kb	5' gccactaaaagagcccatgc 3'	5' gcttagggccaacctgtcac 3'
Q+220 kb	5' tggcagtgatggccacatac 3'	5' ttgcccactgagctgtcttg 3'
Q+270 kb	5' ttgaaaaccgacaaccagcc 3'	5' ccagcgtccaaggtaagtgc 3'
Q β-actin	5' ccataggcttcacaccttcctg 3'	5' gcactaacactaccttcctcaaccg 3'
Q-eGFP	5' ctctgttatccagggccgc 3'	5' cggtgaacagctcctcgc 3'
wt allele	5' cgccgctgcgagtgtaagtttg 3'	5' cgaggctgctcggcgccac 3'
GFP allele	5' agcaagggcgaggagctgttcacc 3'	(same as wt allele)

Table 3.2: Progeny from F2 *Gata2*$^{+/-}$ X *Gata2*$^{+/-}$: Tg^{G2BAC} intercrosses

Line	Tg/Total	+/+ : +/- : -/- *
2.2	57/112	35/68/9
2.12	58/98	25/59/14

*These numbers include both Tg^{G2BAC}-positive and -negative pups. Of the *Gata2*$^{-/-}$ progeny, only those bearing the Tg^{G2BAC} are born, as *Gata2*$^{-/-}$ embryos die during mid-gestation.

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Chapter 4

In Pursuit of the Elusive Hemangioblast

Introduction

The "hemangioblast," hypothesized to exist almost a century ago, is a cell capable of differentiating into both hematopoietic and endothelial cells. During primitive hematopoiesis, these two distinct cell types arise simultaneously from the blood islands of the yolk sac (Murray, 1932; Sabin, 1920). In definitive hematopoiesis, hemangioblasts are believed to differentiate from the ventral mesoderm surrounding the floor of the dorsal aorta (Garcia-Porrero et al., 1995; Tavian et al., 1996; Tavian et al., 1999), from which hematopoietic precursors can be seen budding into the lumen of the dorsal aorta from the endothelium. This has been demonstrated in chick (Dieterlen-Lievre, 1975; Dieterlen-Lièvre and Le Douarin, 2004; Jaffredo et al., 1998), mice (Cumano et al., 2001; Dieterlen-Lievre et al., 1994; Ling and Dzierzak, 2002; Taoudi and Medvinsky, 2007), as well as humans (Tavian et al., 2001).

Evidence for the existence of hemangioblasts is supported by studies of genes that affect both the vasculature and hematopoiesis. Null mutant zebrafish for the gene *cloche* lack an endocardium, and have a reduced number of blood cells (Stainier et al., 1995). The receptor tyrosine kinase Flk1 was originally believed to be endothelial specific (Millauer et al., 1993); however, *Flk1*-null mutant mice present a dual phenotype: they die between embryonic days 8.5-9.5 from a lack of organized blood vessels along with a severely reduced number of hematopoietic progenitors (Shalaby et al., 1995). Conversely, the transcription factor SCL was originally shown to be required for hematopoietic development in mice (Robb et al., 1995; Shivdasani et al., 1995), but upon hematopoietic rescue of *Scl*-null mutant embryos, SCL was also found to be required for remodeling of the yolk sac vasculature (Visvader et al., 1998), and has since been shown

to be important for vasculogenesis (Patterson et al., 2005), including endothelial cell migration and morphogenesis (Lazrak et al., 2004).

Most of our information on the possible origin of hemangioblasts has been provided through *in vitro* studies. Embryonic stem cell-derived embryoid bodies, in response to vascular endothelial growth factor (the ligand for Flk1), give rise to blast colony-forming cells (BL-CFC), which in turn can directly differentiate into hematopoietic and endothelial precursors (Choi et al., 1998; Park et al., 2005). Furthermore, BL-CFC's were enriched in a $Flk1^{+}SCL^{+}$ cell population (Chung et al., 2002). $Brachyury^{+}Flk\text{-}1^{+}$ cells isolated from the primitive streak of a developing mouse embryo show a similar bipotential differentiation pattern in culture (Huber et al., 2004). The only *in vivo* lineage tracing study to date comes from single-cell-resolution fate maps in zebrafish, which show that individually marked cells along the ventral mesoderm can contribute to both hematopoietic and endothelial lineages (Vogeli et al., 2006).

The question remains as to whether definitive hemangioblasts give rise directly to hematopoietic and endothelial cells as is seen *in vitro* and in the blood islands of the yolk sac, or whether the hemangioblast first differentiates into a "hemogenic endothelium" lining the dorsal aorta, where the endothelium itself has hematopoietic potential (Jaffredo et al., 2005; Marshall and Thrasher, 2001). Recent evidence supports the latter hypothesis, as blast colony forming cells have recently been found to have an intermediate, transiently adherent population that would be consistent with expectations from the *in vivo* hemogenic endothelium model (Lancrin et al., 2009). Direct labeling of mesodermal origin cells *versus* the endothelium in mice has also shown that the budding hematopoietic cells arise specifically and uniquely from the endothelial cell population (Zovein et al., 2008).

A number of genes have now been shown to have dual requirements for endothelial and hematopoietic development, including *Flk1, Scl,* and *Runx1*. The fact that a number of these factors are known to interact with the hematopoietic transcription factor GATA-2, and that GATA-2 plays a role in vascular development (Chapter 2) (Dorfman et al., 1992; Khandekar et al., 2007; Umetani et al., 2001), it seemed reasonable to speculate that GATA-2 might play a role in the definitive hemangioblast or hemogenic endothelium development as well.

Multiple organ systems require GATA-2 expression for proper development. GATA-2 is critical for the establishment of primitive hematopoiesis (Tsai et al., 1994), proper urogenital patterning (Khandekar et al., 2007), and V2-interneuron development (Zhou et al., 2000), among many other developmental contributions. Genetic information specified within the 300 kilobase pairs (kbp) surrounding the *Gata2* genetic locus contains regulatory elements critical for proper embryonic development (Brandt et al., 2008), with other (presumably less critical) *Gata2* regulatory elements known to reside outside these boundaries (Khandekar et al., 2004).

A *Gata2* enhancer element was recently described that confers vascular-specific expression (Chapter 2) (Khandekar et al., 2007). Interestingly, this same element had already been shown to drive GATA-2 expression in erythroid cell lines (Grass et al., 2006). Enhancer elements having a dual role in hematopoietic and endothelial development are not without precedent; for example, the transcription factor *Scl* contains an element with similar specificities (Göttgens et al., 2004). To better understand the role of this *Gata2* enhancer element, as well as to address the possible developmental roles of GATA-2 in hemangioblast and endothelial development, we generated transgenic mice in which the *Gata2* endothelial enhancer was flanked by insulator elements in a plasmid in which the fluorescent reporter protein mCherry and CRE/ERT2 (the cre recombinase gene fused in frame to a second generation tamoxifen-sensitive estrogen receptor ligand binding domain) were expressed from the Herpes Simplex Virus (HSV) Thymidine Kinase promoter. By feeding pregnant dams tamoxifen early in embryogenesis, I anticipated that this transgene would specifically mark the hemangioblast population of cells – along with progeny hematopoietic and endothelial cells - when crossed to a reporter strain of mice.

These transgenic mice were first used to examine the role of *Gata2* specifically in vasculogenesis, not only through the hemangioblast, but also potentially through a separate angioblast pathway. This alternate pathway is hypothesized because endothelial cells lining the dorsal aorta appear to develop normally in *Gata2* null mutant mice (Tsai et al., 1994), while broadly expressing GFP when knocked-in to the translational start site of *Gata2* (Suzuki et al., 2006). Given the early embryonic lethality of *Gata2* null mutant mice, transgenic mice in which the *Gata2* vascular enhancer directed cre recombinase

expression could be used to conditionally delete *Gata2* specifically in the vasculature at various stages of embryonic or postnatal development by tamoxifen induction. The results of these experiments should help to elucidate the role played by GATA-2 in vasculogenesis, as well as lead to detection of a phenotype associated with hematopoietic cells derived from the putative hemangioblast should it exist.

This work is the first to directly address the role of GATA-2 in hemangioblast and vascular development in a mammalian model *in vivo*. The results of these experiments could redefine our knowledge of the earliest phases of blood and vascular development, as well as provide new insights into the function of this transcription factor. Finally, given the fact that GATA-2 is known to be vital for cell proliferation (Tsai and Orkin, 1997), these results could also play a critical role in understanding the propagation of certain types of cancer, such as hemangioblastomas.

In the present experiments we show that a transgenic mouse has been generated in which the *Gata2* gene can be conditionally ablated in the vasculature; this mouse was created in a way that allows simultaneous fluorescent marking of all endothelial cells. The data show that GATA-2 plays a significant role in vascular development, predominantly through secondary branching of the primary vasculature. These studies address the possibility of a common developmental origin for the hematopoietic system and the vasculature, and provide evidence of the molecular properties that could contribute to defining the hemangioblast, hemogenic endothelium and early definitive hematopoietic cells. This study, though not without its own caveats, adds weight to the hypothesis that the definitive hemangioblast exists.

Materials and Methods

Insulated *Gata2* Vascular Enhancer Transgene Construction

Sac1 and HindIII sites from the multiple cloning site of pNEB193 (New England Biolabs) were blunted sequentially to augment future cloning steps. Duplicated chicken HS4 insulators were then removed from pJC13-1 (Chung et al., 1993) with BamH1 and EcoR1 and blunt-end ligated into the Sma1 site of the modified pNEB193 plasmid. A second set of chicken HS4 insulators was subsequently blunt-end ligated into the Pme1

site of the modified pNEB193 plasmid to generate a general Insulator Vector (IV) transgene.

To generate the vascular enhancer construct, a previously described transgene construct, called pTKSXβ (Khandekar et al., 2007), was generated by adding the *Gata2* vascular enhancer to pTKβ (Clontech). pTKSXβ was digested with Not1, removing the LacZ sequence. *CreERT2*, taken from plasmid pCreERT2 (Feil et al., 1997; Hayashi and McMahon, 2002) by digestion with EcoR1, was blunt-end ligated into the Not1 site of pTKSXβ, generating the construct Vascular Enhancer – CreERT2 (VEcre). To generate a reporter construct, the fluorescent reporter *mCherry-N1* (Shaner et al., 2005) was digested with BamH1 and Not1 and blunt-end ligated into the same Not1 location of pTKSXβ, generating Vascular Enhancer – Cherry (VEcherry).

Both the VEcre and VEcherry constructs were then digested with PvuII and Sph1 and blunt-end ligated into the Pac1 site of IV to generate insulated versions of each construct: iVEcre and iVEcherry, respectively. All ligation points were verified by sequencing.

Transgenic and Knock-out Mice

To generate transgenic animals, the iVEcre and iVEcherry expression constructs were digested with Fsp1, purified, and microinjected at equal molar ratios into fertilized eggs obtained by mating (C57BL/6 x SJL)F1 female mice with (C57BL/6 x SJL)F1 male mice. Pronuclear injection was performed as described (Nagy et al., 2003). To identify transgenic animals, DNA from yolk sac or tail snip of founder animals was used for genotyping by PCR for both Cre and mCherry sequences. Cre primers used are: sense 5'-ggctgccacgaccaagtgac-3' and anti-sense 5'-cgtgagatatctttaaccctgatc-3'. mCherry primers are sense 5'-acggtactcgaggaactgaa-3' and anti-sense 5'-gtgggaggtgatgtccaact-3'. For stable lines expressing both iVEcre and iVEcherry (hereafter referred to simply as iVE), F2 and subsequent generations of progeny were examined for transgene copy number by quantitative real-time PCR as previously described (Brandt et al., 2008). Heterozygous and homozygous *cre* knock-in animals were used for copy number controls.

Gata2^{gfp} (Suzuki et al., 2006) and *Rosa26R*^{LacZ} (Soriano, 1999) mice have been previously described.

For collection at embryonic day 10.5 (e10.5), approximately 2.5 mg of tamoxifen was administered to the pregnant dam via gavage for four days starting at e6.5. Administration of 5 mg per day resulted in almost complete resorption of the embryos by e10.5 (data not shown). X-gal staining was performed as previously described (Lakshmanan et al., 1999).

For the lineage trace, 2.5 mg of tamoxifen was administered to the pregnant dam via gavage on embryonic days 6.5, 7.5, and 8.5. The fetal liver and spleen of e17.5 embryos were then collected and made into a single cell suspension. Beta-galactosidase activity in single cells was detected with fluorescein di-β-D-galactopyranoside (FDG, Invitrogen). FDG- loaded cells were incubated with FcBlock (BD Biosciences) and then incubated with lineage antibodies for 20 minutes. The following antibodies were used: CD31-APC (clone 390, 0.5ul), B220-APC (RA3-6B2, 0.6ul), CD3-biotin (145-2C11, 0.5ul), Gr1-biotin (RB6-8C5, 0.1ul), Mac1-biotin (M1/70, 0.1ul) and TER119-APC (TER-119, 0.6ul) (all concentrations are per 1 million cells; all antibodies are from eBiosciences). Biotin-conjugated antibody samples were rinsed and counterstained with streptavidin-APC (0.15μl; BD Pharmingen) for another 20 minutes. All cells were subsequently counterstained with DAPI for dead cell exclusion.

Immunohistochemistry

Embryos were harvested at the times indicated in the text and figure legends, and processed for immunohistochemistry as previously described (Khandekar et al., 2004). Cryosections were stained with rabbit anti-GFP (1:500; Molecular Probes), rat anti-Flk1 (1:500; BD Pharmingen), rat anti-PECAM-1 (1:500; BD Pharmingen), rat anti-CD144 (1:500; BD Pharmingen), and rat anti-CD41 (1:500; BD Pharmingen) antibodies overnight at 4°C and were subsequently detected using the appropriate fluorochrome-conjugated secondary antibodies at 1:200 dilution. Digital images were recorded as previously described (Khandekar et al., 2004).

Results

Generation and Characterization of Insulated *Gata2* Vascular Enhancer-directed Transgenic Mice

To better address the role of GATA-2 in endothelial - and potentially in hemangioblast - development, a conditional knockout strategy for *Gata2* inactivation was desired in order to circumvent the early hematopoietic deficiency seen in germline *Gata2* null mutant mice (Tsai et al., 1994). To this end, the *Gata2* vascular enhancer (described in Chapter 2) was used to generate a tamoxifen inducible, vascular-specific *cre* construct. The HSV thymidine kinase promoter was chosen because it does not have intrinsic enhancer activity (Majumder and DePamphilis, 1994), unlike the *Gata2* 1S and 1G promoters (Minegishi et al., 1998). CreERT2 was chosen to specifically delete *Gata2* in the vasculature because it is insensitive to endogenous estrogens and is four-fold more sensitive to tamoxifen than the original Cre/ER fusion protein (Feil et al., 1997). Duplicated chicken HS4 insulator elements (Chung et al., 1993) were placed at either end of the insert in an attempt to prevent position of integration effects. An identical construct carrying the mCherry reporter gene instead of Cre/ERT2 was also generated, providing a convenient marker for transgene expression, along with a vasculature-specific reporter (Fig. 4.1, *A* and *B*).

Pronuclear injection of equal concentrations of both constructs into (C57BL/6 x SJL) zygotes resulted in 44 live births. Of these, 13 carried both transgenes, as determined by PCR for both Cre and mCherry sequences. Copy number analysis of the stably transmitting lines was performed for Cre using quantitative real time PCR (qPCR). Based on this assay, the number of insulated vascular enhancer/cre (iVEcre) transgene copies integrated into the genome ranged from five (iVE62) to approximately forty-seven copies (iVE457) (Fig. 4.1*C*). Since the iVEcre and iVEcherry transgenes are approximately the same length and were co-injected at equal molar ratios, we expected that they would be approximately the same copy number; however, we did not determine the number of mCherry transgene copies. The iVE56, iVE62, and iVE73 lines showed endothelium-specific staining based on whole mount e10.5 embryo mCherry expression (data not shown). The iVE62 line expressed mCherry specifically in the vasculature at a

level that was approximately four times brighter than the other two lines (Fig. 4.1*E*). All subsequent experiments were carried out using this line of mice, and it will be referred to subsequently as Tg^{iVE}.

Given that insulators, by definition, shield genes (or transgenes) from integration position effects, it was expected that the copy number of the iVE constructs would directly reflect the protein expression level in transgenic mice. However, all high copy number lines showed little to no mCherry expression (Fig. 4.1*D* vs. 4.1*E*). The negative mCherry expression was not due to cellular toxicity, as transgene positive embryos were recovered in a normal Mendelian distribution and appeared to be physiologically normal. Adult mice were also recovered from high copy number expressing lines and did not display any phenotypic abnormalities. Other lines containing a similar low copy number to iVE62 did not express mCherry at similar levels (Fig. 4.1*E* vs. 4.1*F*). These data indicate that the chicken HS4 insulators are ineffective in preventing position of integration effects for these transgenes.

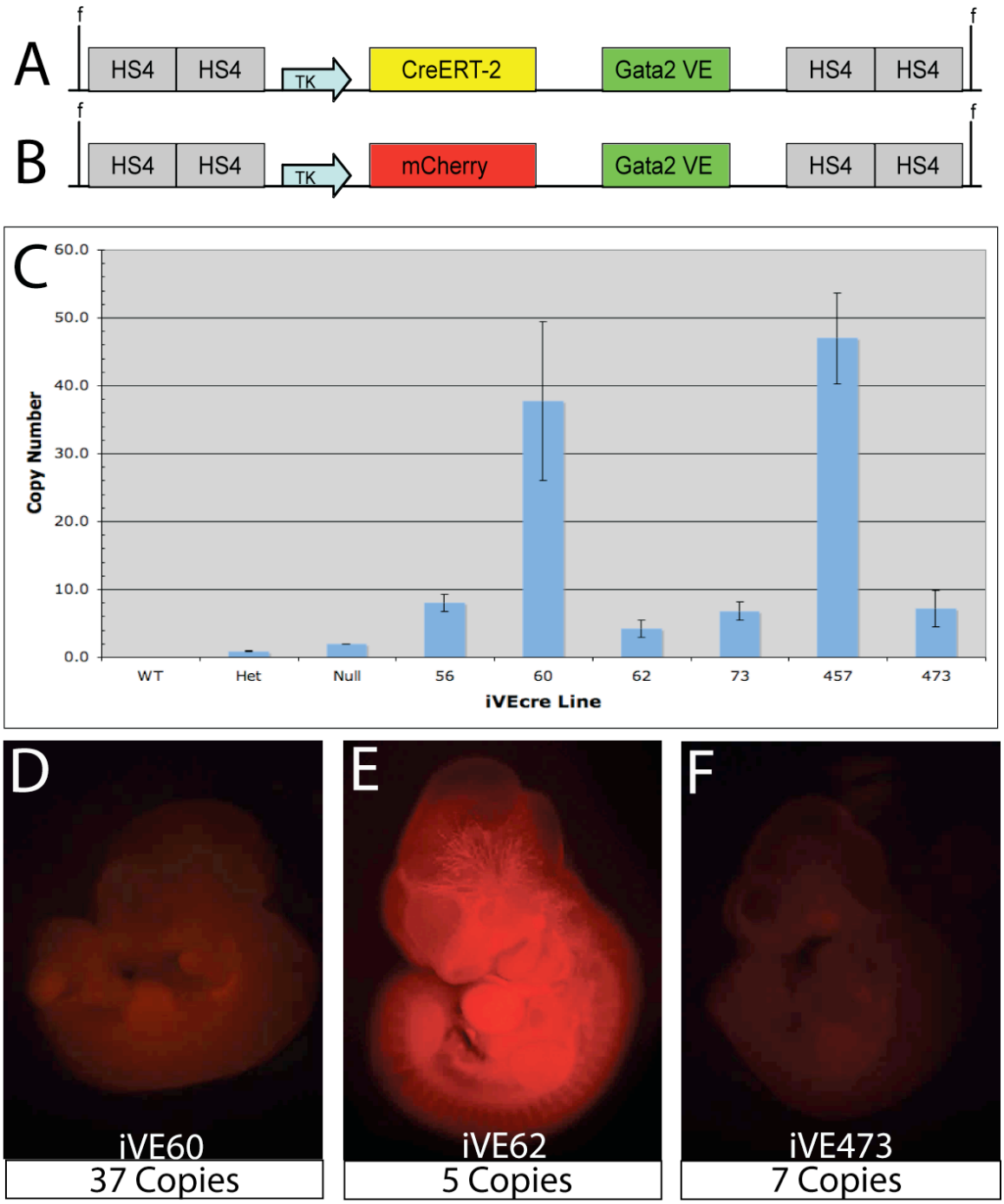


Figure 4.1 – Generation and characterization of iVE transgenic mice. (A and B) Transgenes created to express Cre/ERT2 (A) or mCherry (B) under the control of the *Gata2* vascular enhancer (VE) using the Herpes Simplex Virus Thymidine Kinase (TK) promoter. These transgenes are insulated by duplicated chicken HS4 insulator elements. The constructs were digested with Fsp1 (f) and co-injected into fertilized eggs to generate Tg^{iVE} founder animals. **(C)** Copy number of the Cre transgene was analyzed using quantitative real-time PCR from DNA prepared from each of the iVE transgenic lines. **(D-F)** Whole mount mCherry expression in founder embryos. (D) iVE60 has the highest copy number, but displays low fluorescent mCherry expression. (E) iVE62 has the lowest copy number, but expresses mCherry the strongest of all of the transgenic lines in an endothelial specific manner. (F) Transgenic line iVE473 has a similar copy number to iVE62, but does not express mCherry. WT – wild-type.

To confirm our original impression that the iVE transgenes were expressed throughout the vasculature, and to compare the vascular patterning profile of a wild-type animal to a *Gata2* null mutant animal, confocal microscopy analysis was performed. The Tg^{iVE}:$Gata2^{+/-}$ mouse was crossed with a $Gata2^{gfp/+}$ animal, which produces eGFP in all GATA-2 expressing cells (Suzuki et al., 2006). Confocal analysis of an e10.5 transgenic embryo expressing the iVE transgene (red) and eGFP (green) is shown in Figure 4.2. Specific staining can be seen in the endothelium throughout the intersomitic vasculature of the tail bud (Fig. 4.2*A*) as well as the head region (Fig. 4.2*B*) of an e10.5 embryo. GATA-2/mCherry and GFP expression overlap in the head vasculature, while GFP expression outside the vasculature can be seen in the developing central nervous system (Fig. 4.2*B*).

The vasculature of the *Gata2* null mutant mice showed a significant divergence from the heterozygous control animals. *Gata2* null mutant animals appear to produce the large vessels of the intersomitic vasculature of the tail bud, but fail to undergo vascular remodeling or angiogenesis (Fig. 4.2*A* vs. *C*). A similar phenotype is observed in the head vasculature, where remodeling of the vasculature appears to be normal in heterozygous controls; in these mice, large vessels can be seen extending into the furthest reaches of the brain and branching into the fine vasculature (Fig. 4.2*B*). However, the vasculature of *Gata2* null mutant embryos appears to be aberrant, with the large vessels failing to remodel into the fine vasculature (Fig. 4.2*D).* This robust phenotype was surprising as the effect of GATA-2 on vascular patterning was expected to be subtle at best. The caveat to these results is that to date this is the only embryo I have been able to examine through this process. More samples will need to be analyzed before the validity of the *Gata2* null mutant results can be confirmed.

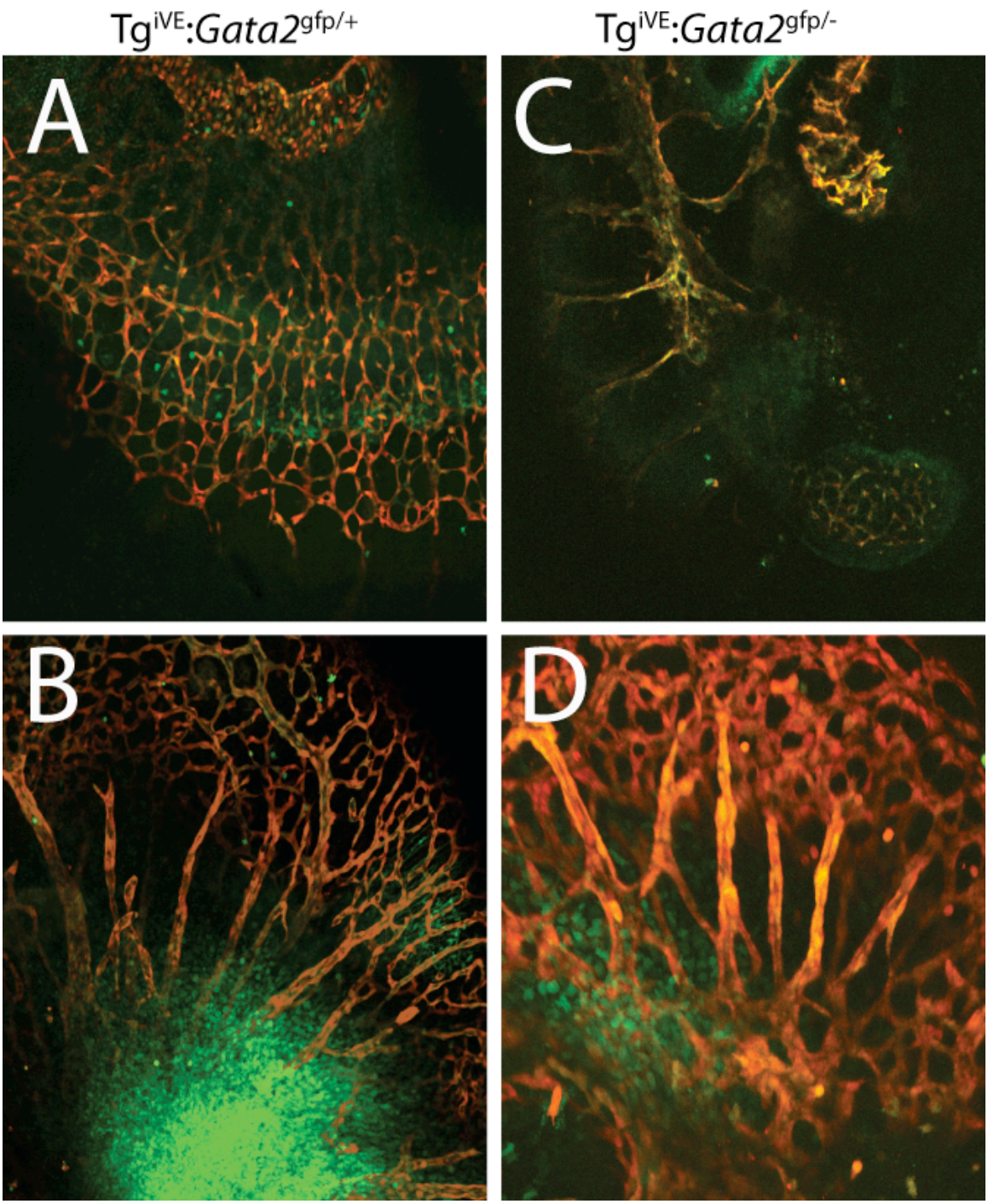


Figure 4.2 – Vasculature of e10.5 *Gata2* heterozygous *versus* homozygous null mutant embryos. (A and C) Confocal microscopy of the intersomitic vasculature in the tail bud of Tg^{iVE}:$Gata2^{gfp/+}$ (A) and Tg^{iVE}:$Gata2^{gfp/-}$ (C) e10.5 embryos, with the fluorescence of mCherry from the iVE transgene analyzed in red and the fluorescence of GFP from the *Gata2* knock-in (null) allele in green. **(B and D)** Confocal analysis of the head vasculature of Tg^{iVE}:$Gata2^{gfp/+}$ (B) and Tg^{iVE}:$Gata2^{gfp/-}$ (D) e10.5 embryos. GFP (green) represents the entire GATA-2 expression profile, while iVE (red) expression reflects GATA-2 driven from the vascular enhancer. While co-labeling of mCherry and GFP is seen throughout the vasculature, independent GFP staining is also visible.

Cre Activity in iVE Transgenic Mice

While mCherry appeared to be expressed specifically in the vasculature, the Cre expression pattern required independent validation. To test the efficacy of the iVEcre transgene, iVE male mice were mated to *Rosa26R*^{lacZ} females (Soriano, 1999). e10.5 embryos were collected and stained with X-gal overnight. The gross expression pattern of *lacZ* clearly mimics that of mCherry (Figure 4.1*E* vs 4.3*A*) using the current tamoxifen protocol. Cre/ERT2 is capable of activating β-gal expression in the endothelium of the dorsal aorta, as shown by transverse sections of X-gal stained e10.5 dpc embryos. Interestingly, it appears that a few hematopoietic cells are also lacZ positive (Figure 4.3*B;* arrows). Flow Cytometry analysis was also performed in order to compare the expression pattern of mCherry to CD31 (a pan-endothelial marker) in whole e10.5 embryo single cell suspensions (Fig 4.3*C*). In this experiment, an average of 68% of mCherry positive cells express the endothelial specific marker CD31. The excision efficiency of Cre/ERT2 could not be confirmed due to technical issues in this experiment, but new experiments circumventing these limitations are currently under investigation.

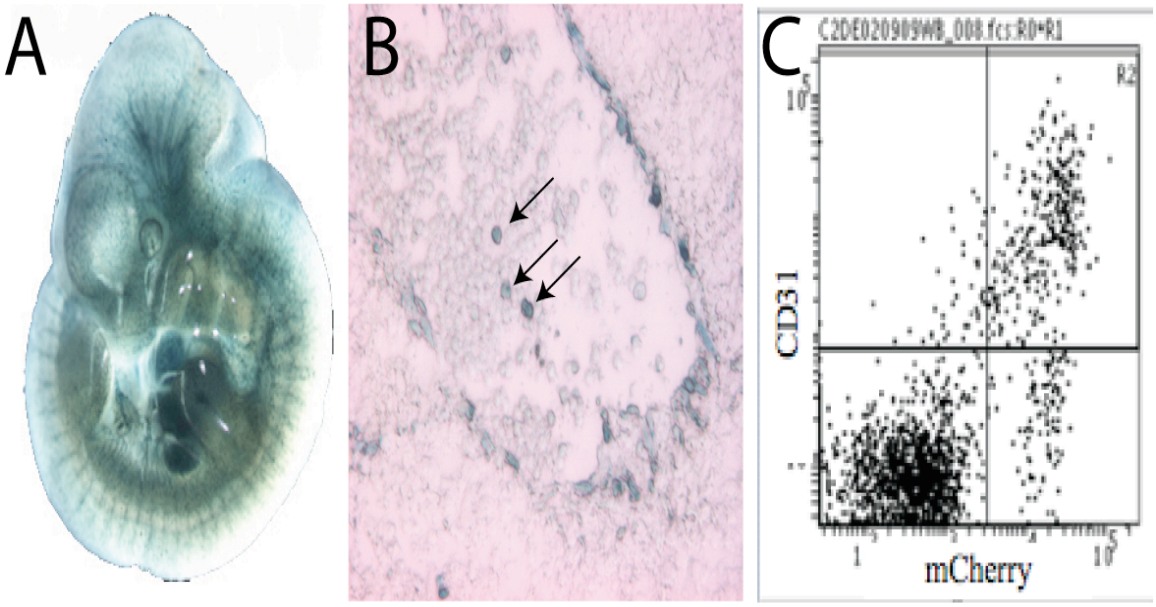


Figure 4.3 – Characterization of Cre activity in iVE transgenic mice. (A) An e10.5 embryo from a cross of Tg^{iVE}:*Gata2*$^{+/+}$ with *Rosa26R*^{lacZ} treated with 2.5 mg of tamoxifen from e6.5 to e9.5 dpc and stained with X-gal solution overnight. **(B)** Transverse section of the Tg^{iVE}:*Rosa26R*^{lacZ} embryo showing lacZ staining in the endothelium of the dorsal aorta. The neural tube is oriented to the top of the image. **(C)** FACS analysis of whole e10.5 embryo single cell suspension shows that 68% of the mCherry positive cells express the endothelial-specific marker CD31.

Vascular and Hematopoietic cell Staining in the e10.5 Dorsal Aorta

GATA-2 is predominantly expressed in hematopoietic precursors, including the budding hematopoietic clusters of the embryonic dorsal aorta (Khandekar et al., 2007). A number of other transcription factors have also been shown to be critical for hemangioblast development, including Flk1 (Shalaby et al., 1995), VE-Cadherin (Breier et al., 1996), SCL (Göttgens et al., 2004), and RUNX1 (Lacaud et al., 2002). We attempted to localize the presumptive hemangioblast, and hemogenic endothelium, with a combination of antibodies that recognize proteins thought to be important for hemangioblast development. Based on expression studies in embryonic stem cells, GATA-2 expression is elevated upon BMP4 induction, followed temporally by Flk1 and SCL (Lugus et al., 2007). There is also some evidence that BMP4 regulates the differentiation of hemangioblasts by controlling the level of c-kit (Marshall et al., 2007). Unfortunately, an antibody specific for BMP4 in immunohistochemistry is not available. GATA-2 expression was analyzed using the previously described *Gata2*^{gfp} knock-in mice.

Flk1 and Gata2

Flk1 (fetal liver kinase, also known as Vegfr2) is a receptor tyrosine kinase responsive to vascular endothelial growth factor (VEGF) and is expressed primarily on proliferating endothelial cells (Millauer et al., 1993), but is also found on hematopoietic precursors *in vitro* (Kabrun et al., 1997). *Flk1*-null mutant mice die around e8.5 with defects in both hematopoietic and vascular development (Shalaby et al., 1995). Flk1 is also a direct target of the GATA factors (Kappel et al., 2000). In attempting to delineate the role of *Flk1* in hemangioblast and hemogenic endothelium development, we analyzed Flk1 expression in the dorsal aorta of e10.5 embryos in the presence and absence of GATA-2. To do this, the *Gata2* GFP knock-in was employed to generate *Gata2* heterozygous and homozygous null mutant mice. Analysis of these mice with respect to Flk1 and GATA-2 (GFP) expression revealed three interesting observations.

First, the number of endothelial cells, along with the expression level of GFP and Flk1, is significantly increased in the absence of GATA-2 (Fig. 4.4*ii*) when compared to *Gata2* heterozygous (Fig. 4.4*i*) mutant mice at e10.5. Second, budding hematopoietic

cells do not express Flk1, but robustly express GFP (Fig. 4.4*iii*). Interestingly, the level of Flk1 in the endothelium surrounding the budding hematopoietic cells seems to be reduced as well (Fig. 4.4*iii* vs *iv*), similar to the expression level of Flk1 in the roof of the dorsal aorta (Fig. 4.4*i*). Third, Flk1 is expressed in cells invading the endothelium of the dorsal aorta, while GFP is either not expressed, or is expressed at a lower level, in both *Gata2* heterozygous (Fig. 4.4*iv*) and homozygous null (Fig. 4.4*v*) mutant mice. Flk1 expression in the endothelium does not appear to be reduced in *Gata2* null mutant embryos (Fig. 4.4*iv* vs *v*). No hematopoietic cells can be seen budding from the dorsal aorta in *Gata2* homozygous null mutant animals (Fig. 4.4, data not shown).

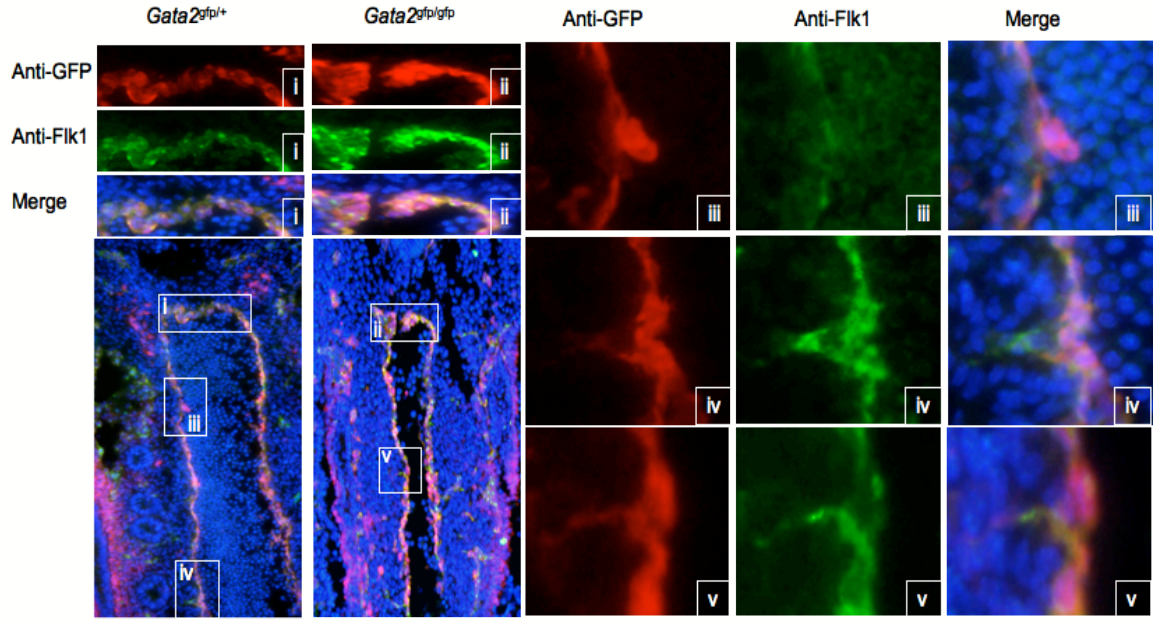


Figure 4.4 – Staining of GATA-2 (GFP) and Flk1 in the e10.5 dorsal aorta in *Gata2* heterozygous and homozygous null mutant mice. (i and ii) GFP and Flk1 expression are compared in the roof of the dorsal aorta of *Gata2*$^{gfp/+}$ (i) and *Gata2*$^{gfp/gfp}$ (ii) embryos. Both antigens are more highly expressed in the *Gata2* null mutant background, and appear to generate a larger number of endothelial cells. **(iii)** GFP is expressed in cells budding into the lumen of the dorsal aorta while Flk1 is restricted to the endothelium. **(iv and v)** Flk1 is expressed in cells invading the endothelium of the dorsal aorta while GFP is either not expressed, or only weakly so, in both the *Gata2* heterozygous (iv) and homozygous (v) null mutant background. All sections are oriented with the neural tube at the top of the image.

VE-cadherin and Gata2

VE-Cadherin (vascular endothelial cadherin, also known as CD144) is expressed exclusively on endothelial cells, and more specifically at the cell-cell junctions to promote cell adhesion, vascular integrity, and to control vascular permeability (Breier et al., 1996; Dejana et al., 1999). Early studies have shown that VE-Cadherin is not

expressed in hematopoietic precursors (Lampugnani et al., 1992), but is found in the *in vitro* equivalent of the hemogenic endothelium, which gives rise to hematopoietic precursors of the AGM (Fraser et al., 2002; Nishikawa et al., 1998). VE-Cadherin was also reported to co-localize with the hematopoietic specific factor CD45 under certain conditions (Fraser et al., 2003; Kim et al., 2005; Taoudi et al., 2005), but this was not true *in vivo* in our hands (data not shown).

In fact, VE-Cadherin is not expressed on budding hematopoietic cells either, in contrast to GFP expression (Fig. 4.5*iii*). However, VE-Cadherin is still strongly expressed in the endothelium around the budding hematopoietic cells, in contrast to Flk1 (Fig. 4.5*iii* vs. Fig. 4.4*iii*). VE-Cadherin is expressed on cells invading into the endothelium of the dorsal aorta, where GFP is only weakly expressed, in both *Gata2* heterozygous (Fig. 4.5*iv*) and homozygous null (Fig. 4.5*v*) mutant mice. The roof of the dorsal aorta in the *Gata2* null mutant embryos again contains a larger number of endothelial cells than wild type embryos, and expresses GFP and VE-Cadherin at a much higher level in *Gata2* null mutants (Fig. 4.5*ii*) than in the heterozygous control (Fig. 4.5*i*).

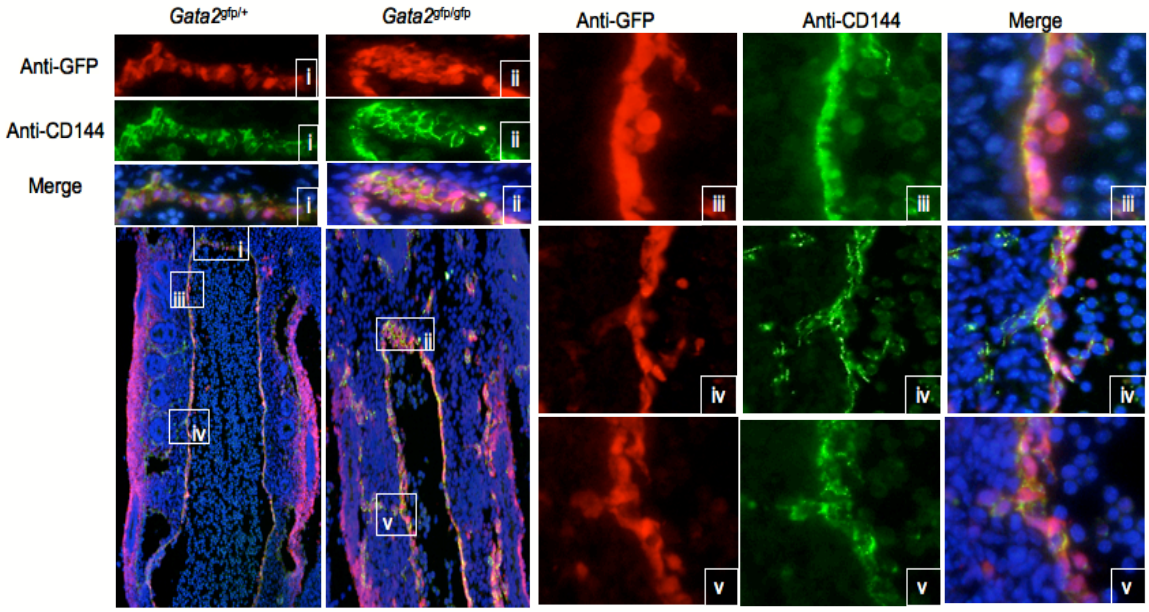


Figure 4.5 – Staining of GATA-2 (GFP) and VE-Cadherin in the e10.5 dorsal aorta in *Gata2* heterozygous and homozygous null mutant mice. **(i and ii)** GFP and VE-Cadherin expression are compared in the roof of the dorsal aorta of *Gata2*$^{gfp/+}$ (i) and *Gata2*$^{gfp/gfp}$ (ii) embryos. Both antigens are more highly expressed in the *Gata2* null mutant background, and contain more numerous endothelial cells. **(iii)** GFP is expressed in cells budding into the lumen of the dorsal aorta while VE-Cadherin is restricted to the endothelium. **(iv and v)** VE-Cadherin is expressed in cells invading the endothelium of the dorsal aorta while GFP is either not expressed, or only weakly so, in both the *Gata2* heterozygous (iv) and homozygous (v) null mutant background. All sections are oriented with the neural tube at the top of the image.

Pecam1 and Gata2

PECAM-1 (platelet-endothelial cell adhesion molecule, also known as CD31) is expressed on both mature hematopoietic and endothelial cells (Watt et al., 1995). This protein functions in cell adhesion (Gumina et al., 1997), promotes cell migration, and assists in inflammatory processes associated with wound healing (Watt et al., 1995). GATA-2 is required for proper expression of PECAM-1, at least in the megakaryocytic lineage (Gumina et al., 1997).

Similar to Flk1 and VE-Cadherin, both GFP and PECAM-1 are induced in intensity coordinately with an increased number of endothelial cells in the roof of the dorsal aorta of *Gata2* null mutant embryos (Fig. 4.6*ii*) when compared to heterozygous controls (Fig. 4.6*i*). PECAM-1 appears to be expressed in budding hematopoietic cells, but appears to be reduced coincident with the release of the hematopoietic precursor cell into the lumen (Fig. 4.6*iii*). GFP and PECAM-1 both appear to be expressed in a cell invading the dorsal aorta endothelium of a *Gata2* null mutant embryo (Fig. 4.6*iv*). No cells were seen invading into the endothelium of the *Gata2* heterozygous embryo, but this was not uncommon given the rare appearance of such cells.

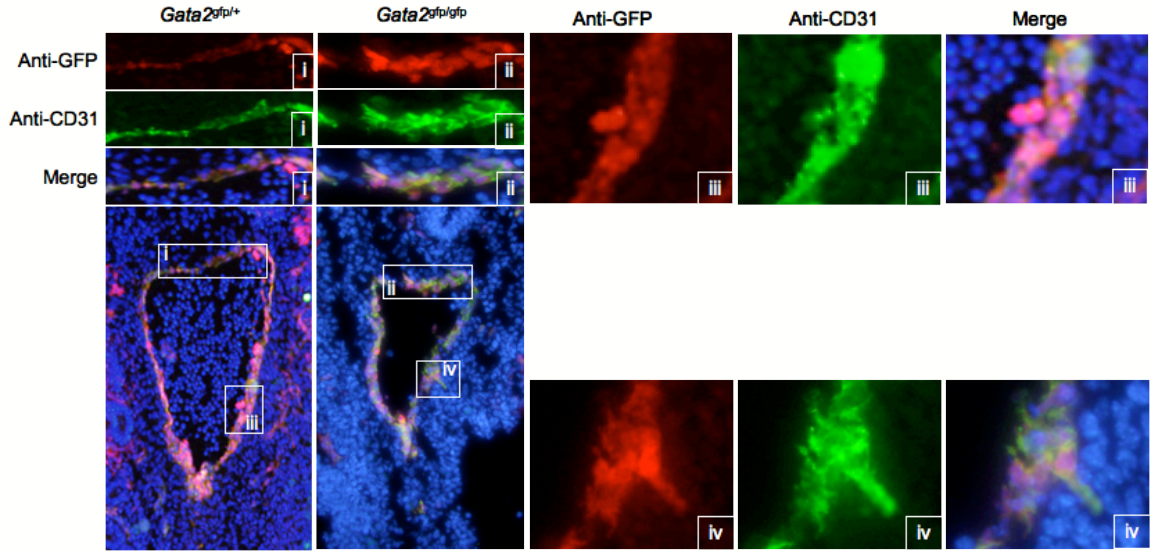


Figure 4.6 – Staining of GATA-2 (GFP) and PECAM-1 in the e10.5 dorsal aorta of *Gata2* heterozygous and homozygous null mutant mice. (i and ii) GFP and PECAM-1 expression are compared in the roof of the dorsal aorta of $Gata2^{gfp/+}$ (i) and $Gata2^{gfp/gfp}$ (ii) embryos. Both antigens are more abundantly expressed in the *Gata2* null mutant background, and contain a greater number of endothelial cells. **(iii)** GFP is expressed in cells budding into the lumen of the dorsal aorta while PECAM-1 is restricted to the endothelium. **(iv)** PECAM-1 is expressed in cells invading into the endothelium of the dorsal aorta while GFP also appears to be expressed, albeit at a lower level than PECAM-1, in the homozygous (v) null mutant background. All sections are oriented with the neural tube at the top of the image.

CD41 and Gata2

CD41 (also known as Itga2b) is one of the developmentally earliest known hematopoietic-specific markers (Ferkowicz et al., 2003; Li et al., 2005), and is expressed prior to the pan-hematopoietic marker CD45 (Mikkola et al., 2003). CD41 contains two subunits, which, in the presence of calcium, act together with CD61 to form a functional adhesive receptor protein (Ferkowicz et al., 2003). In later stages of hematopoiesis, CD41 is expressed in megakaryocytes and platelets (Li et al., 2005). In early development, definitive hematopoietic cells appear to require CD41 for differentiation from hemogenic endothelium (Li et al., 2005).

In accord with expectations from the literature, CD41 is expressed solely in the budding hematopoietic cells of the dorsal aorta, while GFP is expressed in the budding hematopoietic cells as well as in the underlying endothelium (Fig. 4.7*iii*). GATA-2 is also weakly expressed in cells invading into the endothelium, while CD41 is not (Fig. 4.7*iv*). GATA-2 is expressed more broadly in the roof of the dorsal aorta in *Gata2* null mutant mice (Fig. 4.7*ii*) than in the heterozygous control (Fig. 4.7*i*), whereas CD41 is not expressed in either case (the green spot in the CD41 panel of Fig. 4.7*ii* is artifactual).

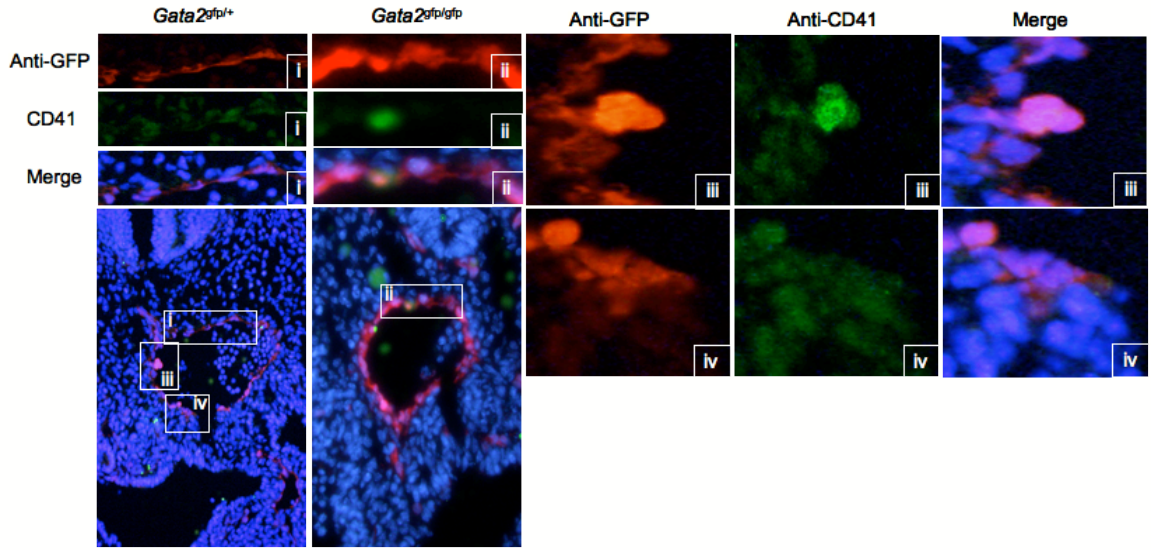


Figure 4.7 – Staining of GATA-2 (GFP) and CD41 in the e10.5 dorsal aorta of *Gata2* heterozygous and homozygous null mutant mice. (i and ii) GFP and CD41 expression are compared in the roof of the dorsal aorta of *Gata2*$^{gfp/+}$ (i) and *Gata2*$^{gfp/gfp}$ (ii) embryos. GFP expression is more highly expressed in the *Gata2* null mutant background, and contains a greater number of endothelial cells. The green cell in CD41 (ii) is artifactual. No CD41 expression was found in the roof of the dorsal aorta in either case. **(iii)** GFP and CD41 are expressed in cells budding into the lumen of the dorsal aorta while only GFP is found in the endothelium. **(iv)** CD41 is not found in cells invading into the endothelium of the dorsal aorta while GFP appears to be weakly expressed in the heterozygous (iv) background. All sections are oriented with the neural tube at the top of the image.

CD45 antibody staining does not show CD45 expression in the endothelium or the budding hematopoietic cells of the dorsal aorta; however, it is found on random cells throughout the embryo, presumably on monocytes and macrophages (data not shown) as previously described (Cuadros et al., 1992; Jaffredo et al., 1998). In *Runx1* lacZ-transgenic mice, LacZ expression is restricted to the floor of the dorsal aorta, along with budding hematopoietic cells (Nottingham et al., 2007). Unfortunately, antibody staining for RUNX1 has been unsuccessful under all conditions attempted (N. Speck, personal communication, and data not shown). Similarly, SCL, a helix-loop-helix transcription factor known to be critically important for hemangioblast determination (Lugus et al., 2007) and differentiation (Lancrin et al., 2009) *in vitro*, does not display a decipherable pattern with currently available SCL antibodies. Elucidation of a genuine hemangioblast through experiments like these might be possible if these reagents were generally available. Until that time, the iVE transgenic mice that were generated should aid in elucidating the role of GATA-2 in early vascular development.

Gata2 iVE in the Hemangioblast

While the vascular enhancer of *Gata2* has already been shown to regulate GATA-2 expression in the developing vasculature (Khandekar et al., 2007; Wozniak et al., 2007), it can also robustly activate luciferase expression in erythroid precursor cells (Grass et al., 2006), and is also expressed in the budding hematopoietic stem cells of the dorsal aorta (Khandekar et al., 2007). These observations indicate that the vascular enhancer of *Gata2* may direct *Gata2* expression in the precursor to these two cells types – the hemangioblast.

Preliminary observations are in accord with this hypothesis, as mCherry is robustly expressed in the dorsal aorta of e10.5 mouse embryos (Fig. 4.8*A*), is expressed in budding hematopoietic cells (Fig. 4.8*A*, arrows), and appears to be expressed in circulating hematopoietic cells in the head vasculature (Fig. 4.8*B*). By examining a confocal microscope z-stack, it became apparent that mCherry was expressed in rounded cells lining the floor and ventral walls of the dorsal aorta, while the dorsal walls and roof appear to display reduced mCherry expression (Fig. 4.8*A*), except in the budding hematopoietic cells (Fig. 4.8*A*, arrows).

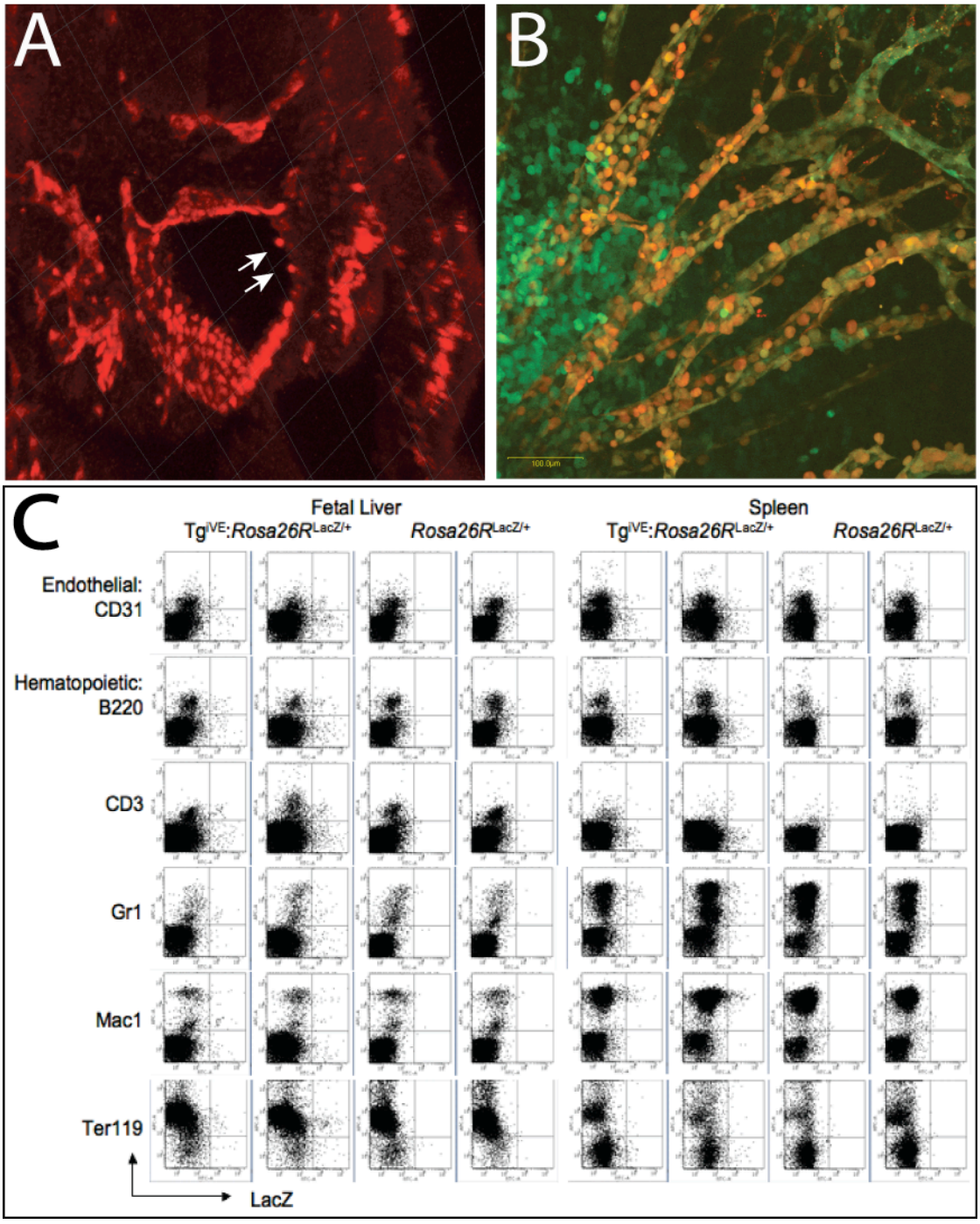


Figure 4.8 – Analyzing the *Gata2* iVE in the hemangioblast. (A) Transverse image of a confocal z-stack to analyze mCherry expression in the dorsal aorta. Expression is seen in the endothelium of the dorsal aorta as well as in budding hematopoietic cells (arrows). The neural tube is oriented at the top of the image. **(B)** A saggital image using a confocal microscopic z-stack to analyze mCherry and GFP expression in the head vasculature. Hematopoietic cells appear to express mCherry in the lumen of the blood vessels. **(C)** Flow cytometry analysis of Tg^{iVE}:$Rosa26R^{lacZ}$ e17.5 fetal liver and spleen. FDG (lacZ expression) is analyzed on the x-axis, and APC-conjugated antibodies to the endothelial and hematopoietic markers listed are assayed on the y-axis. Double positive cells can be seen in all lineages in Tg^{iVE}:$Rosa26R^{LacZ/+}$ (left two plots, upper right chamber), while no double-positive cells are seen in the $Rosa26R^{LacZ/+}$ controls (right two plots, upper right chamber).

To directly address hemangioblast function in the developing mouse embryo, a lineage tracing study was performed. A pregnant dam harboring Tg^{iVE}:$Rosa26R^{lacZ}$ embryos was fed 2.5 mg of tamoxifen per day via gavage on embryonic days 6.5, 7.5 and 8.5. This was expected to activate LacZ expression in the putative hemangioblast, but not in hematopoietic cells, as definitive hematopoietic precursors are not generated in the embryo until approximately e10.5. The fetal liver and spleen were collected on e17.5 and co-stained for LacZ along with antibodies for definitive, lineage-restricted hematopoietic cells, including B-cell surface marker B220 (Coffman, 1983; Morse et al., 1982), T-cell marker CD3 (Leo et al., 1987; Samelson et al., 1987), myeloid marker Gr1 (Hestdal et al., 1991), the marker Mac1, which is expressed on macrophages, NK cells, granulocytes, activated lymphocytes, and mouse B-1 cells (Springer et al., 1979), and the erythroid specific marker Ter119 (Kina et al., 2000; Vannucchi et al., 2000), along with the endothelial specific marker CD31 (Baldwin et al., 1994). While the overall percentage of labeled cells was low, double positive cells containing each of these markers co-stained with the LacZ substrate FDG were found in both the fetal liver and spleen when analyzed by flow cytometry (Fig. 4.8*C*), and could explain the population of mCherry positive, CD31 negative cells observed in a single cell suspension of a whole e10.5 embryo (Fig. 4.3*C*, lower right panel). This is the first *in vivo* data in a mammalian model demonstrating that precursor cells from the mesoderm give rise to at least a subset of all definitive, lineage-restricted hematopoietic cells and the endothelium exists, and that the *Gata2* vascular enhancer regulates gene expression in this cell population.

Discussion

The results of this study indicate that: a transgenic mouse has been generated that is capable of specifically labeling, and conditionally ablating, *Gata2* in the vasculature; GATA-2 indeed plays a significant role in vascular development, primarily through the secondary branching of the primary vasculature; and that through further manipulation of the iVE/CreERT2 transgenic mice, the *in vivo* identification and characterization of the definitive hemangioblast is almost a reality.

Gata2 iVE Transgenic Mice

The primary constraints in elucidating the vascular phenotype regulated by GATA-2 function have been two-fold. First, *Gata2* null mutant mice die at embryonic day 10.5, only allowing a cursory evaluation of the vasculature at this stage. The second is that the vascular phenotype in the *Gata2* null mutant background originally appeared to be rather subtle. Both of these issues have been addressed by creation of the iVE/CreERT2 transgenic mice. The fluorescent protein mCherry provides a very clear and robust fluorescent signal specifically localized to the vasculature, providing a useful and sensitive reporter. Based on *Rosa26R*^{lacZ} reporter staining, it appears that the CreERT2 transgene specifically ablates *Gata2* in the vasculature, allowing normal development of the early hematopoietic (Zhou et al., 1998) and urogenital (Khandekar et al., 2004) systems that lead to early lethality in complete *Gata2* null mutant mice.

Transgenes succumb to two primary difficulties upon insertion into the genome: position of integration effects, in which a transgene is influenced by regulatory elements in the DNA surrounding the integration site; and transgene silencing, where the transgene over time adopts a closed conformation. Given the small size of the *Gata2* vascular enhancer, we hypothesized it would be easily influenced by position effects. For this reason we attempted to generate transgenes that would be protected from these chromatin site of integration effects by including chicken HS4 insulators flanking the expression cassette. These insulators have been shown previously to effectively protect transgenes from such position of integration effects in flies and mice (Emery et al., 2000; Sarkar et al., 2006). Unfortunately, the insulator elements failed to protect the transgene from its surrounding chromatin environment. Through the analysis of future generations of the iVE62 line of transgenic mice, we will be able to determine if the insulator elements can prevent gene silencing.

The Role of GATA-2 in Vascular Development

Gata2 null mutant animals carrying the iVE transgene were assessed in an attempt to observe a noticeable vascular phenotype using mCherry as a marker of the vasculature. Confocal microscopy was used to create optical sagittal sections in e10.5 embryos from

Gata2 heterozygous and homozygous null mutant animals to look for altered vascular development. The apparent lack of angiogenesis in the *Gata2* null mutant embryo observed in this study was surprising, as a striking vascular phenotype has not been observed in *Gata2* null mutant animals. However, I have only been able to analyze one embryo to date. A larger number of *Gata2* null mutant embryos will need to be examined before any definitive conclusions can be drawn about the role of GATA-2 in vascular development.

As a vascular phenotype for GATA-2 loss or gain of function has been difficult to address, the ultimate goal of the iVE transgenic mice is to conditionally ablate GATA-2 in the vasculature. We plan to mate the iVE transgenic mice to *Gata2* conditional knockout mice ($Gata2^{f}$), which contain *loxP* sites flanking the *Gata2* exon 5 zinc finger (Charles et al., 2006). Mice of the genotype Tg^{iVE}:$Gata2^{f/-}$ should be viable. Tamoxifen will be administered to pregnant dams carrying embryos of this genotype, which will then be analyzed for vascular defects at various stages of development. Our hypothesis is that these mice will display angiogenic deficiencies, but not as severe as those detected in the *Gata2* null mutant mice in this study (Fig. 4.2).

The Molecular Signature of a Hemangioblast?

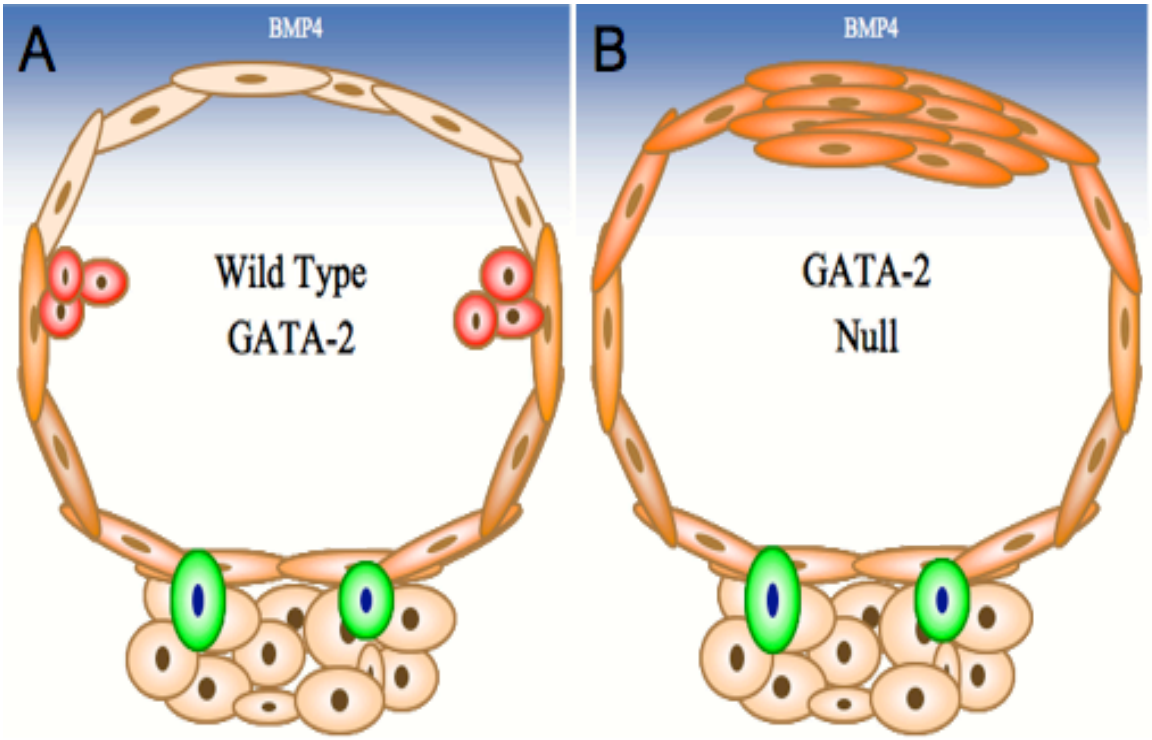
A recent review by Jaffredo *et al* (Jaffredo et al., 2005) contains a schematic representation of a transverse section through the dorsal aorta of a developing mouse embryo. This schematic has artificially colored hemangioblasts, differentiating them from the mesoderm, hemogenic endothelium, and surrounding non-hemogenic endothelium. Our goal was to generate a similar image using mouse confocal sections as the canvas; if we could not, this would suggest that the current model of the hemangioblast was deficient.

During this process, I made several interesting discoveries. Historically, hematopoietic cells were thought to bud primarily from the ventral walls of the dorsal aorta in accord with a model in which the hemangioblast directly generates hematopoietic precursor cells. However, in this study hematopoietic cells were always found budding from the walls of the dorsal aorta, sometimes ventrally but often laterally as well. Second, invading hemangioblast cells were always found ventral to the budding hematopoietic

cells in the endothelium. The invading hemangioblast cell and the budding hematopoietic cell were never seen in the same location along the dorsal aorta, implying that an intermediate state (the hemogenic endothelium) between a common progenitor (the hemangioblast) and final product (committed hematopoietic cells) migrates as it differentiates. This putative hemogenic endothelium compartment probably responds, through either negative or positive regulation, to a global dorsal-ventral patterning signal such as Bone Morphogenetic Protein 4 (BMP4) to induce budding of the definitive hematopoietic precursors (Fig. 4.9*A*, Fig. *4i*-4.7*i*). In *Gata2* null mutant animals, the data shown here suggest that the hemangioblast invades into the endothelium defined by the dorsal aorta, but in the absence of GATA-2 protein, the hemogenic endothelium cannot respond to the dorsal-ventral patterning signal(s) and therefore cannot differentiate and release hematopoietic precursors. As a result, these defective hemangiogenic endothelial cells appear to accumulate in the roof of the dorsal aorta (Fig. 4.9*B,* Fig. 4.4*ii*-4.7*ii*).

The staining pattern of the e10.5 dorsal aorta proved to be informative. Flk1, VE-Cadherin, and PECAM-1 all appear to be active in the mesenchymal hemangioblast precursor, along with low GATA-2 levels. Flk1, VE-Cadherin, PECAM-1, and GATA-2 are all broadly expressed in the endothelium of the dorsal aorta. In the hemogenic endothelium surrounding the budding hematopoietic precursors, Flk1 is down regulated, VE-Cadherin is stable, and PECAM-1 appears to be the last to maintain contact with the budding hematopoietic cells (among the cell adhesion molecules analyzed here). GATA-2 is the only protein analyzed in this study that is broadly expressed in both the endothelium and budding hematopoietic cells. CD41, the earliest known hematopoietic specific marker, begins to express in the budding hematopoietic precursors along with continued GATA-2 expression. These results are summarized in Figure 4.9*C.*

Other proteins thought to be expressed in both the hemogenic endothelium and budding hematopoietic precursors are SCL and RUNX1; however, suitable antibodies for immunohistochemistry in mice are not currently available. If the hemangioblast, or hemogenic endothelium, cannot be localized using the available protein antibodies, it may prove worthwhile to analyze these sections using *in situ* hybridization probes to SCL and/or RUNX1.



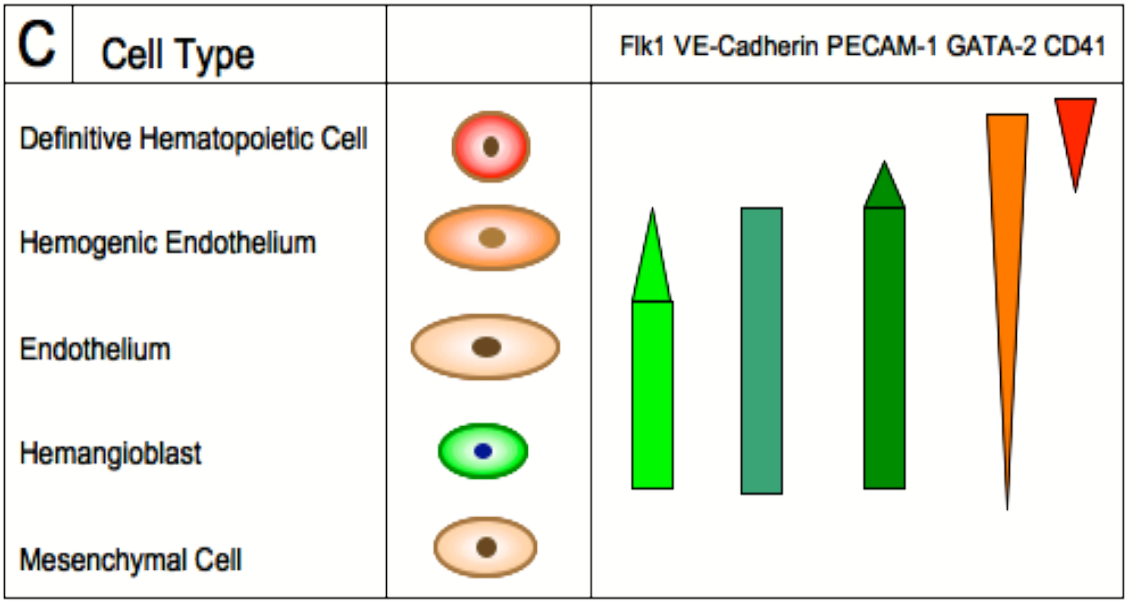


Figure 4.9 – A model for hemangioblast development and differentiation. (A) In *Gata2* heterozygous or wild type animals, hemangioblasts arise from the mesoderm surrounding the floor of the dorsal aorta around e10.5 and invade into the endothelium. The endothelium then migrates up the walls of the dorsal aorta and differentiates into hematopoietic cells upon recognition of a global patterning protein, such as Bone Morphogenetic Protein 4 (BMP4). **(B)** In *Gata2* null mutant animals, hemangioblasts still arise from the mesoderm surrounding the floor of the dorsal aorta around e10.5 and invade into the endothelium. The endothelium then migrates up the walls of the dorsal aorta, but fails to recognize the global patterning protein, resulting in accumulation of the precursor cells in the roof of the dorsal aorta. **(C)** Description of the various cell types represented in (A) and (B), along with the proposed expression pattern of the proteins in these cell types as observed in Figures 4.4 through 4.7. The neural tube is oriented at the top of both models.

The GATA-2 iVE and the Hemangioblast

Previous studies have demonstrated that the *Gata2* vascular enhancer element regulates GATA-2 expression in the endothelium as well as in erythroid cells. It therefore seemed reasonable that this enhancer element might be able to direct *Gata2* expression in the presumptive definitive hemangioblast. Accordingly, mCherry expression has been found in the dorsal aorta, budding hematopoietic cells, and possibly even in circulating hematopoietic cells (see Fig. 4.3*B*, arrows). Lineage tracing experiments have now confirmed these observations. Tamoxifen was administered to a pregnant *Rosa26R*^{lacZ} dam, after mating to a Tg^{iVE}:*Gata2*$^{+/+}$ male, from embryonic day 6.5 through 8.5, when no definitive hematopoietic precursor cells yet exist. Interestingly, when fetal liver and spleen were collected at embryonic day 17.5, a subset of all definitive hematopoietic lineage cells, along with endothelial cells, co-express LacZ and the lineage specific marker. These data indicate that the CreERT2 transgene excised the floxed stop codon preempting LacZ expression in a progenitor cell that gave rise to both endothelial and definitive, lineage-restricted hematopoietic cells, providing the first *bona fide* evidence for the existence of the hemangioblast *in vivo* in mammals. Whether this enhancer element is required for all hematopoietic cells is still unclear. Optimization of the tamoxifen administration protocol both for concentration and timing may augment the number of definitive hematopoietic-restricted cells that co-express the *Gata2* iVE generated LacZ marker.

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Chapter 5

Summary and Future Directions

Transcription factors are known to play critical roles in development, but only more recently have we realized that the same transcription factor pathways used for development are being abused in the generation or elaboration of various disease states. For this reason it has become increasingly important to discover the biological mechanisms that regulate the development of an organism, and more specifically, to discover how specific genes and gene networks are regulated. The results of these studies will benefit both the basic science and clinical communities.

GATA-2 is a transcription factor that is known to be crucial for a variety of developmental processes, including hematopoietic and urogenital development. My work has been focused on distinct aspects of mammalian development involving GATA-2, including the elucidation of the functional boundaries of the *Gata2* locus in addition to the initial experiments reported here in which we have attempted to define a role for this factor in the generation or maintenance of the hemangioblast, hemogenic endothelium, and the vasculature.

A *Gata2* Vascular Enhancer

Chapter two focused on the regulation of *Gata2* in the endothelium of the developing mouse embryo. While the original analysis of the *Gata2* knockout mouse indicated that GATA-2 does not have a functional role in vascular development (Tsai et al., 1994), we have shown here that GATA-2 is broadly expressed in endothelial cells of an E10.5 days post coitum (dpc) embryo. Using deletion constructs of a YAC spanning the *Gata2* locus, we identified an endothelial specific enhancer of *Gata2* located in intron 4 of the *Gata2* gene.

Further analysis of this enhancer allowed us to localize the vascular endothelial

regulatory domain to a 322 base pair (bp) enhancer element. A core 167 bp enhancer element was able to autonomously confer broad endothelial expression throughout the E10.5 dpc embryo, except in the endocardium. The 155 bp immediately 5' to this minimal domain contained a non-autonomous regulatory element necessary for endocardial expression of GATA-2. By generating site-specific mutations in the core VE element, it was shown that binding sites for multiple Ets factors as well as an e-box transcription factor are important for the regulation of this enhancer. I went on to determine that the transcription factor SCL+E12 can bind to this e-box in the endothelial enhancer element.

The *Gata2* vascular enhancer element is intriguing for a couple of reasons. First, GATA-2 is known to be expressed in endothelial cells (Dorfman et al., 1992; Yamamoto et al., 1997), and yet it still has not been reported to have a discernable vascular phenotype in mice (De Val and Black, 2009), even though GATA-2 is expressed throughout the embryonic vasculature (Chapter 2). Second, this same enhancer was originally reported to be required for hematopoietic development (Grass et al., 2006).

The transcription factor *Scl* has a similar developmental profile and physiological requirement as *Gata2*, including embryonic lethality from its germ line loss at E10.5 dpc from hematopoietic failure (Robb et al., 1995). To circumvent the early embryonic lethality of *Scl* null mutant mice, *in vitro* angiogenesis and matrigel implant *in vivo* angiogenesis studies were undertaken, elucidating a role for SCL in the migration, but not proliferation, of endothelial cells undergoing angiogenesis (Lazrak et al., 2004). Similar experiments for GATA-2 could be performed using adenoviral vectors to introduce GATA-2, and/or a dominant negative form of GATA-2, to primary endothelial cells, and assess their ability to undergo angiogenesis. Given that GATA-2 and SCL seem to play such similar roles in development, and interact to regulate early hematopoietic processes (Wozniak et al., 2008), I would expect a similar result to the *Scl* study in the absence of GATA-2. Specifically, one might predict that the GATA-2 dominant negative mutant would inhibit the process of angiogenesis in primary endothelial cell and matrigel implants. However, unlike the effects due to alteration in SCL expression, I would additionally expect the GATA-2 dominant negative protein to abrogate the proliferation of primary endothelial cells, as GATA-2 has repeatedly been shown to be important for

cell proliferation (Briegel et al., 1993; Kitajima et al., 2002; Tsai and Orkin, 1997). If successful, the next step would be to identify the factors that interact with GATA-2 to mediate these effects in the vasculature. Candidate factors that have already been identified (as discussed above) include SCL, Ets factors, and GATA factor auto- or cross-regulation, and can be tested using Chromatin Immunoprecipitation (ChIP) analysis.

Another interesting experiment would be to remove the vascular enhancer of GATA-2 by homologous recombination to generate *Gata2VE* conditional null mutant mice. It is expected that these mice would survive beyond the lethality seen at embryonic day 10.5 from the gene knockout, as hematopoiesis would proceed normally, and *Gata2* null mutant mice generate initial vascular patterning of the embryo even in the absence of GATA-2 protein. I would expect to see vascular remodeling defects similar to that of $Gata4^{+/-}$:$Gata6^{+/-}$ compound heterozygous mutant embryos, which display myocardial hypoplasia as well as disorganized patterning of the cranial and intersomitic vessels and hemorrhaging. Interestingly, the *Gata4/Gata6* compound heterozygous mutant mice displayed a 90% reduction in the mRNA level of the myocardial specific factor Mef2c (Xin et al., 2006), which is a potential regulator of *Gata2* through the MEF2 binding site in the endocardial vascular enhancer (Martin et al., 1993). A signaling pathway can be envisioned where GATA-4 and GATA-6 regulate the levels of Mef2c, which in turn induces GATA-2 expression, resulting in myocardial proliferation. This hypothesis could be tested perhaps most easily through gain and loss of function experiments of GATA-4, GATA-6, Mef2c, and GATA-2 in a myocardial cell line.

The Ets factor family of winged helix proteins, which are known to be important for vascular development (De Val et al., 2008; Sumanas and Lin, 2006), are required for proper endothelial-specific expression of GATA-2 in founder assays (Chapter 2). All three Ets factor binding sites are in close proximity to basic helix-loop-helix (bHLH) transcription factor binding sites (two GATA sites, one Scl) in the *Gata2* vascular enhancer. Mutations of these binding sites reduces GATA-2 expression. To elucidate the Ets factors function in the *Gata2* vascular enhancer, ChIP experiments could be conducted to confirm the *in vivo* binding of GATA factors and SCL to their respective binding sites. Second, co-immunoprecipitation experiments could be performed to confirm the interaction of GATA or SCL with Ets proteins, and more specifically which

Ets proteins bind these elements. This would begin to define potential combinatorial endothelial-specific regulation conferred by Ets factors in collaboration with bHLH transcription factors. One candidate Ets factor is *Fli1*, which appears to act upstream of GATA-2 in regulating hemangioblast expression in zebrafish (Liu and Patient, 2008).

This same *Gata2* intronic enhancer can drive high levels of luciferase expression in the erythroid cell line G1E. This expression is critically dependent on the GATA binding sites, as mutation of these sites abolishes luciferase activity. This enhancer also undergoes a "GATA switch" as shown by quantitative ChIP analysis with GATA-1 displacing GATA-2 in the vascular enhancer upon GATA-1 induction (Grass et al., 2006). One possibility is that GATA-2 binds to this region during normal hematopoietic development, and then GATA-1 displaces GATA-2 in myeloid (erythroid, megakaryocytic, mast and eosinophilic) lineages to promote differentiation.

The current data supports a model in which the *Gata2* intronic enhancer appears to play a dual role in development, regulating GATA-2 expression in both endothelial and hematopoietic precursor cells. The question this raises is whether GATA-2 is expressed in, or necessary for, hemangioblast (a cell that gives rise to both hematopoietic and endothelial cells) development or differentiation. One way to address this question would be to conditionally delete the *Gata2* intronic enhancer from within the endogenous gene through homologous recombination with a construct in which the *Gata2* vascular enhancer (as discussed above) is flanked by loxP sites, breeding to a vascular-specific cre transgene followed by analysis of homozygous *Gata2*$^{f/f}$ mutant mice. A simpler way to address this question was to create a *Gata2* transgenic mouse using the intronic vascular enhancer to drive Cre recombinase expression. Crossing this mouse into a conditional *Gata2* knockout line revealed the specific role of this enhancer element in the earliest stage of mesodermal/hematopoietic development. Since an inducible form of cre was used, the particular role of this enhancer element was addressed by gene ablation from the vasculature at various stages of very early embryonic development through to the adult vasculature.

Localizing the Functional Boundaries of the *Gata2* Locus

Chapter three focused on the rescue of *Gata2* null mutant mice using a linked bacterial artificial chromosome (BAC) transgene. The goal of this experiment was to determine the functional boundaries of the *Gata2* locus, or failing that to uncover a previously unseen development defect associated with abrogated *Gata2* expression. The original attempt for this experiment involved rescue of the hematopoietic failure seen in *Gata2* homozygous null mutant mice by introducing a yeast artifical chromosome (YAC) containing the hematopoietic enhancers of *Gata2* - located between 40 and 170 kbp 5' to the structural gene - into a *Gata2* null mutant environment. These mice showed robust hematopoietic expression and survived to birth. However, lethal hydroureternephrosis and other genitourinary abnormalities developed, leading to the conclusion that *Gata2* was vitally required for not only embryonic hematopoiesis but for embryonic kidney development as well (Zhou et al., 1998). In this manner, a critical secondary requirement for GATA-2 in the proper patterning of the ureter-bladder junction was discovered (Zhou et al., 1998). Subsequent BAC mapping experiments revealed the existence of three separate urogenital enhancers, two in the urogenital mesenchyme located approximately 100 kbp 3' to the structural gene, and one in the ureteric epithelium that has not yet been specifically localized (Khandekar et al., 2004).

While elucidation of the regulatory elements required for *Gata2* urogenital expression was incomplete, a second *Gata2* rescue was attempted, but unfortunately would not be as simple. Multiple technical issues had come to light involving YACs, and the distance between the upstream hematopoietic enhancers and the downstream urogenital enhancers was too large to be spanned by a single BAC. To overcome this limitation, two overlapping BACs were linked together utilizing the differential recombination efficiency of distinct *loxP* sequences and cre-mediated homologous recombination.

This linked BAC (containing approximately 150 kbp of 5' sequence information, and both known hematopoietic enhancers as well as 220 kbp of the genome encompassing the structural gene and the 3' urogenital enhancers, hereafter referred to as *G2BAC*) was used to create transgenic mice and mated into the *Gata2* null mutant background. There were several scenarios that were envisioned might result from this

experiment. First, the rescued mice could manifest a urogenital defect similar to the earlier rescue attempt, since the ureteric epithelial enhancer had still not been localized. Second, even if the urogenital patterning was normal - presumably due to inductive effects from the mesenchyme or compensation by another GATA factor - the mice could succumb to a third critical, as yet unknown, developmental role for GATA-2, as this transcription factor is known to play a role in various processes in which the enhancers have not yet been localized, including the generation of placental trophoblasts, adipocytes, the pituitary, and skin development. A third scenario is that the mice would be physiologically normal, indicating that the *G2BAC* transgene is capable of rescuing all developmental processes that vitally require GATA-2 expression.

Upon analysis of the *G2BAC*-rescued *Gata2* null mutant mice, no observable behavioral or reproductive anomalies were found. Upon further inspection, we were surprised to find that not only was the gross morphology of the ureteric epithelium unaltered, GATA-2 protein was expressed at normal levels as well. This expression was not seen when analyzing either the original 5' YAC or the 3' BAC individually (which together encompass all of the sequence within the rescuing *G2BAC*). One potential explanation for this discrepancy is that a binary synthetic enhancer could regulate GATA-2 expression in the ureteric epithelium, with two or more regulatory domains separately located in each of the individual BACs that only becomes functional once the BACs are linked, as in the normal genomic configuration. Another potential explanation is that a single ureteric epithelial enhancer exists, but requires either certain domain boundary elements that are present only in the *G2BAC* for expression, or requires specific spacing from the *Gata2* promoter, which was abrogated in the BAC-trap study, but is maintained in the *G2BAC*. Discovering the mechanism(s) underlying this discrepancy will be informative, as it should elucidate a unique transcriptional regulatory process.

The original YAC that rescued *Gata2*-regulated hematopoiesis spanned from -174 to +73 kbp relative to the *Gata2* translational start site, and one of the original BACs (33I12) used for the BAC-trap study spanned +60 to +268 kbp (Khandekar et al., 2004). However, when we converted from attempting to use YACs to BACs for gene rescue, the 5' BAC (115E9) chosen spanned from -187 to -10 kbp and the 3' BAC (81F7) employed in the linking experiment spanned from -34 to +226 kbp. The original BAC-trap study

used a *Gata2* promoter sequence that started 4 kbp 5' to the alternate first exon through the translational start site which was fused to a *lacZ* reporter gene (Khandekar et al., 2004). While this fragment contained both the *Gata2* specific (1S) and general (1G) promoters, along with three GATA-motif dependent enhancer elements immediately 5' to this region (Wozniak et al., 2007), it remains possible that regulatory elements lying outside these boundaries, or the spatial organization of these elements relative to the *Gata2* ureteric epithelial enhancer, are necessary for its functionality. The 81F7 BAC, which contains sequences from 34 kbp 5' to the translational start site to 100 kbp 3' to the most distant known urogenital enhancer, should account for both of these potentialities. The original BAC studied (33I12) originated 60 kbp downstream of the translational start site, potentially disrupting the spatial orientation of the enhancer and its promoter, along with removing 90 kbp of potential regulatory DNA within and surrounding the *Gata2* structural gene. Therefore, the first experiment that should be done is a BAC trap experiment similar to those performed previously (Khandekar et al., 2004), this time using BAC 81F7 to look for *Gata2* ureteric epithelial expression. Since the *G2BAC* is known to confer ureteric epithelial expression to *Gata2* in transgenic mice, a subsequent experiment would be to delete segments of the *G2BAC* to localize the necessary regulatory elements in a similar manner to the localization of the *Gata2* endothelial enhancer (Khandekar et al., 2007).

Another important experiment that is yet to be performed is characterization of the localized urogenital enhancers. Analysis of the sequence for the urogenital enhancers UG2 and UG4 should reveal the identity of putative transcription factor binding sites that are potentially critical for activation of these enhancers. Once presumptive binding factors are discovered, mutation of these sites followed by founder transgenic experiments can be performed to reveal their importance in urogenital transcription. Since UG2 and UG4 are both a significant distance 3' to the *Gata2* gene (at +73 and +113 kbp, respectively) and yet are spatially and functionally distinct, these experiments could help uncover the functional relevance of their unique roles in development. It seems likely that the enhancers are responsive to different transcriptional regulatory programs.

The final interesting aspect of these experiments is that the *G2BAC*-rescued transgenic *Gata2* null mutant mice have a normal phenotypic appearance, even though

some enhancer elements have either not been localized (*i.e.* for giant cell trophoblast, pituitary or adipocyte specificity) or are known to lie outside the boundaries of the *G2BAC* (*i.e.* whisker follicles). For the cell types known to express GATA-2, simple analysis of these tissue types in *G2BAC* transgenic *Gata2* null mutant mice for GATA-2 expression will help to initially refine the position of the various regulatory elements (either within or outside the 413 kbp encompassed by the linked BAC). Looking at X-gal staining in the BAC-trap mice for these cell types should also help to localize the regulatory elements, as the original BAC-trap assays encompassed more than one megabase surrounding the *Gata2* locus (Khandekar et al., 2004). These experiments may reveal a subtle phenotype that was previously overlooked, or may reveal a compensatory regulatory mechanism, possibly complemented by other GATA factor family members expressed in the same tissue.

GATA-2 in the Vasculature and the Hemangioblast

Chapter 4 deals with the possible transcriptional regulatory roles for GATA-2 in the vasculature and in an even earlier cell, the hemangioblast. Delineation of a regulatory pathway where GATA-2 plays some role in the hemangioblast and/or hemogenic endothelium was attempted by comparison of the staining patterns of various transcription factors known to be important for hematopoietic and endothelial development in the dorsal aorta of E10.5 dpc *Gata2* wild type and null mutant embryos. A recent review by Jaffredo *et al.* presented a diagram of the dorsal aorta representing the various cell types that might exist or be generated there with distinct colors, including the mesenchyme, mesenchyme containing hematopoietic potential (putative hemangioblasts), endothelium, hemogenic endothelium, and budding hematopoietic stem cells (Jaffredo et al., 2005). I attempted to investigate the validity of this image *in vivo* in mouse embryo sections by following the expression of combinations of marker proteins.

A number of conclusions can be drawn from these studies. First, Flk1, VE-Cadherin, and PECAM-1 can all be seen in presumptive hemangioblasts - cells that are invading the endothelium of the dorsal aorta. GATA-2 is either not expressed, or only weakly so, in this cell population. The endothelium robustly expresses Flk1, VE-Cadherin, PECAM-1, and GATA-2. Interestingly, Flk1 appears to be diminished in the

endothelium surrounding the site of budding hematopoietic cells, while the other markers continue to be robustly expressed. GATA-2 and CD41 are the only markers expressed in the presumptive budding hematopoietic cells, though remnants of PECAM-1 expression can still be seen where the budding hematopoietic cells still attach to the endothelium. Unfortunately, antibodies to other known critical players in the development and differentiation of the putative hemangioblast, including SCL and RUNX1, are not currently available for this assay.

GATA-2, Flk1, and SCL are all expressed throughout the endothelium of the dorsal aorta, even though the hemogenic endothelium and budding hematopoietic cells were previously thought to be restricted to the ventral floor. Given that endothelial specificity is known to require a unique combination of multiple transcription factors (De Val and Black, 2009), it seems reasonable that these factors are conferring a unique identity to the hemogenic endothelium even though their expression appears to be uniform throughout the tissue. Lending credence to this hypothesis, analysis of a three dimensional image of a transverse section through an E10.5 dpc embryo shows that the floor of the dorsal aorta appears to consist of small, rounded cells that strongly express the marker protein, while the walls and roof of the dorsal aorta contain cells that are elongated and express the marker protein less abundantly, except in the budding hematopoietic cells. Thus, while we are not yet at the stage where we can completely distinguish between the various cell types (from the mesenchyme through the definitive hematopoietic cells) that contribute to this complex early developmental process, the stage is set. Complete elucidation may require characterization of factors that have not yet been included in the analysis, or an understanding of unique, currently unknown molecules that associate with the factors that have been hypothesized to function in the hemogenic endothelium. One such factor is RUNX1. This transcription factor is expressed most abundantly in the floor of the dorsal aorta, but not in the roof, as assayed by *in situ* hybridization. An antibody for RUNX1 would greatly augment our ability to characterize these distinct cell types. Without this reagent, it may still be possible to co-stain the same section using immunohistochemistry for GATA-2 and *in situ* hybridization for RUNX1.

GATA-2 is known to bind to an enhancer of *Runx1*, along with an Ets protein and

SCL, to regulate *Runx1* expression (Nottingham et al., 2007). We have shown that the *Gata2* vascular enhancer also relies on Ets factor binding sites for proper expression of GATA-2 in the vasculature (Chapter 2). ChIP or EMSA assays analyzing these regulatory regions of *Runx1* and *Gata2* for the Ets factors (potentially *Fli1*) regulating these elements may provide a new target for the immunohistochemistry experiment as well.

While attempting to address the role of GATA-2 in the hemangioblast in a *Gata2* null mutant background has its merits, it also has its limitations. A conditional knockout of *Gata2* would allow us to analyze the role of GATA-2 specifically in the hemangioblast or hemogenic endothelium cell populations without the overriding hematopoietic and urogenital deficiencies. To this end, a transgene using the *Gata2* intronic endothelial enhancer (Chapter 2) was generated to drive a tamoxifen inducible form of cre recombinase (Cre-ERT2). Hemangioblast/endothelial specific GATA-2 deficiencies can then be directly assessed when crossed to a *Gata2* conditional knockout mouse. To test the efficacy of this enhancer and generate a robust reporter, an identical construct with the fluorescent protein mCherry in the place of the Cre-ERT2 was generated. To attempt to prevent position integration effects, these constructs were also insulated with duplicated chicken HS4 insulator elements.

Equal concentrations of each construct were co-injected into zygotes to create transgenic mice. Three lines were found to transmit both transgenes and robustly express mCherry in a vasculature specific manner. If the chicken HS4 elements were functioning properly, one would expect the copy number of this transgene to directly correlate with the expression level of mCherry. However, most transgene positive embryos expressed mCherry very weakly, if at all, from mice containing a whole range of transgene copy numbers. The lack of expression is not due to cellular toxicity, as the high copy number mice can reach adulthood, reproduce, and do not display any obvious phenotypic abnormalities. These results indicate that the chicken HS4 insulators are not able to prevent position of integration effects. A second effect often seen in transgenic mice is reduced expression over time due to chromatin silencing. The ability of the insulators to prevent this transgene silencing can be assessed after these transgenic mice have been bred for multiple generations.

With an endothelial-specific fluorescent marker at our disposal, mice bearing both transgenes were backcrossed into a *Gata2* null mutant background to assess the formation of the early vasculature. The primary vasculature is properly formed, but the angiogenic process appears to be disrupted, though additional studies are required before any firm conclusions about how this process is defective can be reached. Since we can now follow this phenotype past embryonic day 10.5 in conditional *Gata2* knockout mice, we should be able to secure informative results. To this end, we also addressed the question of whether cre activity would have the same expression profile as mCherry. The iVE transgenic mice were crossed to the Rosa26^{lacZ} reporter strain to test the expression profile of the iVE/creERT2 transgene. Upon tamoxifen induction, we found that LacZ is robustly expressed in a pattern that significantly overlaps that of the mCherry reporter, indicating that the tamoxifen administration protocol is working efficiently. Furthermore, fluorescence activated cell sorting (FACS) analysis indicated that a significant proportion of mCherry-positive cells co-express the endothelial specific marker CD31, indicating that they are indeed endothelial specific. However, while LacZ-positive cells were not detected that were not also mCherry positive, the percentage of LacZ expressing cells in mCherry positive cells was rather low, indicating either that a better protocol for tamoxifen administration may still be required or that the lacZ labeling protocol for endothelial cells may be less than complete. There were multiple technical issues with the way the LacZ flow cytometry experiment was performed, and confirming the excision efficiency of Cre is dependent upon accounting for each of these complications. We hope to avoid these technical issues altogether by using Rosa26^{YFP} reporter mice in place of Rosa26^{lacZ}, so that co-expression of mCherry and YFP fluorescence can be assessed directly.

Another experiment I performed, which is less dependent on the absolute activity of the creERT2 fusion protein, is a lineage trace following the activation of LacZ in Rosa26^{lacZ} mice. More specifically, tamoxifen was administered to a pregnant dam harboring Tg^{iVE}:*Rosa26*^{lacZ} embryos on embryonic days 6.5, 7.5, and 8.5 - well before definitive hematopoietic cells begin to emerge in the embryonic AGM at E10.5. The fetal liver and spleen of E17.5 embryos was collected and analyzed. The various lineage-restricted definitive hematopoietic cell types were then characterized by co-expression

with LacZ. Interestingly, all of the markers analyzed in this study, including the B-cell marker B220, the T-cell marker CD3, the myeloid marker Gr1, the macrophage marker Mac1, and the erythroid marker Ter119 contained a small but clear subset of cells that co-express LacZ. Endothelial cells also contained an infrequent population of cells that double stained for CD31 and LacZ. Insofar as I am aware, this is the first study to demonstrate that labeling of the mesoderm prior to the existence of definitive hematopoietic cells gives rise to all definitive hematopoietic lineages *and* endothelial cells *in vivo* in mammals, arguing that the definitive hemangioblast indeed exists and is functional. It also indicates that the *Gata2* vascular enhancer is active in this precursor cell population.

This result raises a number of other interesting questions. For instance, to what percentage of hematopoietic and endothelial cells does the definitive hemangioblast contribute? In our study, only approximately 1-2% of lineage-marker positive cells expressed LacZ; however, as discussed previously, the excision efficiency by cre recombinase under the conditions I developed for tamoxifen administration is not yet known. Second, if the definitive hemangioblast only contributes to a restricted subset of hematopoietic and endothelial cells, do they carry out a unique developmental function? Third, what role does GATA-2 play in this process? Conditional ablation of the hemangioblast population in Tg^{iVE}:$Gata2^{f/-}$ mice will begin to address these questions, along with the role GATA-2 plays in hemangioblast, hemogenic endothelium and vasculature development.

Conclusions

The embryonic development of an organism is a complex, intricately organized process that sometimes relies on the subtlest of changes to enact broad differentiation events. The same transcription factor can be independently utilized to regulate dozens of unique developmental processes. To accomplish this specificity, a gene sometimes requires regulatory elements up to a megabase away from the structural gene.

Understanding the molecular mechanisms by which transcription factors can affect such a broad array of developmental processes will greatly enhance our ability to diagnose, treat, and possibly even prevent a large number of diseases that currently

plague humanity. We could enact these changes either through understanding of the mechanisms that go awry through DNA damage at various stages of life and how to compensate for them, or through understanding of the molecular and cellular pathways that regulate development, allowing *ex vivo* regulation of these processes (i.e. organ formation, stem cell transplants, *etc*) to repair a defective *in vivo* system.

In this dissertation, I have attempted to focus on a developmental process involving one such broad mediator, the zinc finger transcription factor *Gata2.* We now know where the functional boundaries of the *Gata2* locus absolutely required for survival reside. We have also identified a previously unknown role for GATA-2 in the vasculature, and have developed a new mouse model that should help to clarify one of biologies oldest questions regarding the role of the hemangioblast in definitive hematopoiesis and vasculogenesis. Given the plethora of tools and information available to biologists today, key discoveries are being made at an ever-increasing rate. The next major milestone will be to put all of the analysis of individual genes and proteins together to understand developmental processes at a systems level, and to define how each of these individual proteins interact to generate a particular functional output (*e.g.* generation of a healthy organ). Only then will we be able to truly state that we understand how diseases that plague society (such as cancer) are regulated, and that we understand how to effectively place them in permanent remission.

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