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A transgene-assisted genetic screen identifies essential regulators of vascular development in vertebrate embryos

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Abstract

Formation of a functional vasculature during mammalian development is essential for embryonic survival. In addition, imbalance in blood vessel growth contributes to the pathogenesis of numerous disorders. Most of our understanding of vascular development and blood vessel growth comes from investigating the Vegf signaling pathway as well as the recent observation that molecules involved in axon guidance also regulate vascular patterning. In order to take an unbiased, yet focused, approach to identify novel genes regulating vascular development, we performed a three-step ENU mutagenesis screen in zebrafish. We first screened live embryos visually, evaluating blood flow in the main trunk vessels, which form by vasculogenesis, and the intersomitic vessels, which form by angiogenesis. Embryos that displayed reduced or absent circulation were fixed and stained for endogenous alkaline phosphatase activity to reveal blood vessel morphology. All putative mutants were then crossed into the *Tg(flk1:EGFP)*^{s843} transgenic background to facilitate detailed examination of endothelial cells in live and fixed embryos.

We screened 4015 genomes and identified 30 mutations affecting various aspects of vascular development. Specifically, we identified 3 genes (or loci) that regulate the specification and/or differentiation of endothelial cells, 8 genes that regulate vascular tube and lumen formation, 8 genes that regulate vascular patterning, and 11 genes that regulate vascular remodeling, integrity and maintenance. Only 4 of these genes had previously been associated with vascular development in zebrafish illustrating the value of this focused screen. The analysis of the newly defined loci should lead to a greater understanding of vascular development and possibly provide new drug targets to treat the numerous pathologies associated with dysregulated blood vessel growth.

Keywords: Zebrafish; Mutagenesis; Vascular development

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Introduction

The vasculature needs to undergo continuous modification and remodeling to accommodate the diverse needs for growth, regeneration, and repair during an organism's life. This dynamic modulation of the vascular system is achieved by intricate interactions between two distinct mechanisms, vasculogenesis (*de novo* assembly of vessels) and angiogenesis (modification and expansion of pre-existing vessels) (reviewed by Risau, 1997; Risau and Flamme, 1995).

Failure to regulate vasculogenesis and angiogenesis has been implicated in a wide variety of pathological conditions. Excessive vascular formation is usually associated with cancer, psoriasis, arthritis, and blindness, while insufficient vascular formation is involved in a variety of inherited diseases, as well as heart and brain ischemia, neurodegeneration, and osteoporosis (reviewed by Carmeliet, 2003, 2005; Carmeliet and Jain, 2000). The pathological consequences of dysregulated vascular formation have provided the impetus to understand the underlying principles of vascular system development and function, resulting in the identification of various signaling pathways and their downstream effectors (reviewed by Rossant and Howard, 2002).

The earliest event in this developmental cascade is the specification of endothelial cells, the cells lining all blood vessels. Initially, factors such as Bone Morphogenic Proteins (BMPs) (Gupta et al., 2006; Park et al., 2006) and Wnts (Lindsley et al., 2006; Wang et al., 2006) appear to define the number of potential endothelial progenitors within the nascent mesoderm. Subsequently Sonic Hedgehog (SHH) (Lawson et al., 2002; Vokes et al., 2004), Vascular Endothelial Growth Factor (VEGF) (Carmeliet et al., 1996; Ferrara et al., 1996; Cleaver and Krieg, 1998), and Notch (Krebs et al., 2000; Lawson et al., 2001) signaling pathways, as well as transcription factors including Ets family members (Dube et al., 1999; Sumanas and Lin, 2006; Pham et al., 2007), Scl/Tal1 (Kallianpur et al., 1994; Visvader et al., 1998; Patterson et al., 2005), and Coup-TFII (You et al., 2005) play critical roles in the differentiation of endothelial progenitors into arterial and venous endothelial cells. Endothelial cells then migrate towards the midline to form an aggregate known as the vascular cord, which subsequently lumenizes to form functional blood vessels (Torres-Vazquez et al., 2003; Jin et al., 2005). Extracellular matrix proteins such as Fibronectin, and mediators of cell movements such as Rac and Cdc42 provide critical functions during the migration of endothelial cells and vascular lumen formation (Jiang et al., 1994; Wijelath et al., 2002; Kamei et al., 2006). Nascent vascular networks then recruit vascular smooth muscle cells and pericytes, a process that requires the function of Platelet Derived Growth Factor (PDGF) signaling and EphrinB2–EphB4 interaction (Jain, 2003; Betsholtz et al., 2005).

Despite the progress in identifying some of the key factors required for vascular development, the functions of many signaling pathways and the interactions between them are poorly understood due to technical limitations of commonly used model systems. It is technically challenging to study the mechanisms of vascular development since the intricate architecture and context of vascular networks are difficult to reproduce *in vitro* and the development of the vascular system is strongly influenced by interactions between vessels and neighboring tissues. In addition, inaccessibility of mammalian embryos during development makes *in vivo* analyses of vascular formation a difficult task. Furthermore, the indispensable function of the placental vasculature during mammalian development constitutes another obstacle for studying the effect of genetic mutations within the developing embryo.

To understand the fundamental principles of vascular development and identify essential genes in this process, we chose to utilize the zebrafish system, since it offers a unique opportunity to overcome the aforementioned technical difficulties. In addition to its well-documented amenability to forward genetic screens (Driever et al., 1996; Haffter et al., 1996; Amsterdam et al., 1999), the externally fertilized and optically clear embryos enable one to analyze the development of the vasculature *in vivo* at a cellular level. Recently generated vascular specific transgenic lines, such as *Tg(flk1:EGFP)*^{s843} (Jin et al., 2005), facilitate the analyses. Although blood circulation starts at 24 hpf, zebrafish embryos can survive up to 7 dpf without a functional vasculature or heart beat (Stainier, 2001; Stainier et al., 1995; Sehnert et al., 2002), allowing one to study defects in vascular formation and patterning in the developing embryo over an extended period of time.

Here we present the results of a three-step forward genetic screen in zebrafish. Initially, we identified mutants with vascular defects by observing their blood circulation and subsequently stained them for endogenous alkaline phosphatase activity, which labels endothelial cells. These analyses were followed by an *in vivo* analysis using a transgenic endothelial specific GFP-reporter line (*Tg(flk1:EGFP)*^{s843}).

We identified 30 distinct genetic loci that regulate vascular development. Mutations in 11 of these loci interfere with vasculogenesis by causing changes in the number of endothelial cells or in vascular tube and lumen formation. The other 19 mutations affect angiogenesis by disturbing vessel patterning, remodeling, integrity, and/or maintenance.

A majority of the identified mutations cause vascular defects in the context of otherwise unaffected embryos. Many of the vascular defects are similar to known human conditions such as hemangioma, aortic dissection, arterio-venous malformation, cerebral cavernous vascular malformation, and cerebral hemorrhage. Given the paucity of genetic models for these human vascular conditions, these zebrafish mutants should help understand the molecular and cellular etiology of these disorders as well as provide novel insights into vascular development.

Materials and methods

ENU mutagenesis and screening

Mutagenesis was performed by treating zebrafish (*Danio rerio*) males with the chemical mutagen *N*-nitroso-*N*-ethylurea, to induce mutations in premeiotic germ cells. Founder males were subjected to three to five treatments with ENU at weekly intervals and used to generate F2 generations, as previously described (Muto et al., 2005).