MECHANISMS AND ANALYSIS OF INTRANEURAL BLOOD VESSEL PATTERNING IN THE QUAIL NEURAL TUBE.

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ABSTRACT

JENNIFER M. JAMES: Mechanisms and Analysis of
Blood Vessel Patterning in the Quail Neural Tube
(Under the direction of Victoria L. Bautch)

Neurovascular development requires communication between two embryonic organ systems, the neuroepithelium and blood vessels. During neural tube development, blood vessels enter at stereotypical locations from the surrounding peri-neural vascular plexus, forming an intraneural vascular pattern. We first investigated the role of VEGF-signaling from the neural tube in blood vessel ingression and pattern formation. Localized, ectopic expression of heparin-binding VEGF165 or VEGF189 from the avian neural tube resulted in supernumerary vessel sprouts and disrupted vessel patterning. Conversely, localized loss of endogenous VEGF-A signaling in the neural tube, via ectopic expression of the VEGF inhibitor sFlt-1, locally blocked blood vessel ingression. Thus, we demonstrated that neural-derived VEGF-A has a direct role in the spatially localized molecular crosstalk required for neurovascular development and vessel patterning in the neural tube.

Though necessary and sufficient for blood vessel sprout formation, neural tube-derived VEGF was not sufficient to explain why blood vessel sprouts form at specific times and highly stereotypical locations within the neural tube. The neural tube is also a patterned structure, and we hypothesized that normal processes of neural tube development, such as programmed neurogenesis and dorsoventral patterning of neurons,

influence the blood vessel pattern. To test this, we manipulated both the timing of neurogenesis and dorsoventral neuronal patterning via electroporation of genes known to regulate these processes. We demonstrated that the specific time of neuronal differentiation within the neural tube is important for regulating the timing of angiogenic sprout ingression. Furthermore, perturbations in neuronal cell fate specification along the dorsoventral axis of the neural tube predictably altered the locations where angiogenic sprouts ingressed. This allowed us to identify pro- and anti-angiogenic regions along the dorsoventral axis of the neural tube. This work demonstrates that neural tube development and blood vessel patterning are linked and that the neural tube directs both the timing and spatial distribution of ingressing blood vessels in quail.

To my family, and Dan.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Chapter III.

Chapter IV.

LIST OF ABBREVIATIONS

BMP	Bone Morphogenetic Protein
CNS	Central Nervous System
DRG	Dorsal Root Ganglia
DV	Dorsal-Ventral
E	Embryonic day
EC	Endothelial Cell
eGFP	enhanced Green Fluorescent Protein
ES	Embryonic Stem
Flk	Fetal Liver Kinase
(m/s) Flt1	(membrane/soluble) *fms*-like Tyrosine Kinase
HH	Hamburger and Hamilton
HIF	Hypoxia Inducible Factor
IZ	Intermediate Zone
MNR2	Motor Neuron Restricted Gene-2
MZ	Marginal Zone
NC	Notocord
NRP1	Neuropilin-1
PBS	Phosphate Buffered Saline
PNVP	Peri-Neural Vessel Plexus
PNS	Peripheral Nervous System
PSM	Pre-Somitic Mesoderm
QH1	Quail monoclonal antibody
Sema3	Semaphorin III
Shh	Sonic Hedgehog
VEGF	Vascular Endothelial Growth Factor-A
VZ	Ventricular Zone

CHAPTER I

INTRODUCTION

The work presented in this dissertation is based on observations and analyses made quite a long time ago, as scientists from the early 1900's painstakingly extracted patterns from what they saw in the intraneural blood vessels of many different organisms (Sterzi, 1904a). They discovered that the vasculature was not a tangled disarray of tubes, but instead exhibited a high degree of order. Neural blood vessels within organisms of the same species patterned reproducibly in both space and time; however, vessels within a single organism patterned differently depending on the region of the central nervous system (CNS) in which the vessels were found (Craigie, 1920; Feeney and Watterson, 1946; Sterzi, 1913). Following these observations, an idea emerged that the CNS may be able to shape the vasculature to suit its own metabolic needs. What scientists did not understand at that time was *how* the neural tissue was able to establish communication with the vasculature to direct blood vessel growth and patterning. Here, in the present study, we are focused on answering this question in one specific developmental niche of the CNS—the avian neural tube, as vessels first surround and invade the embryonic neural tissue.

Neural vessels assemble via two major processes: vasculogenesis and angiogenesis (Cleaver and Kreig, 1999; Risau, 1997). Vasculogenesis is the process by which endothelial cell (EC) precursors called angiogblasts assemble into primitive vascular beds. Angioblasts are derived from mesodermal tissue, and can either undergo *in situ* differentiation to form vascular cords in a process called vasculogenesis type I or converge into blood vessels through migration and differentiation—a process known as vasculogenesis type II. Once angioblasts and ECs have coalesced to form a primitive vessel bed, they undergo angiogenesis, or the growth of new blood vessels from pre-

existing vessels. Sprouting angiogenesis is the process in which ECs emerge from the parent vessel in response to pro-angiogenic signals in the surrounding environment, forming a blood vessel sprout. In the avian neural tube, these processes are reproducibly coordinated in both time and space, forming a blood vessel pattern.

How do the neural vessels pattern? The neural tube and the blood vessels arise from different germ layers, the ectoderm and the mesoderm, *respectively*. This presents an interesting problem for the neural tube—it does not contain blood vessels or blood vessel precursor cells (Nakao et al., 1988) and instead must rely solely on recruiting angioblasts and ECs from the surrounding mesodermal tissue (Fig 1.1 A). The first neural vessels form as migratory angioblasts and ECs coalesce into a ring of vessels surrounding the neural tube known as the peri-neural vessel plexus (PNVP) (Fig 1.1 B). Angioblasts arise in many regions within the embryo, and they are able to migrate extensively to incorporate into various vascular beds (Noden, 1989; Wilting et al., 1997). Grafted angioblasts have been shown to reproducibly migrate and incorporate into specific host vascular beds (Pardanaud et al., 1996; Wilms et al., 1991; Wilting et al., 1995). For example, mouse-avian chimeras containing mouse mesodermal grafts show that mouse angiogblasts migrate extensively and incorporate into the host's PNVP (Ambler et al., 2001). These results suggested that angioblasts were able to respond to local environmental signals that reproducibly induced migration and blood vessel formation in specific embryonic locations. Furthermore, ectopic, mouse-derived neural tube grafts also directed PNVP formation in avian hosts (Hogan et al., 2004). Taken together, these observations led to the hypothesis that the neural tube is the source of a diffusible blood vessel patterning signal that is sent out into the surrounding mesodermal

tissue to establish positive communication with angioblasts and ECs—resulting in the recruitment of a PNVP.

Subsequent experiments revealed that a major component of this neural tube-derived signal was Vascular Endothelial Growth Factor-A (VEGF). Grafted mouse ECs mutant for Flk1, the receptor that mediates intracellular VEGF-signaling, were unable to migrate and incorporate into an avian host's PNVP (Ambler et al., 2003). Furthermore, explanted mouse pre-somitic mesoderm (PSM), a tissue rich in angioblasts, was able to form a blood vessel plexus when co-cultured in a collagen matrix with avian neural tubes; however, this plexus did not form when the PSM was cultured with neural tubes in the presence of a VEGF inhibitor (Hogan et al., 2004). These experiments demonstrate that neural tube-derived VEGF is important for PNVP formation. Proper patterning of the PNVP is crucial for CNS function in the adult, as blood vessels comprising the PNVP have an important role in the formation of the blood brain barrier (Bar, 1980; Risau et al., 1986a; Risau et al., 1986b; Risau and Wolburg, 1990). PNVP vessels have another role that is central to the work presented in this thesis; they are the source of ECs that subsequently invade and vascularize the CNS.

While VEGF is clearly important for regulating PNVP formation, less is known about its role in the next step of neural tube vascularization—the angiogenic invasion of PNVP vessels into the neural tube. Blood vessels form the PNVP approximately one day before angiogenic sprouts invade the neural tube, and vessel sprouts entering the neural tube do so in highly stereotypical locations. Two works have illustrated much of what is known about the intraneural vascular pattern in the avian neural tube. Feeney and Watterson (1946) described sixteen distinct blood vessels that form reproducibly and at

somewhat regular intervals along the anterior-posterior axis of the chick neural tube before it becomes uniformly vascularized. Each vessel forms along both spatial and temporal axes, and earlier vessels build a scaffold onto which subsequent vessels will either anastomose with or sprout from. The first three intraneural blood vessels (#1, #2, and #3 according to Feeney and Watterson) that form are of particular importance to this work. The first angiogenic sprouts (#1) to invade the neural tube do so ventrally, adjacent to the floor plate (Fig. 1.1 C). The next two vessels (#2 and #3) sprout medio-laterally (Fig. 1.1 D) within 12-24 hours of ventral sprouting events. The discovery that a hierarchal system of intraneural blood vessel growth and patterning exists, suggests that neural tube angiogenesis is highly regulated by the neural tissue, and the specific order and region in which each vessel forms likely serves an important role in proper neural development.

The second body of work by Kurz and Christ (1996) focuses on a narrower window of embryonic development, during which only these first three blood vessels enter the quail neural tube. They utilized an antibody recognizing a sugar moiety on the surface of quail angioblasts and ECs, called QH1 (Pardanaud et al., 1987), to label the quail vasculature. This tool allowed Kurz and Christ to add to what was already known about vascular patterning in the avian neural tube because it allowed them visualize vessels that did not yet have blood flow, such as new vessel sprouts and individual angioblasts and ECs. They found that the first vascular cells to enter the neural tube were invasive angioblasts that immigrated into the dorsal neural tube (Fig. 1.1, C; arrowhead). Additionally, they confirmed that angiogenic sprouts do not invade the dorsal neural tube in quail as they do in mouse (Nakao et al., 1988). Like Feeney and

Watterson, Kurz and Christ also believed that the neural tube directed intraneural vessel growth and patterning, but it was unclear how the neural tissue orchestrated these patterning events. We were first interested in understanding how VEGF-signaling from the neural tube contributes to this stage of vessel patterning, as angiogenic sprouts from the PNVP invade the neuroepithelium.

The VEGF signaling pathway has well characterized roles in vascular development (Bautch and Ambler, 2004; Carmeliet and Collen, 1999; Olsson et al., 2006). VEGF binds two receptors expressed on the surface of ECs, Flt1 (VEGFR1) and Flk1 (VEGFR2). VEGF binding to Flk1 mediates positive downstream effects such as angioblast differentiation into ECs, as well as EC proliferation, survival, and migration. The Flt1 receptor is alternatively spliced into two isoforms, a membrane-bound form (m-Flt1), and a soluble form (s-Flt1). Both Flt isoforms are thought to act as a ligand sink to negatively modulate the amount of VEGF available to bind Flk1 (Kendall and Thomas, 1993). Flt1 mutant mice and embryonic stem (ES) cells display a vessel overgrowth phenotype resulting in early embryonic lethality (Fong et al., 1995), and the formation of vascular sheets in ES cell cultures (Kearney et al., 2002). The vessel overgrowth phenotype can be rescued by s-Flt1 expression and partially rescued by m-Flt1 expression in the ES cell cultures (Kappas et al., 2008). These vascular defects may be caused by a VEGF gain of function, as elevated amounts of VEGF expression in embryos also results in vascular overgrowth (Miquerol et al., 2000). The major components of the VEGF-signaling pathway are shown in Fig. 1.2.

VEGF expression is regulated, in part, by hypoxia (Pugh and Ratcliffe, 2003; Shweiki et al., 1992). Quail embryos incubated in hypoxic conditions globally

upregulate VEGF (Nanka et al., 2006). In the context of the developing neural tube, VEGF is upregulated early, as rapidly proliferating neural progenitor cells become hypoxic. This VEGF diffuses into the surrounding mesodermal tissue, presumably forming a gradient. The concept of a VEGF gradient is important for understanding how VEGF can act both as a long range signal, attracting angioblasts that are quite a distance from the neural tube, and also as a short-range blood vessel patterning cue to mediate local blood vessel patterning events. The VEGF gradient can be broken down into two major components: 1) Concentration of VEGF, and 2) Alternative splicing of *Vegf*, leading to the generation of VEGF isoforms with different affinities for the extra-cellular matrix.

1) Concentration of VEGF: The level of VEGF expression in the developing embryo is crucial for embryonic survival. Inactivation of one *Vegf* allele results in embryonic lethality in mice between embryonic (E) day 11 and 12 (Carmeliet et al., 1996; Ferrara et al., 1996). Blood vessels and ECs fail to develop properly in these mutant embryos. Conversely, moderate increases in VEGF signaling, produced by insertion of a modified *vegf* gene into the endogenous locus, result in embryonic lethality due to cardiovascular defects and vessel overgrowth at E12.5 (Miquerol et al., 2000). Proper levels of CNS-derived VEGF are important for regulating the density of blood vessels in the brain. A severe reduction, or targeted deletion, of CNS-derived VEGF in mouse resulted in early postnatal lethality, reduced blood vessel density in the brain, increased neuronal apoptosis, and severe degeneration of the cerebral cortex (Haigh et al., 2003; Raab et al., 2004). These experiments suggest that a narrow range of VEGF levels, allowing for proper blood vessel growth and patterning, exists in developing embryos. If

sufficient levels aren't met, not enough vessels form to allow proper development of embryonic organs, and if too much VEGF is expressed, vessel overgrowth is the result; however, these studies did not extensively examine the effects of modulating VEGF expression levels in the neural tube at the time of vessel ingression.

2) Alternative splicing of vegf. VEGF can act both as a long-range vessel patterning cue, as well as a short-range signal to locally guide vessel sprouts (Carmeliet et al., 1999; Ruhrberg et al., 2002; Stalmans et al., 2002). It can achieve this signaling range because it is alternatively spliced into at least 6 isoforms, three of which are abundantly expressed in the mouse embryo: VEGF120, VEGF164, and VEGF188. In the quail, there are four major isoforms: VEGF122, VEGF146, VEGF166, and VEGF190 (Finkelstein and Poole, 2003). Each isoform interacts differently with the extracellular matrix via heparin binding sites (Houck et al., 1992). VEGF188 has two heparin-binding sites and is the least soluble, while VEGF120 has no matrix-binding sites and is completely soluble. VEGF165 has one heparin-binding site and maintains intermediate properties (Park et al., 1993). VEGF isoform mRNA expression analysis in mouse shows that all three major splice variants are expressed in the CNS, but they are present in greatly varying amounts. VEGF165 is the most abundant transcript, comprising approximately 75% of the total mRNA, while VEGF120 and VEGF188 make up 20% and 5% of the remaining transcripts, *respectively* (Ng et al., 2001). Mice generated to express only one of the three major isoforms have intraneural vessel patterning defects. $VEGF^{120/120}$ neural tubes display delayed ingression and reduced sprout number. $VEGF^{188/188}$ mouse neural tubes have hyper-branched, thin vessels, while $VEGF^{165/165}$ mice have phenotypically normal blood vessels (unpublished data, J.M.J. and V.L.B.).

These observations are consistent with reports describing vessel branching and morphogenesis defects in other regions of the isoform mutant mice (Ruhrberg et al., 2002).

Neuropilin-1 (NRP1) is also important for mediating isoform-specific, VEGF-signaling effects. NRP1, a co-receptor for VEGF, is expressed in endothelial cells and has been shown to enhance VEGF-Flk1 interactions *in vitro* (Soker et al., 1998) by complexing with Flk-1. NRP1 is also highly expressed on neuronal axons and acts as a co-receptor for Semaphorin molecules, forming a complex with Plexin receptors (Kolodkin et al., 1997). Although NRP1 can bind all three major VEGF isoforms, VEGF120 is too small to bridge the gap between Flk1 and NRP1 (Pan et al., 2007), thus precluding complex formation. This is thought to partially account for the severity of blood vessel defects in the $VEGF^{120/120}$ mice. Mice mutant for NRP1 display embryonic blood vessel patterning defects (Kawasaki et al., 1999). In these mutant mice, blood vessel ingression and blood vessel density within the embryonic hindbrain appear normal; however, there are defects in blood vessel branching as vessel sprouts interface the ventricular zone (Gerhardt et al., 2004). Endothelial-specific deletion of Neuropilin-1 has a different effect, resulting in the formation of large, un-branched vessels within the neural tube (Gu, 2003), which is reminiscent of the $VEGF^{120/120}$ vessel phenotype.

In Chapter II, we further dissect the role of VEGF-signaling in intraneural blood vessel patterning, demonstrating that neural tube-derived VEGF is required for blood vessel ingression. We also explored how (or if) the spatial distribution of VEGF in the neural tube directs stereotypical blood vessel ingression, by overexpressing individual VEGF isoforms in quail neural tubes in the presence of endogenous quail VEGF. We

found that VEGF isoforms differentially regulate ectopic angiogenesis and that matrix-binding VEGF-expressing cells are able to provide precise patterning information to ingressing vessels, whereas non-matrix-binding VEGF cannot.

While VEGF is clearly important for blood vessel ingression, recent studies have implicated other signaling pathways in intraneural blood vessel patterning. For example, Wnt/ß-catenin signaling regulates sprouting angiogenesis into the mouse neural tube, revealing an important neural-specific role for endothelial ß-catenin in the formation of the blood brain barrier (Daneman et al., 2009; Stenman et al., 2008). Mice mutant for Wnt7a and Wnt7b displayed intraneural vessel hemorrhage, resulting in "bloody neural tubes"; however, the mechanism behind the vessel phenotype remains to be elucidated. Although the general assessment was that Wnt signals emanating from the ventricular zone (VZ) of the neural tube positively mediated blood vessel ingression, blood vessels still ingressed in the Wnt mutant embryos, albeit in reduced numbers. If genes such as Wnt and VEGF are expressed throughout the neruoepithelium, positively regulating blood vessel ingression, then *why do blood vessels enter the neural tube in such highly stereotypical locations?*

In Chapter III we explore the possibility that specific regions of the neural tube may be anti-angiogenic while other regions promote blood vessel ingression. Normal processes of neural development, such as programmed neurogenesis and dorsal-ventral (DV) neuronal cell fate specification, divide the neural tube into specific sub-populations of cells: neural progenitor cells and differentiated neurons, including dorsal interneurons, ventral interneurons, and motor neurons (Jessell, 2000; Lee and Jessell, 1999). We altered the developmental landscape of the neural tube by manipulating the timing of

neurogenesis, as well as DV patterning, to assess whether or not ingressing blood vessels were negatively or positively influenced by certain neural populations. We demonstrate for the first time that these normal processes of neural tube development direct the intraneural vessel pattern. Most intriguingly, we identified motor neurons in the ventral neural tube as a transiently anti-angiogenic cell population that initially blocks vessel ingression into this area. *But what is the mechanism?*

As motor neurons develop, they must project axons out of the neural tube to innervate multiple target tissues. The axonal growth cone is an extremely tactile structure, sending out filopodia to interact with multiple signals in its environment. Signals are used to determine which way to migrate, based on a fine balance of positive and negative axonal guidance cues. Numerous studies show that molecules regulating axonal guidance and patterning also regulate blood vessel patterning, and the tip cell of a growing vessel acts much like an axonal growth cone. There are four main classes of guidance molecules that have roles in both axonal and blood vessel patterning: Semaphorins, Slits, Netrins, and Ephrins (Carmeliet and Tessier-Lavigne, 2005). The Semaphorins and Slits are of particular interest to us because of their motor neuron-restricted patterns (Holmes and Niswander, 2001; Luo et al., 1995). A family of Semaphorin molecules, the Semaphorin III's (Sema3), repels axons when expressed outside of the neural tube, placing motor axons on the correct trajectory toward muscle targets, while Sema3-sigaling within the neural tube sets axon sensitivity to this peripheral Sema expression by regulating Nrp1 expression (Moret et al., 2007). Certain Sema3 family members also negatively regulate angiogenesis and EC migration (Gu et al., 2005; Serini et al., 2003; Torres-Vazquez et al., 2004). Studies suggest that Slit2-

signaling through the Robo4 receptor on ECs, also negatively regulates EC migration (Jones et al., 2008; Park et al., 2003). Slit2 is highly expressed in the motor neurons. These studies highlight possible mechanisms for the negative regulation of blood vessel ingression by the motor neurons demonstrated in Chapter III. In Chapter IV, we begin to explore the possibility that motor neuron cell bodies negatively regulate blood vessel ingression via Semaphorin expression; however, this work is ongoing.

Blood vessels invade the neural tube at a specific time and in highly stereotypical locations. While studies show that the neural tube directs PNVP formation, the role that the neural tube plays in regulating blood vessel ingression has not been explored. We demonstrate here that neural patterning and blood vessel patterning are linked, and that neural tube development directs the intraneural blood vessel pattern in quail.

Figure 1.1: Major blood vessel patterning events in the avian neural tube.

(A) Angioblasts and ECs in the surrounding mesodermal tissue migrate toward the neural tube in response to secreted signals produced by the neural tube. **(B)** Migratory cells begin to coalesce and form the PNVP as early as HH14 (Day 2). **(C)** At HH22 (Day 3.5), ventral sprout formation commences (#1), and sprouts invade the neural tube lateral to the floor plate (FP) (arrow). Ventral sprouts continue on a dorsal trajectory, migrating along the ventricular zone (VZ); however, they never invade this region. Single angioblasts immigrate into the dorsal region of the neural tube at this stage (arrowhead). **(D)** Approximately 12-24 hours after ventral sprouts ingress, medio-lateral sprouts form (#2 and #3, arrowheads). At HH25 (Day 5), the stage depicted here, medio-lateral sprouts branch laterally within the intermediate zone (IZ) as they interface the VZ. DRG, dorsal root ganglia; MZ, marginal zone; NC, notocord.

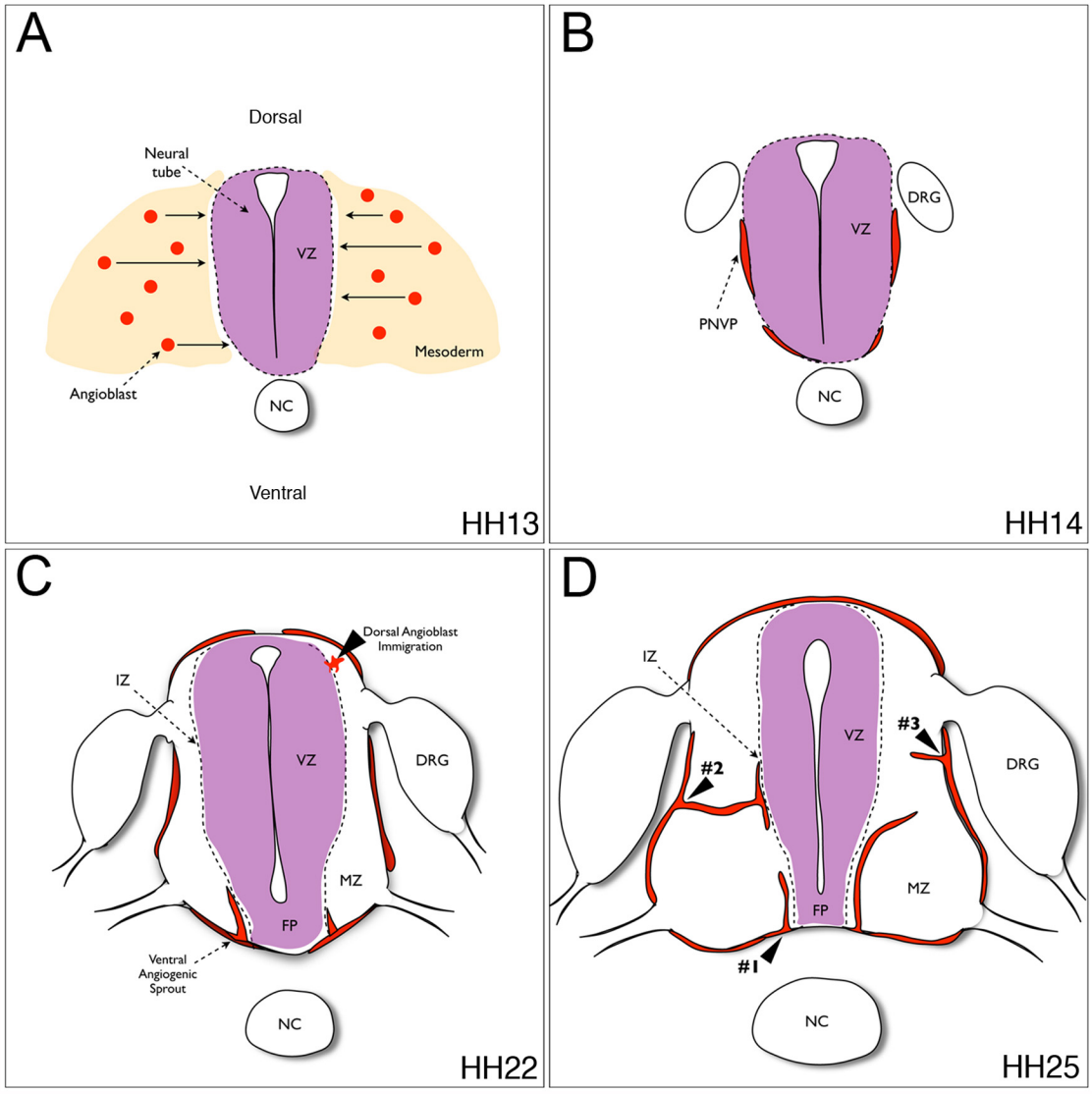
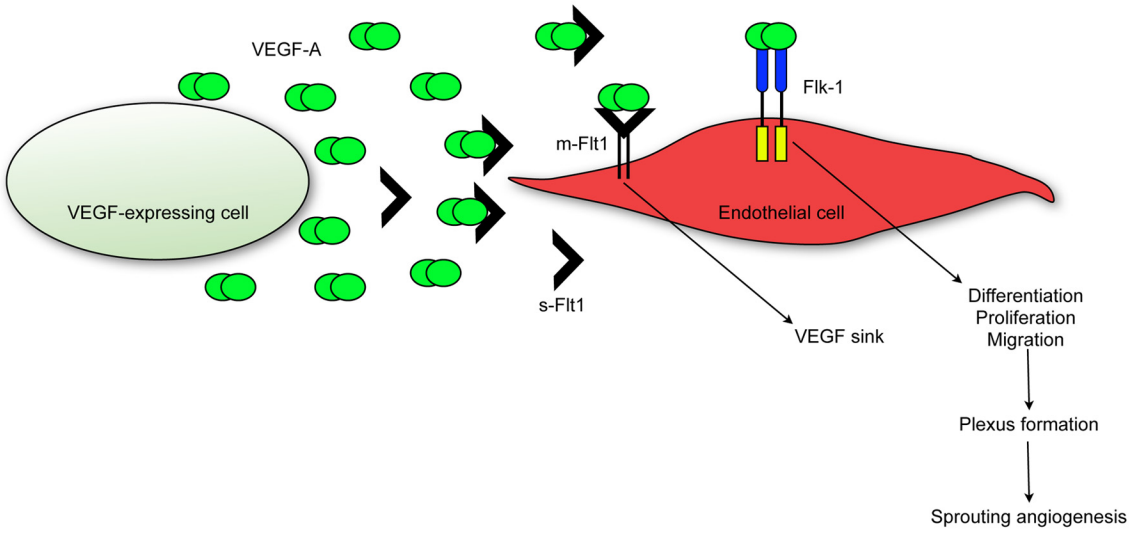

A
Dorsal
Neural tube
VZ
Mesoderm
Angioblast
NC
Ventral
HH13
B
DRG
VZ
PNVP
NC
HH14
C
Dorsal Angioblast Immigration
IZ
VZ
DRG
MZ
FP
Ventral Angiogenic Sprout
NC
HH22
D
IZ
VZ
#3
#2
DRG
MZ
FP
#1
NC
HH25

Figure 1.2: VEGF signaling in the neural tube.

(A) Overview of major VEGF-signaling components. VEGF, expressed by neural cells, interacts with Flk1 and Flt1 receptors expressed by endothelial cells (ECs). VEGF binding Flk1 mediates positive downstream effects such as EC survival, proliferation, and migration, resulting in vessel plexus formation and angiogenesis. Both s-Flt1 and m-Flt1 bind free VEGF, preventing VEGF interaction with Flk1 by acting as a ligand sink.


VEGF-A
VEGF-expressing cell
m-Flt1
Flk-1
Endothelial cell
s-Flt1
VEGF sink
Differentiation
Proliferation
Migration
Plexus formation
Sprouting angiogenesis

CHAPTER II*

NEUROVASCULAR DEVELOPMENT USES VEGF-A SIGNALING TO REGULATE BLOOD VESSEL INGRESSION INTO THE NEURAL TUBE

*Reproduced with permission from: Jennifer M. James, Cara Gewolb, and Victoria L. Bautch (2009). Neurovascular Development Uses VEGF-A Signaling to Regulate Blood Vessel Ingression Into the Neural Tube. *Development*, 136; 833-841.

A. SUMMARY

Neurovascular development requires communication between two developing organs, the neuroepithelium and embryonic blood vessels. We investigated the role of VEGF-A signaling in the embryonic crosstalk required for ingression of angiogenic vessel sprouts into the developing neural tube. As the neural tube develops, blood vessels enter at specific points medially and ventrally from the surrounding peri-neural vascular plexus. Localized ectopic expression of heparin-binding VEGF165 or VEGF189 from the developing avian neural tube resulted in supernumerary blood vessel ingression points and disrupted vessel patterning. By contrast, localized, ectopic neural expression of non-heparin-binding VEGF121 did not produce supernumerary blood vessel ingression points, although the vessels that entered the neural tube became dysmorphogenic. Localized loss of endogenous VEGF-A signaling in the developing neural tube via ectopic expression of the VEGF inhibitor sFlt-1 locally blocked blood vessel ingression. The VEGF pathway manipulations were temporally controlled and did not dramatically affect neural tube maturation and dorsal-ventral patterning. Thus, neural-derived VEGF-A has a direct role in the spatially localized molecular crosstalk that is required for neurovascular development and vessel patterning in the developing neural tube.

B. INTRODUCTION

A properly patterned network of blood vessels is crucial to embryonic development, as this network supplies nutrients and oxygen to developing organ systems within the embryo. Embryonic blood vessels pattern reproducibly in both space and time, indicating that molecular cues emanating from other embryonic organs shape the vasculature and are also highly regulated (for reviews, see (Hogan and Bautch, 2004); (Coultas et al., 2005)). Although it is known that the developing vasculature responds to spatial cues from other embryonic structures to produce a functional vessel network, relatively little is known about how this crosstalk is established and regulated.

The central nervous system (CNS) is initially devoid of blood vessels and blood vessel precursors, so communication between the developing CNS and the developing vasculature outside the CNS is essential for proper development of the brain and spinal cord. The brain and neural tube recruit blood vessels by inducing the proliferation, migration and differentiation of angioblasts and endothelial cells from the adjacent presomitic mesoderm and the lateral plate mesoderm (Ambler et al., 2001; Hogan et al., 2004; Klessinger and Christ, 1996; Kurz et al., 1996; Pardanaud and Dieterlen-Lievre, 1993; Pardanaud et al., 1996; Wilting et al., 1995). A ring of vessels, known as the peri-neural vascular plexus (PNVP), initially forms around the CNS. Subsequently, vessels invade the neural tissue through angiogenic sprouting, and in avian neural tubes single angioblast migration into the dorsal neural tube also contributes to neural vascularization (Kurz et al., 1996). Thus, a vessel network is established within the developing CNS to

support further growth and development. As blood vessels enter the neural tube, they migrate along radial glia to move inwards from the lateral surface (Virgintino et al., 1998). In some areas of the CNS, the pattern of blood vessel ingression is highly stereotypical, as originally described by Feeney and Watterson (Feeney and Watterson, 1946), suggesting that neural-derived spatial cues regulate the patterning of ingressing vessels. Although there is recent evidence that endothelial cells may respond to intrinsic transcription factor programs to pattern in the telencephalon (Vasudevan et al., 2008), this paradigm is unlikely to be operative in the neural tube where internal vessels arise from nearby surface vessels.

Several signaling pathways are involved in embryonic vascular patterning, including VEGF-A (VEGF), Notch, ephrin and semaphorins (for reviews, see (Carmeliet and Tessier-Lavigne, 2005; Eichmann et al., 2005; Hogan and Bautch, 2004). Target tissues produce ligands that interact with receptors expressed on angioblasts or endothelial cells, and these interactions impart attractive or repulsive cues that pattern blood vessels. This paradigm, however, is difficult to demonstrate in the developing nervous system, because most of the relevant signaling pathways have roles in both the neural and vascular compartments (for reviews, see (Carmeliet and Tessier-Lavigne, 2005; Lambrechts and Carmeliet, 2006). For example, genetic manipulation of components of the VEGF-A (VEGF) signaling pathway indicates a positive role for the pathway in neurovascular crosstalk, but the exact role of VEGF signaling in vascular versus nervous tissue is unclear. Global deletion of VEGF or its major signaling receptor Flk-1 (VEGFR-2) is embryonic lethal early in development (Carmeliet et al., 1996; Ferrara et al., 1996;

Shalaby et al., 1995), precluding analysis of neurovascular interactions. Reduction of VEGF signaling from the developing CNS can be achieved by conditional deletion of VEGF-A using a nestin-Cre deletor strain. This produces a reduced density of blood vessel branching with moderate reduction of VEGF-A signal, while a more profound reduction leads to neuronal apoptosis and lethality (Haigh et al., 2003; Raab et al., 2004). Genetic deletion of a VEGF-A co-receptor, Neuropilin-1 (NRP1), results in appropriate vessel ingression but reduced lateral branching in the sub-ventricular zone (Gerhardt et al., 2004), whereas endothelial-specific deletion of NRP1 results in large unbranched vessels in the brain, indicating a role for NRP1 in vessel branching and morphogenesis after ingression (Gu et al., 2003).

VEGF-A RNA is alternatively spliced to yield several major isoforms, and different isoforms have differential affinity for the extracellular matrix. VEGF165 and VEGF189 interact moderately or strongly with the matrix via heparin-binding domains, whereas VEGF121 does not have heparin-binding properties and is more diffusible (Park et al., 1993). Analysis of mice that express individual VEGF-A isoforms shows that vessel morphogenesis is affected by these perturbations (Ruhrberg et al., 2002; Stalmans et al., 2002). $VEGF^{120/120}$ embryos have larger diameter vessels that branch less often than normal, whereas $VEGF^{188/188}$ embryos have smaller diameter vessels that branch more often than normal. However, embryos expressing single VEGF-A isoforms supported vessel ingression into the CNS.

We have previously shown that endothelial cells of presomitic mesoderm origin make a significant contribution to the PNVP (Ambler et al., 2001), and that VEGF

signaling is important in this process (Ambler et al., 2003; Hogan et al., 2004). Ectopically grafted neural tubes recruited a PNVP, and an explant model was used to show that neural tube derived VEGF-A is required for formation of a vascular plexus from presomitic mesoderm. Here, we address the role of VEGF-A signaling in the next step of neurovascular communication: the ingression of blood vessels into the developing neural tube. We show that locally mis-expressed heparin-binding VEGF-A isoforms induce ectopic ingression of blood vessels into the neural tube, and that local blockade of endogenous VEGF-A prevents vessel ingression in a spatially restricted manner. These perturbations are temporally controlled and do not dramatically affect the patterning of the neuronal populations of the developing neural tube, indicating that direct communication between neural tissue and developing vessels via VEGF-A signaling is crucial to proper and patterned blood vessel ingression into the neural tube.

C. MATERIALS AND METHODS

Expression Vectors

The human VEGF121, VEGF165 and VEGF189 cDNAs (gift of J. Abraham) (Tischer et al., 1991) were inserted into the pCAGGS-IRES2-nucEGFP (pCIG) vector (Megason and McMahon, 2002) (gift of L. Pevny and A. McMahon) between the *Pst*I and *Sma*I sites (VEGF121 and VEGF165) or the *Eco*RI and *Pst*I sites (VEGF189) to make the VEGF expression vectors pCIG-VEGF121, pCIG-VEGF165 and pCIG-VEGF189. Mouse soluble Flt1 cDNA (Kappas et al., 2008) was inserted into the *Eco*RI site of the pCIG vector, generating pCIG-sFlt-1.

In ovo quail electroporation

Hamburger and Hamilton stage 16-18 (HH 16-18) Japanese quail embryos (Coturnix japonica, Ozark Egg Company, Stover, MO) were electroporated *in ovo* as described previously (Itasaki et al., 1999), with modifications. Briefly, the pCIG control vector (0.5 µg/µl), VEGF121, VEGF165, VEGF189 (0.2 µg/µl), or sFlt-1 (0.5 µg/µl) DNA was suspended in DMEMF12 media (GIBCO, Grand Island, NY) containing 1 penicillin/ streptomycin and 50 ng/ml Fast Green (Sigma, St Louis, MO). Approximately 1 nl was injected, to fill the posterior lumen of the neural tube. Electroporation was with three pulses (50 milliseconds each) of 20 mV using a BTX ECM830 Square Electroporator (Harvard Apparatus, Holliston, MA) equipped with 3 mm gold-tipped, L-shaped BTX genetrode electrodes (Genetronics) that flanked the neural tube. Eggshells were taped and embryos developed an additional 48 hours at 37°C before dissection into

cold PBS. Embryos were viewed under an Olympus IX-50 epifluorescence microscope (Opelco, Sterling, VA) to visualize DNA incorporation via GFP expression.

Immunofluorescence and Analysis of Ingression Patterns

At stage HH 25-26, embryos were dissected from the yolk and fixed for 1-2 hours in 4% PFA (paraformaldehyde)/PBS at 4°C, rinsed in cold PBS, then washed for 1 hour in PBS on ice. Embryos were incubated in 30% sucrose/PBS overnight at 4°C, then embedded in OCT media (TissueTEK, Sakura Finetek, Torrance, CA), and 12 μm sections through the upper-limb level (thoracic neural tube just posterior to the heart) were cut with a cryostat (Microm HM505E, Germany). Frozen sections were washed in PBS, briefly blocked in antibody staining solution containing: 1X PBS, 0.1% Triton X-100 (Sigma), 1% heat-inactivated goat serum (GIBCO), then incubated in antibody staining solution and primary antibody overnight at 4°C. Monoclonal mouse antibodies to Pax7, Pax6 and MNR2 were used at a concentration of 1:50 [obtained from the Developmental Studies Hybridoma Bank (DSHB) developed under the auspices of the NICHD and maintained by The University of Iowa Department of Biological Sciences, Iowa City, IA 52242]. QH1 (DSHB), transitin (DSHB) and anti-β-Tubulin type III (Tuj1, Covance, Emeryville, CA) were used at a concentration of 1:500. Sections were rinsed twice in antibody staining solution, then incubated with anti-mouse IgG conjugated Cy3 (Sigma) for 2 hours at room temperature at a concentration of 1:250. Mounted sections were visualized with an Olympus IX-50 epifluorescence microscope and images were acquired with an Olympus DP71 digital camera (Center Valley, PA).

Ingression patterns were analyzed quantitatively as follows. A 1 mm region of

quail neural tube, starting at the upper limb, was sectioned. Cryostat sections (12 μm) were taken at intervals of approximately 72 μm and stained with QH1. Vessel ingression points were scored when a clear vascular connection from the PNVP into the neural tube was noted. Angle measurements of ingressing angiogenic sprouts were calculated for each image by drawing a center line through the lumen of the neural tube from the floor plate to the roof plate and locating the center point of this line. To determine the location of angiogenic sprouts, we plotted the angle of ingression from the initiation point of each sprout to the center, then down to the ventral-most point. Angles were then measured using Metamorph software. The ventral-most point along the center line is labeled 0°, whereas the dorsal-most point is 180° (Fig. 2A). Angles were binned into each 10° of arc. In each group, five embryos were analyzed and the total number of ingression points in each 10° of arc plotted on a graph.

In situ hybridization

cDNAs were generated from quail genomic DNA obtained from whole embryos (HH 23). Published quail VEGF (qVEGF) primer sequences were used to amplify fragments of qVEGF166 cDNA (Flamme et al., 1995). The fragment was gel-extracted and ligated into the pCR-Blunt II-TOPO vector (Invitrogen, Carlsbad, CA). Quail VEGF166 antisense probe was amplified using the SP6 promoter according to manufacturer's instructions (Roche, Indianapolis, IN). In situ hybridization was performed as described (Colbert et al., 1995), with minor modifications, on 20 μm transverse sections cut with a cryostat.

RNA Analysis

Neural tubes electroporated on day 3 (HH16-17) were harvested on day 5 (HH25), and three to five neural tubes/construct were pooled. Total RNA was isolated using Trizol (Invitrogen) and cDNA was generated as described (Kappas et al., 2008). Equivalent amounts of cDNA were amplified using human VEGF-A (hVEGF-A) primers that spanned the alternative splice region, and PCR products were visualized by agarose gel electrophoresis and normalized to GFP. The primers used were:

forward hVEGF-A, 5-CTGCTGTCTTGGGTGCATTGG-3;

reverse hVEGF-A, 5 -TCACCGCCTCGGCTTGTC-3;

eGFP forward, 5 -CCTACGGCGTGCAGTGCTTCAGC-3;

eGFP reverse, 5 -CGGCGAGCTGCACGCTGCGTCCTC-3.

D. RESULTS

Blood vessel patterning around and within the quail neural tube

Feeney and Watterson described stereotypical vessel ingression into the embryonic chick neural tube at the cervical level from days 4-16 of development using dye injection (Feeney and Watterson, 1946). Our studies focused on the thoracic level of the quail neural tube between days 3-5 of development. To establish a developmental time frame for major blood vessel patterning events in this area of the neural tube, we performed a time-course analysis of blood vessel patterning in transverse sections at stages HH 16-27, using QH1 immunostaining to visualize both patent vessels and non-patent sprouts (Hamburger and Hamilton, 1992; Pardanaud et al., 1987) (Fig. 2.1 A, D, G, J, M, P). Similar to initial neurovascular patterning events in mouse (Hogan et al., 2004), the perineural vascular plexus (PNVP) begins to form first along the mid-levels of the lateral (pial) surface of the neural tube (Fig. 2.1 A; arrow). As development proceeds, the PNVP becomes progressively more complete (Fig. 2.1D, G). Dorsal angioblast immigration (Fig. 2.1 G; arrowheads) and ventral sprouting (Fig. 2.1 G; arrows) were first observed at stage HH 22-24, and medial angiogenic sprouting was first observed at stage HH 24-25 (Fig. 2.1 J, M; arrows). Angiogenic sprouting from the PNVP never occurred dorsal to the entry site for the DRG (dorsal root ganglia), and vessels were never seen sprouting into the floor plate. These results are consistent with an earlier description of developmental vessel ingression in the cervical region of the quail embryo (Kurz et al., 1996) and with the work of Feeney and Watterson (Feeney and Watterson, 1946), given that events occur earlier in more anterior regions of the CNS.

β-Tubulin III was visualized by staining with the TuJ1 antibody on adjacent sections to follow neural development. During early development the neural tube exists primarily as a proliferating pool of neural progenitor cells that maintain connections with both the luminal and pial surfaces of the neural tube. As these cells undergo programmed differentiation, they lose contact with the luminal surface of the neural tube and migrate laterally to the pial surface, where they begin to express β-tubulin III (Fig. 2.1 B, E, H, K, N, Q). At these stages, the Tuj1-positive cells are primarily neurons (Nakai and Fujita, 1994). Angiogenic sprouts ingressed into the Tuj1-positive area, but only in specific medial and ventral regions (compare Fig. 2.1 G-R). Once in the neural tube, angiogenic sprouts avoided the Tuj1-negative medial area that contained proliferative progenitor cells, as described for the hindbrain (Gerhardt et al., 2004). These data suggest that the developing neural tube may influence blood vessel ingression.

Blood vessel ingression into the neural tube is highly stereotypical

To determine the spatial patterning of ingressing blood vessels in the developing neural tube, a region of the quail embryo at the upper limb level (thoracic) was serially sectioned in the transverse plane, stained for QH1 and analyzed as described in the Materials and Methods (*n*=5 embryos). Control analyses showed that the ingression points of angiogenic sprouts within the quail neural tube were highly stereotypical (Fig. 2.2 A, B). Approximately 33% of blood vessels ingressed into the neural tube between 70 and 110 degrees, whereas the remaining 66% of vessels ingressed between 10 and 20 degrees. To determine whether electroporation and/or expression of GFP affected the spatial pattern of vessel ingression, quail neural tubes were electroporated with a vector

that only expressed eGFP (Fig. 2.2 C, D). At the time of electroporation (HH stage 16-18, day 3), the DNA enters the cells that line the lumen of the neural tube. The neural cells that acquire DNA are primarily neuroepithelial and radial glia progenitor cells whose descendents form neurons and glial cells such as astrocytes and oligodendrocytes (Gotz and Huttner, 2005; Leber and Sanes, 1995). At this time, no blood vessel sprouts have entered the neural tube, so they are not electroporation targets. Analysis of these embryos at HH stage 25-26 (day 5) showed that the pattern of vessel ingression was similar to that of unperturbed controls, on both the side of the neural tube that expressed eGFP and the contralateral side that received current but no DNA. Thus, these technical manipulations did not affect the spatial pattern of vessel sprouting ingression into the developing neural tube. As *in ovo* electroporation at these developmental stages ultimately delivered DNA to the medial vessel ingression area of the neural tube more efficiently than to the ventral ingression area, medial blood vessel ingression into the neural tube was further analyzed.

Localized mis-expression of matrix-binding VEGF isoforms alters the neural tube blood vessel ingression pattern

We hypothesized that VEGF-A expressed by the developing neural tube was involved in the ingression of angiogenic sprouts into the tube. We confirmed that VEGF-A was expressed in the quail neural tube at the stages analyzed (see Supplementary Fig. 2.1), consistent with previous reports (Aitkenhead et al., 1998; Nanka et al., 2006). The pattern of VEGF-A RNA expression did not correlate with specific ingression points. Rather, it was fairly uniform throughout the developing neural tube, with a modest

concentration of signal in the floor plate, roof plate and motoneuron area, which are sites blocked to blood vessel ingression (see Supplementary Fig. 2.1). To analyze the role of individual VEGF isoforms in blood vessel ingression into the developing neural tube, we electroporated DNAs that expressed VEGF121, VEGF165 or VEGF189 from the ubiquitous chicken β-actin promoter into stage HH 16-18 quail neural tubes. The DNAs also expressed eGFP from an IRES sequence, so GFP-positive cells corresponded to cells that expressed the VEGF isoforms. The amount of DNA and the electroporation conditions were titrated to achieve moderate and localized expression of the reporter GFP and VEGF isoform cDNAs, so that the phenotypes could be analyzed in a spatial context. Analysis of human VEGF mRNA in electroporated neural tubes by semi-quantitative RT-PCR indicated that equivalent amounts of each transgene were expressed relative to the GFP signal (see Supplementary Fig. 2.2). Expression ofVEGF121 did not alter the vessel ingression pattern (Fig. 2.3 A-D; *n*=5 embryos). The pattern was indistinguishable from that seen on the contralateral non-electroporated side and in the controls, although medial ingression points were scored more frequently on the VEGF121-expressing side of the neural tube (Fig. 2.3 D). In some cases, the vessels that ingressed into neural tubes expressing VEGF121 had an increased diameter (data not shown, Fig. 2.4 A and Fig. 2.5 A). By contrast, expression of similar levels of the heparin-binding VEGF-A isoforms VEGF165 or VEGF189 induced ectopic vessel ingression points along the PNVP (Fig. 2.3 E-L; *n*=5 embryos for each group). Analysis of the ingression points in relation to the cells expressing VEGF165 or VEGF189 showed that ectopic ingression points were localized to areas of the neural tube that contained

cells expressing the heparin-binding VEGF isoform (Fig. 2.4 B, C, E, F). By contrast, neural tubes with cells that ectopically expressed VEGF121 showed blood vessel ingression only in the medial region, despite the presence of VEGF121-expressing cells along the dorsoventral axis of the neural tube (Fig. 2.4 A, D).

We next analyzed several regional markers of neural tube patterning to determine whether ectopic VEGF-A isoform expression affected neural tube development (Fig. 2.5). Pax7 is expressed in population of dorsal neural progenitors, Pax6 is expressed in a group of medial neural progenitors and MNR2 is a marker for motoneuron progenitors, which are localized to the ventral-most region of the developing neural tube. The localized, moderate expression levels of the VEGF isoforms over 48 hours did not significantly affect the expression patterns of these markers (Fig. 2.5 D-F, K-M, R-T). Thus, VEGF isoforms that interact with the matrix induce ectopic blood vessel sprouting from the PNVP into the developing neural tube and perturb vascular patterning, without significantly affecting neural tube development on the dorsoventral axis.

Because blood vessels migrate along radial glia once they enter the neural tube, we asked whether electroporated neural tubes had perturbed patterning of the radial glia. Staining with the radial glia marker transitin showed that expression of moderate levels of any of the VEGF isoforms did not significantly alter the staining pattern (see Supplementary Fig. 2.3). Moreover, the radial glia staining pattern on both the electroporated and control sides of the neural tube suggested that radial glia do not selectively associate with ingression points. Instead, the staining indicated that radial glial processes are spaced relatively evenly along the dorsal-ventral axis of the lateral edge of

the neural tube (see Supplementary Fig. 2.3).

Loss of VEGF-signaling from the neural tube blocks vessel ingression

To assess the effect of loss-of-function of the VEGF signaling pathway, we electroporated a soluble Flt-1 (sFlt-1)-expressing cDNA into the developing neural tube. sFlt-1 is a natural splice form of the Flt-1 receptor that can bind and sequester VEGF-A, thus preventing binding of VEGF-A to Flk-1 and downstream signaling (Kendall and Thomas, 1993). In contrast to the supernumerary blood vessel ingressions seen with VEGF isoform over-expression, localized ectopic expression of sFlt-1 led to a complete blockade of vessel ingression in areas of sFlt-1 expression (Fig. 2.6, n=5 embryos). Relative to the contralateral control side of the neural tube, the sFlt-1-expressing side showed no ingression points in the medial area where sFlt-1 cDNA was expressed, and reduced ingression even in the ventral area of the developing neural tube (Fig. 2.6 A-C, H). Expression of the VEGF-A blocking peptide over a 48-hour period did not significantly affect the dorsoventral patterning of the neural tube (Fig. 2.6 D-G). These findings indicate that endogenous VEGF-A expressed by cells of the developing neural tube is required for the stereotypical ingression of angiogenic blood vessels into the medial and ventral regions.

E. DISCUSSION

Our results show that VEGF-A signaling is crucial to the communication between the developing neural tube and the developing vascular system. Moreover, here we highlight the precise choreography between neural VEGF-A expression and blood vessel patterning, and we show that a very reproducible pattern of blood vessel ingression depends on proper spatial regulation of VEGF signaling from the neural compartment. The essential aspects of our model are shown in Fig. 2.7. We have previously shown that neural tube-derived VEGF-A was required for formation of the PNVP that surrounds the developing neural tube (Ambler et al., 2003; Hogan et al., 2004) (Fig. 2.7 A). By manipulating ectopic expression of VEGF-A isoforms or the sFlt-1 inhibitor in time and space, we now reveal a second requirement for neural-derived VEGF-A in blood vessel ingression into the developing neural tube (Fig. 2.7 B-E). This ingression requires VEGF-A at the ingression sites, as local loss via sFlt-1 expression prevents ingression (Fig. 2.7 E). However, VEGF-A localization does not explain why vessels only ingress at specific points along the dorsoventral axis. We hypothesize that stereotypical vessel ingression into the neural tube also uses negative patterning cues (Fig. 2.7 B-E), and this is discussed further below.

Why is it important that blood vessels sprout into the developing neural tube at specific places? One possibility is that ingressing vessels must coordinate with neural development and maturation to prevent mis-routing of neural connections and disruption of fasciculation of the axon tracts. For example, the motoneurons form in the ventral part

of the neural tube, and their axons subsequently migrate out of the neural tube ventro-laterally to enervate their targets (for a review, see (Price and Briscoe, 2004)). As axons leave the neural tube they form bundles, and perturbation of either their fasciculation or egression compromises their ability to migrate and properly connect to their targets. Thus, extensive vessel ingression at points of motoneuron egression may compromise the function of the nervous system. Stereotypical ingression patterns of vessels into the neural tube may also be important for efficient functioning of the vascular system. Once angiogenic sprouts enter the neural tube at the medial ingression point, they migrate forward until they reach the sub-ventricular zone that separates differentiated neurons from neural progenitors cells. When they reach this border, they branch and migrate in both the dorsal-ventral and rostral-caudal axes. The rostral-caudal migration leads to interconnections within the neural tube and eventual blood flow (Feeney and Watterson, 1946; Nakao et al., 1988). These interconnections are probably made more efficiently and sooner if the vessels are at the same level on the dorsal-ventral axis, which is accomplished by having a defined medial ingression point.

We used a naturally produced inhibitor of VEGF-A signaling, sFlt-1, to downregulate endogenous VEGF signaling. We, and others, have shown that this spliced isoform of the sFlt-1 receptor complexes with VEGF-A and competitively inhibits binding to Flk-1 (VEGFR-2) (Kappas et al., 2008; Kendall and Thomas, 1993; Roberts et al., 2004). Because we were able to direct modest expression of sFlt-1 to localized areas within the neural tube for a specific time period, neural degeneration was minimized and neural development and dorsal-ventral patterning was not significantly affected.

However, even this modest blockade of VEGF-A signaling was sufficient to block normal vessel ingression dramatically in areas of sFlt-1 expression, revealing an absolute requirement for VEGF-A signaling for proper blood vessel ingression into the developing neural tube. Our preliminary results show that, just as the supernumerary sprouts ingressed locally in conjunction with ectopic VEGF-A expression, the block to ingression produced by sFlt-1 expression was also localized and did not extend significantly beyond areas of sFlt-1 expression (J.M.J. and V.L.B., unpublished). The localized nature of the blockade indicates that sFlt-1 is also a local morphogenetic mediator, as suggested by its ability to bind heparin and thus the surrounding matrix (Park et al., 1993).

Localized ectopic expression of the three major VEGF-A isoforms revealed that, although all isoforms perturbed neural tube angiogenesis, VEGF165 and VEGF189 but not VEGF121 were able to induce supernumerary sprouts at locations along the periphery that normally did not allow for sprout ingression. There are two major differences between these VEGF isoforms. VEGF165 and 189 bind heparin and thus can interact with the matrix, whereas VEGF121 does not bind heparin. VEGF165 and presumably VEGF189 can use NRP1 as a co-receptor to enhance signaling through the Flk-1 (VEGFR-2) receptor, whereas VEGF121 binds NRP1 but does not use it as a co-receptor for signaling through VEGFR-2 (Pan et al., 2007; Soker et al., 1998). Deletion of NRP1 affects vascular development (Gerhardt et al., 2004; Gu et al., 2003; Kawasaki et al., 1999). However, ingression of vessels into the CNS is not compromised; the vessel defects in NRP1 mutant neural tubes result from mis-patterning in lateral branching and vessel size increases, indicating that neural tube vessel ingression is not NRP1 dependent

(Gerhardt et al., 2004; Gu et al., 2003). Our preliminary results show that co-electroporation of VEGF165 and a soluble form of NRP1 that is predicted to act as a dominant-negative block to NRP1/Flk-1 interactions does not block ectopic ingression of vascular sprouts (J.M.J. and V.L.B., unpublished). Taken together, these findings suggest that NRP1 interactions are not crucial to blood vessel ingression into the neural tube, and that the heparin-binding properties of VEGF165 and 189 confer on these isoforms the ability to induce ectopic ingression points.

The hypothesis that VEGF isoform interactions with the local matrix within the neural tube are crucial to proper vessel ingression is also supported by our finding that ectopic sprouting ingression is localized to areas of the neural tube that contain cells expressing either VEGF165 or VEGF189. When broad areas expressed heparin-binding VEGF ectopically, there were numerous sprouts in these areas. However, even when only a few cells expressed heparin-binding VEGF DNA, supernumerary sprouts correlated with their placement. This finding strongly indicates that heparin-binding VEGF-A is normally deposited near the cells of origin in the neural tube, and this spatial arrangement of VEGF-A contributes to the stereotypical ingression pattern. It also shows that endothelial cells outside the neural tube can sense sources of VEGF-A within the neural tube, over multiple cell diameters, and can overcome normal restraints to ingression if the positive signal is strong enough. Although it is formally possible that VEGF-A signaling could travel over space via a relay system from a localized ligand source, existing data suggest that the gradient hypothesis of VEGF-A signaling is responsible for our findings. This model proposes that a gradient of VEGF-A protein

emanating from a source provides a haptotactic slope that directs the migration of angiogenic sprouts. Support for this model was provided by Ruhrberg and colleagues, who showed that VEGF-A protein is concentrated at the midline of the developing hindbrain and decreases in lateral areas (Ruhrberg et al., 2002). The use of a VEGF-A gradient for blood vessel ingression into the developing neural tube is also consistent with our finding that ectopic expression of VEGF121 is not capable of inducing supernumerary vessel ingression points, although once in the neural tube vessels exposed to ectopic VEGF121 become dysmorphogenic. However, mice that express only VEGF120 still exhibit ingression of angiogenic vessels into the developing neural tube (Ruhrberg et al., 2002), although ingression is delayed and ingression points are less dense than in controls (J.M.J. and V.L.B., unpublished). This finding contrasts with our results showing that ectopic expression of VEGF121 does not result in supernumerary vessel ingression into the neural tube. One explanation of this paradox is that in the absence of normal VEGF-A isoforms, VEGF120 forms a 'soluble' gradient from its source that can provide instructional information for endothelial sprout migration, although with less efficiency than a gradient formed by heparin-binding VEGF-A isoforms. However, when VEGF121 is overexpressed in the context of a normal gradient it cannot contribute significantly to the positional information conveyed by that gradient.

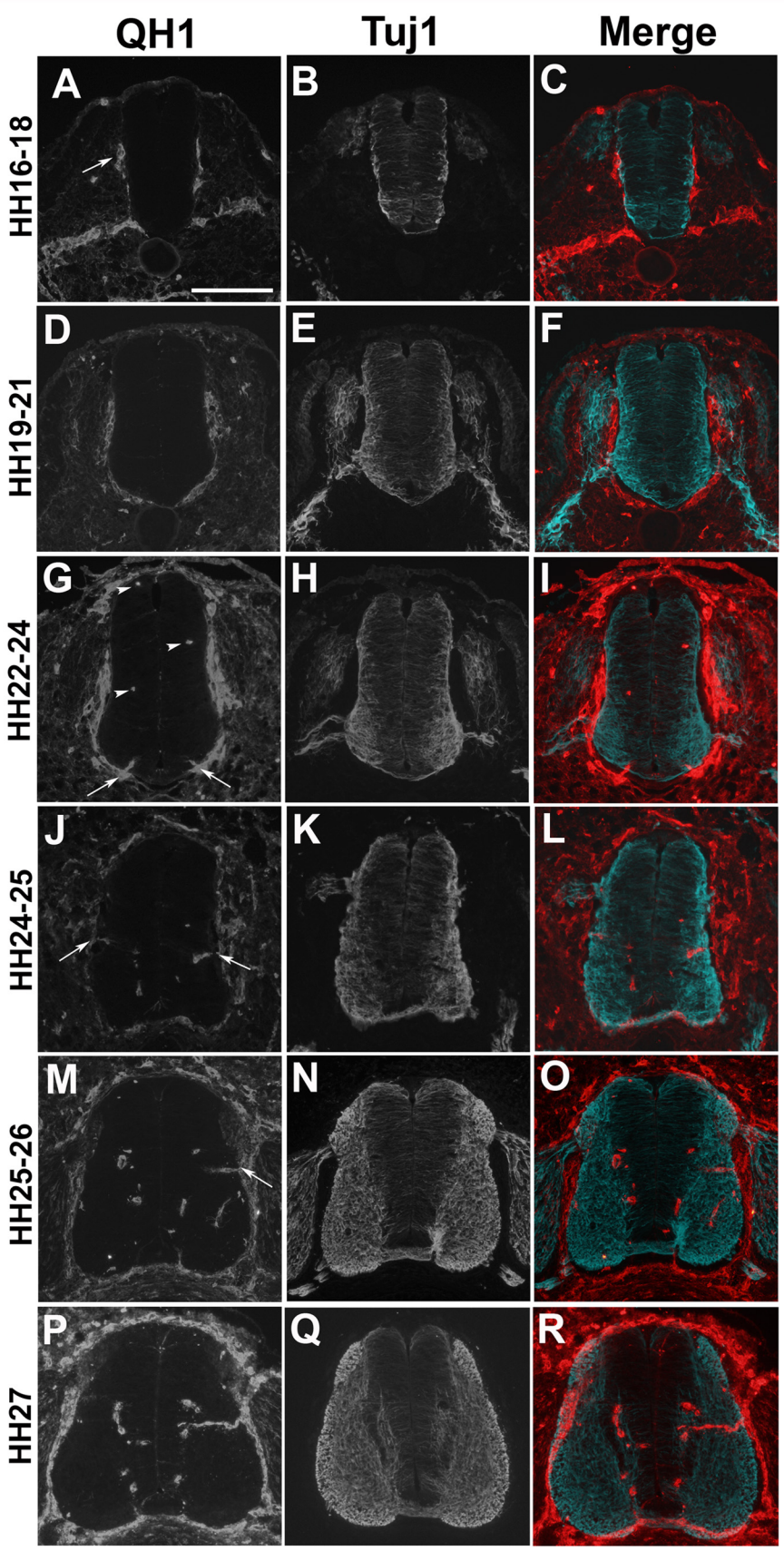
A model of patterned vessel ingression into the neural tube that only considers VEGF-A, however, is obviously not sufficient to explain the stereotypical pattern we observed. VEGF-A RNA expression is not localized to areas of ingression, but is broadly expressed, with no observable differences along the dorsal-ventral axis at stages when

blood vessels ingress at specific medial locations. Moreover, staining for the heparin sulfate proteoglycans that bind VEGF165 and VEGF189 showed uniform expression along the lateral edge of the neural tube, suggesting that VEGF protein is not preferentially localized to ingression points via matrix binding (J.M.J. and V.L.B., unpublished). We thus conclude that VEGF-A is necessary but not sufficient to pattern the angiogenic blood vessels that enter the developing neural tube. Although it is formally possible that the endothelial cells at the ingression points are uniquely able to respond to the VEGF-A signal due to cell-autonomous differences between them and neighboring endothelial cells, our data do not support such a model, as all PNVP endothelial cells seem capable of responding to ectopic expression of heparin-binding VEGF-A. Likewise, a model whereby egression of motor neurons and/or ingression of DRG neurons physically blocks blood vessel ingression does not account for the extensive areas of the floor plate, ventral neural tube, and dorsal neural tube that do not support ingression of the adjacent PNVP vessels. Our data best support a model in which the positive signals emanating from the neural tube are balanced by negative spatial cues that are also produced by the neural tube and prevent ingression both dorsally and ventrally (Fig. 2.7 B-E). Several signaling pathways are candidates to coordinate with VEGF signaling to pattern vessel ingression into the neural tube, based on the expression of the ligands and their ability to negatively influence vessel migration (see review by (Eichmann et al., 2005). Among these are the semaphorins that signal through plexins, the slits that signal through robo receptors and netrins that signal through UNC and DCC receptors. Thus, VEGF-A signaling is predicted to provide a positive spatial cue that, when balanced by a negative

spatial cue, is neutralized. However, this balance can be tipped in favor of VEGF-A and vessel ingression by ectopic expression of VEGF-A. In our model endothelial cells are capable of a sophisticated reading of incoming cues, and of integrating these cues to produce a behavior that leads to proper neurovascular communication. Moreover, pathologies such as the CCMs (cerebral cavernous malformations) disrupt a unique communication between the neural and vascular compartment (for reviews, see (Lok et al., 2007; McCarty, 2005) that begins at the earliest stages of development.

Figure 2.1: Formation of the PNVP and vessel ingression coordinate with neural differentiation in the quail neural tube.

HH stage 16-27 Japanese quail embryos were sectioned in the transverse plane at the thoracic level and stained with either QH1 (red, A, D, G, J, M, P) to label blood vessels or Tuj1 (β-tubulin III, blue, B, E, H, K, N, Q) to label differentiated neurons. Merged images (C, F, I, L, O, R) represent two super-imposed, adjacent 12μm sections at the upper limb level. (**A-C**) At stage HH 16-18, initiation of PNVP formation correlated with the start of neuronal differentiation and migration of Tuj1-positive neurons to the pial surface of the neural tube. (**D-F**) At stage HH 19-21, the PNVP continued to develop around the ventral neural tube, whereas motoneurons extended axonal projections from the motor horn, and DRG axons innervated the neural tube at the dorsal root entry zone. (**G-I**) By stage HH 22-24, PNVP formation was complete. Single QH1-positive angioblasts were noted dorsally and medially (arrowheads), and ventral angiogenic sprouts were seen (arrows) adjacent to the floor plate. (**J-L**) At stage HH 24-25, angiogenic sprouts from the PNVP formed mediolaterally (arrows) along the dorsal-ventral axis of the neural tube (this vessel ingression site was maintained at later stages, see M and P). (**M-O**) At stage HH 25-26, both ventral and medial (arrow) vessel ingression sites were noted, along with continued differentiation of Tuj1-positive neurons. (**P-R**) By stage HH 27, the amount of Tuj1 positive neurons increased, while the vessel ingression pattern established at earlier stages was maintained. Scale bar: 100μm.


QH1
Tuj1
Merge
HH16-18
HH19-21
HH22-24
HH24-25
HH25-26
HH27
A
B
C
D
E
F
G
H
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J
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M
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O
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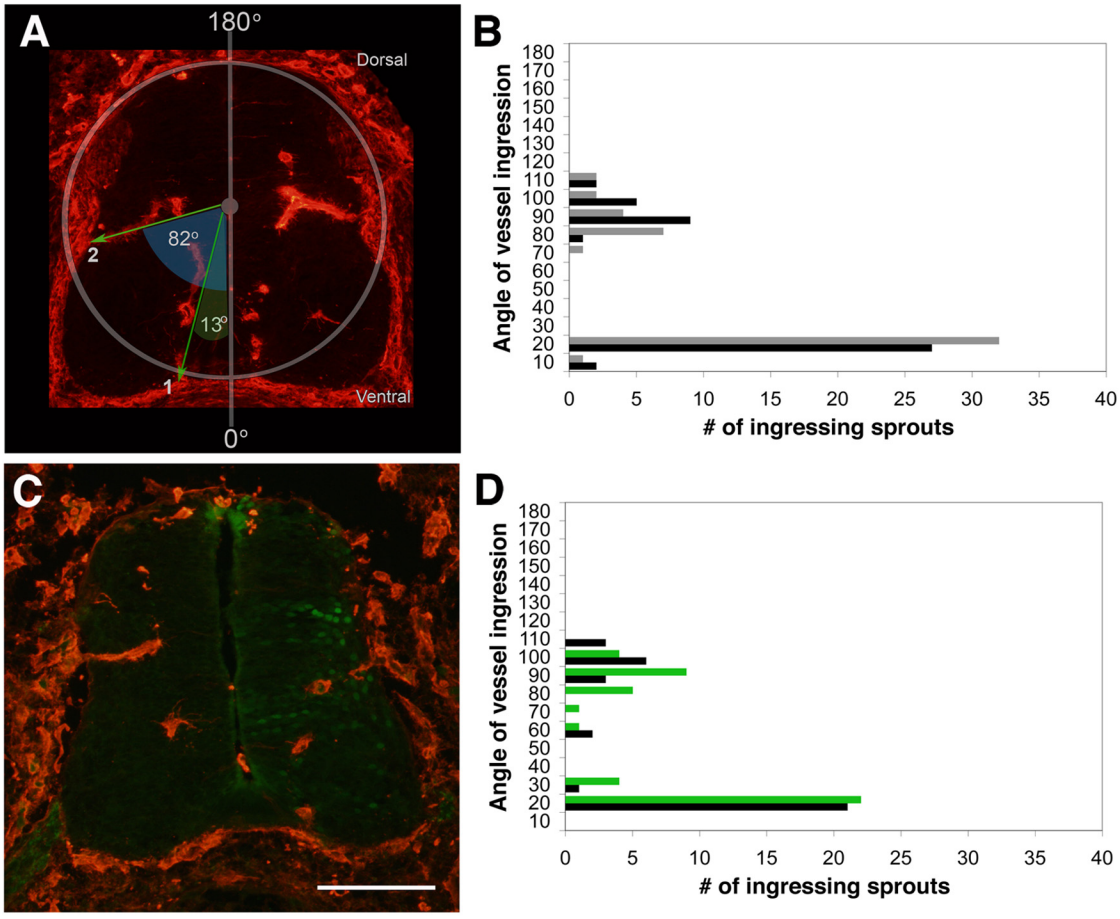
Figure 2.2: Quantitative analysis of angiogenic sprouting into the developing neural tube reveals stereotypical ingression points.

Unperturbed quail embryos and embryos whose neural tubes were electroporated with eGFP control DNA at HH stage 16-18 were serially sectioned at stage HH 25-26 through the upper limb. Every sixth 12μm section was stained for QH1 (red). Fourteen images were analyzed for each embryo as described in the Materials and Methods. (**A**) Unperturbed quail embryo section stained with QH1 to illustrate blood vessel analysis strategy. (**B**) Total number of angiogenic sprouts within the left (gray) and right (black) neural tube halves of five unperturbed embryos. There were concentrations of ingression points between 0-20° (ventral ingression points) and 70-110° (medial ingression points). (**C**) Representative image of a quail neural tube electroporated with eGFP DNA (electroporated side to the right). (**D**) Total number of ingressing angiogenic sprouts within the neural tubes of five control embryos electroporated with eGFP DNA (green); untransfected control contralateral neural tube side (black). Scale bar: 100μm.


A
180°
Dorsal
82°
2
13°
1
Ventral
0°
B
Angle of vessel ingression
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
5
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15
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25
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35
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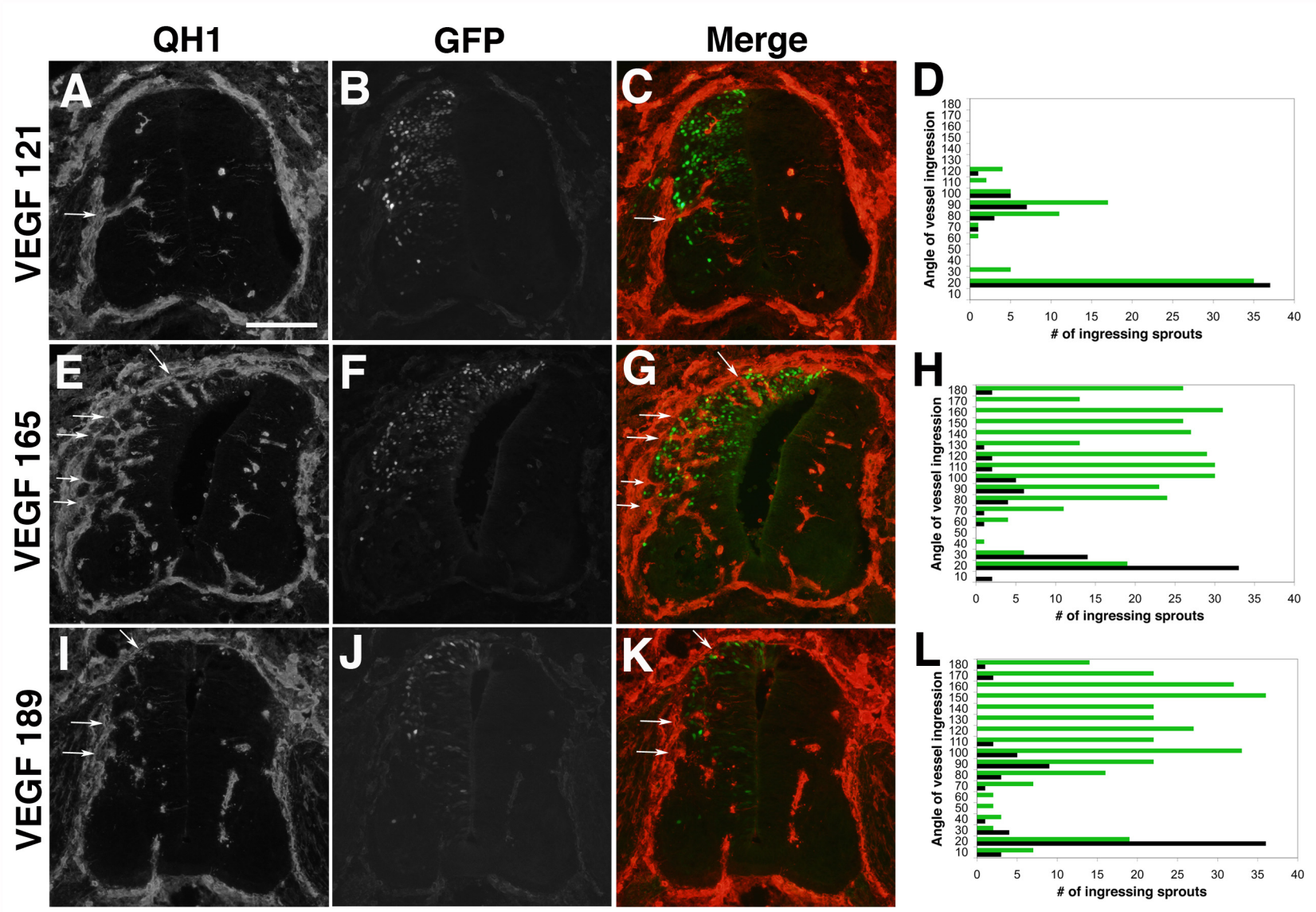
# of ingressing sprouts
C
D
Angle of vessel ingression
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
5
10
15
20
25
30
35
40
# of ingressing sprouts

Figure 2.3: Ectopic expression of heparin-binding VEGF isoforms induces supernumerary vessel ingression points into the developing neural tube.

Quail neural tubes were electroporated with hVEGF121-GFP, hVEGF165-GFP or hVEGF189-GFP DNAs (green, panels B, F, J) on day 3 (HH 16-18) and harvested 48 hours later (HH 25-26). Transverse sections were stained with QH1 antibody (red, panels A, E, I) to visualize vessels, and five embryos from each group were analyzed as described (panels D, H, L; green lines, total ingression points for ectopic VEGF expressing sides of neural tubes at each 10° of arc; black lines, total ingression points for contralateral control sides of the neural tubes at each 10° of arc). C, G and K are a merge of red (QH1) and green (eGFP) channels. (**A-C**) Quail neural tubes electroporated with hVEGF121 DNA displayed a grossly normal distribution of angiogenic ingression points along the dorsoventral axis of the ectopic VEGF-expressing side of the neural tube (arrows in A, C). (**D**) The quantitative analysis showed no change in the distribution of ingression points for sprouts between the control (black) and VEGF121-expressing (green) sides of the neural tube, and a slight increase in the frequency of ingression points in the medial region of the VEGF-expressing side of the neural tubes (*n*=5 embryos). (**E-G**) Quail neural tubes electroporated with hVEGF165 DNA had ectopic dorsal sprouts (arrows in E, G). (**H**) The quantitative analysis showed increased distribution and frequency of vessel ingression points in the dorsal region of the hVEGF165-expressing side of the neural tube (green), where ectopic expression is localized (*n*=5 embryos). (**I-K**) Quail neural tubes electroporated with hVEGF189 DNA had ectopic dorsal sprouts (arrows in I, K). (**L**) The quantitative analysis showed increased distribution and frequency of ingression points in the dorsal region of the hVEGF189-expressing side of the neural tube, where ectopic expression is localized (*n*=5 embryos). Scale bar: 100μm.


QH1
GFP
Merge
VEGF 121
VEGF 165
VEGF 189
A
B
C
D
E
F
G
H
I
J
K
L
Angle of vessel ingression
# of ingressing sprouts
180
170
160
150
140
130
120
110
100
90
80
70
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50
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10
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35
40

Figure 2.4: Localized ectopic expression of heparin-binding VEGF-A isoforms in the developing neural tube correlates with supernumerary vessel ingression points.

Neural tubes processed for QH1 (red) and eGFP (green, reporter for ectopic VEGF-A isoform expression) were examined for the relationship between vessel ingression points and ectopic VEGF-A isoform expression. (**A-C**) Lower power views to show location of normal (A) or supernumerary (B, C) vessel sprout ingressions on the dorsoventral axis of the neural tube. (**D-F**) Higher magnification of the boxed areas in A-C. Several eGFP-positive cells that ectopically express heparin-binding hVEGF165 or hVEGF189 are close to the supernumerary vessel sprouts (arrows in E, F), whereas numerous eGFP-positive cells that ectopically express hVEGF121 (arrowheads in D) do not induce supernumerary vessel ingression points. Scale bar: 100μm in A-C; 50μm in D-F.

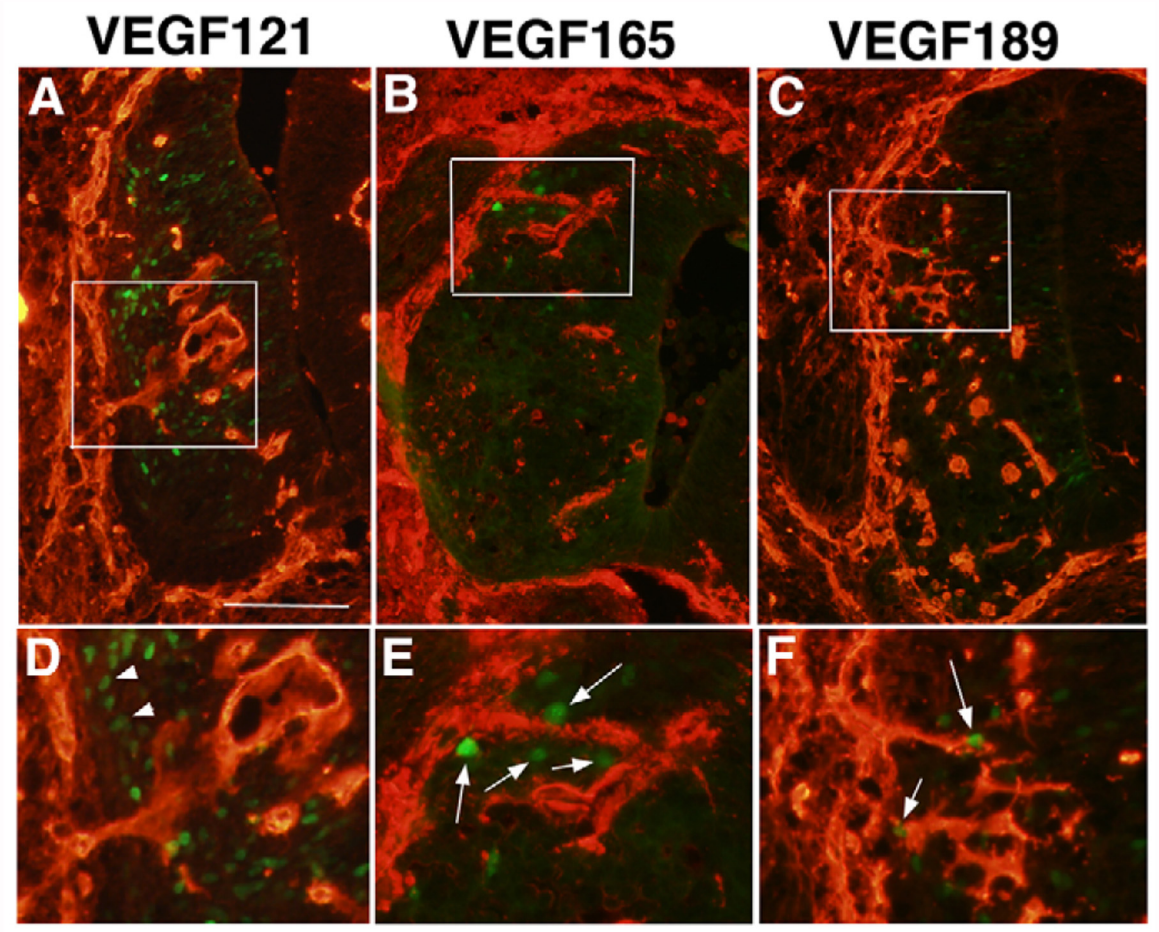

VEGF121
VEGF165
VEGF189
A
B
C
D
E
F

Figure 2.5: Neural patterning is not perturbed in neural tubes that ectopically express VEGF-A isoforms.

(A-U) Quail neural tubes were electroporated with (**A-G**) hVEGF121, (**H-N**) hVEGF165 and (**O-U**) hVEGF189 on day 3 (HH 16-18), and harvested 48 hours later (HH 25-26). Neural tubes were sectioned and adjacent sections were stained with antibodies to: QH1 (red, A, H, O, C, J, Q) to visualize vessels; Pax7 (purple, D, K, R) to visualize dorsal neural precursors; Pax6 (orange, E, L, S) to visualize medial neural precursors; MNR2 (yellow, F, M, T) to visualize ventral motoneuron precursors; and Tuj1 (blue, G, N, U) to visualize differentiated neurons. (B, I, P) eGFP expression (green) illustrates the neural tube side expressing ectopic VEGF-A isoforms (left) versus the control contralateral side (right); (C-G, J-N, Q-U) merges of marker and eGFP channels for each section. Scale bar: 100μm.

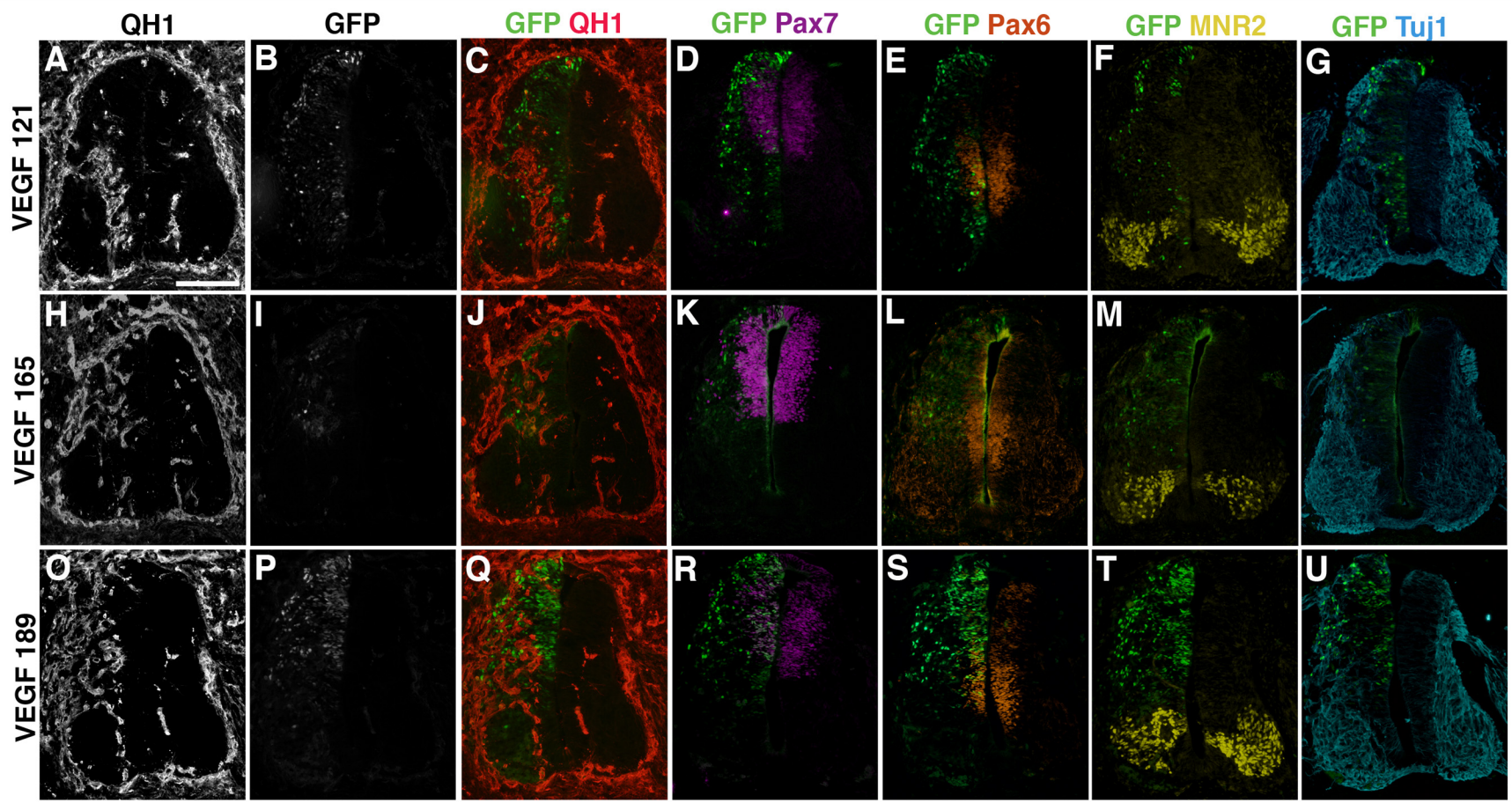

QH1
GFP
GFP QH1
GFP Pax7
GFP Pax6
GFP MNR2
GFP Tuj1
VEGF 121
VEGF 165
VEGF 189
A
B
C
D
E
F
G
H
I
J
K
L
M
O
P
Q
R
S
T
U

Figure 2.6: VEGF signaling from the neural tube is required for blood vessel ingression.

Quail neural tubes were electroporated with sFlt1-GFP and analyzed as previously described. (**A-C**) No medial vessel ingression and little ventral vessel ingression was seen in areas of the neural tube that were eGFP positive. (**D-G**) Neural patterning is not detectably perturbed on the electroporated side of the neural tube (left) based on Pax7 (D, purple), Pax6 (E, orange), MNR2 (F, yellow) and Tuj1 (G, blue) expression patterns. (**H**) Quantitative analysis of five electroporated neural tubes showed no medial and few ventral vessel ingression points in areas of localized sFlt1 expression (green), compared with the control contralateral side (black) (*n*=5 embryos). Scale bar: 100μm.

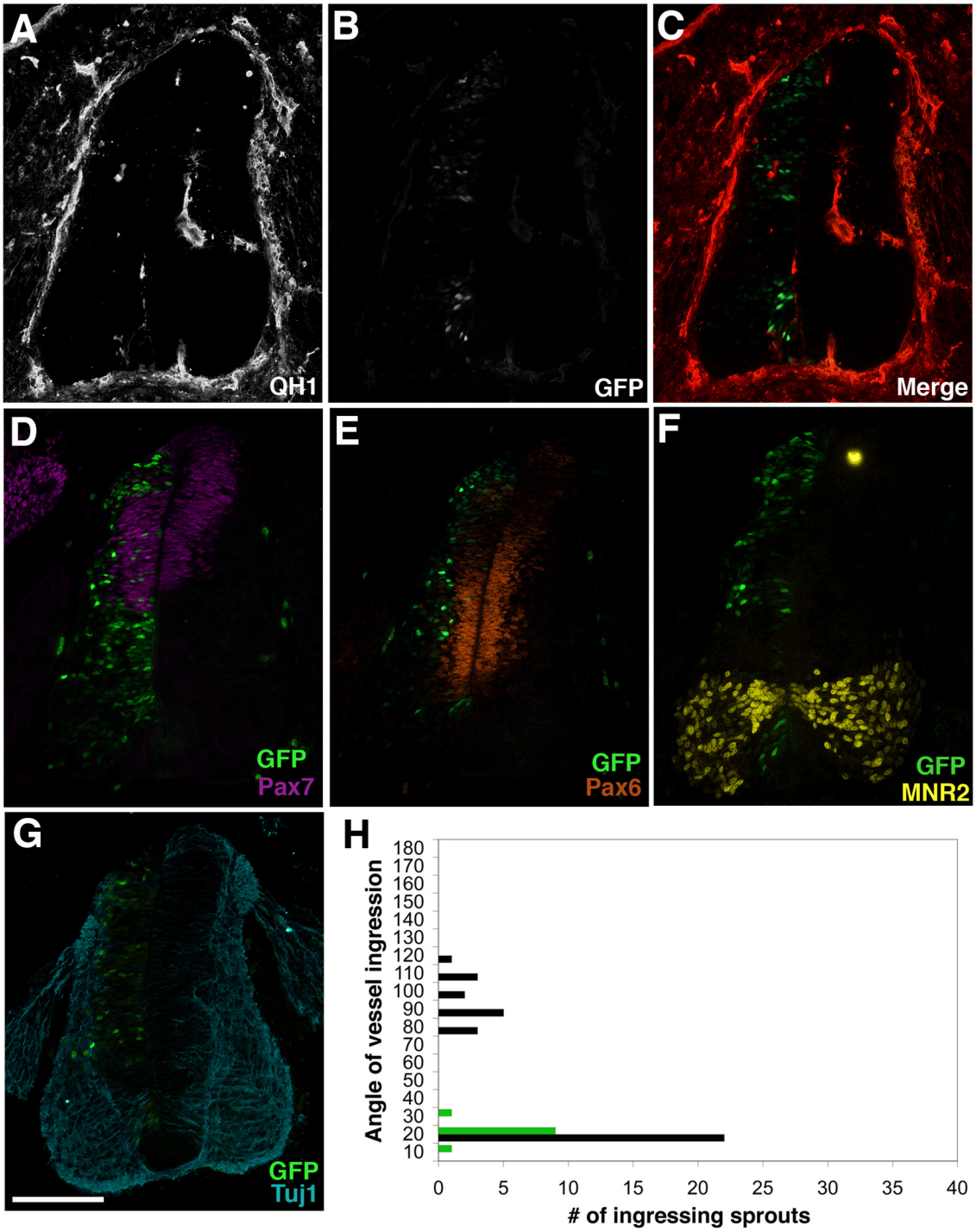
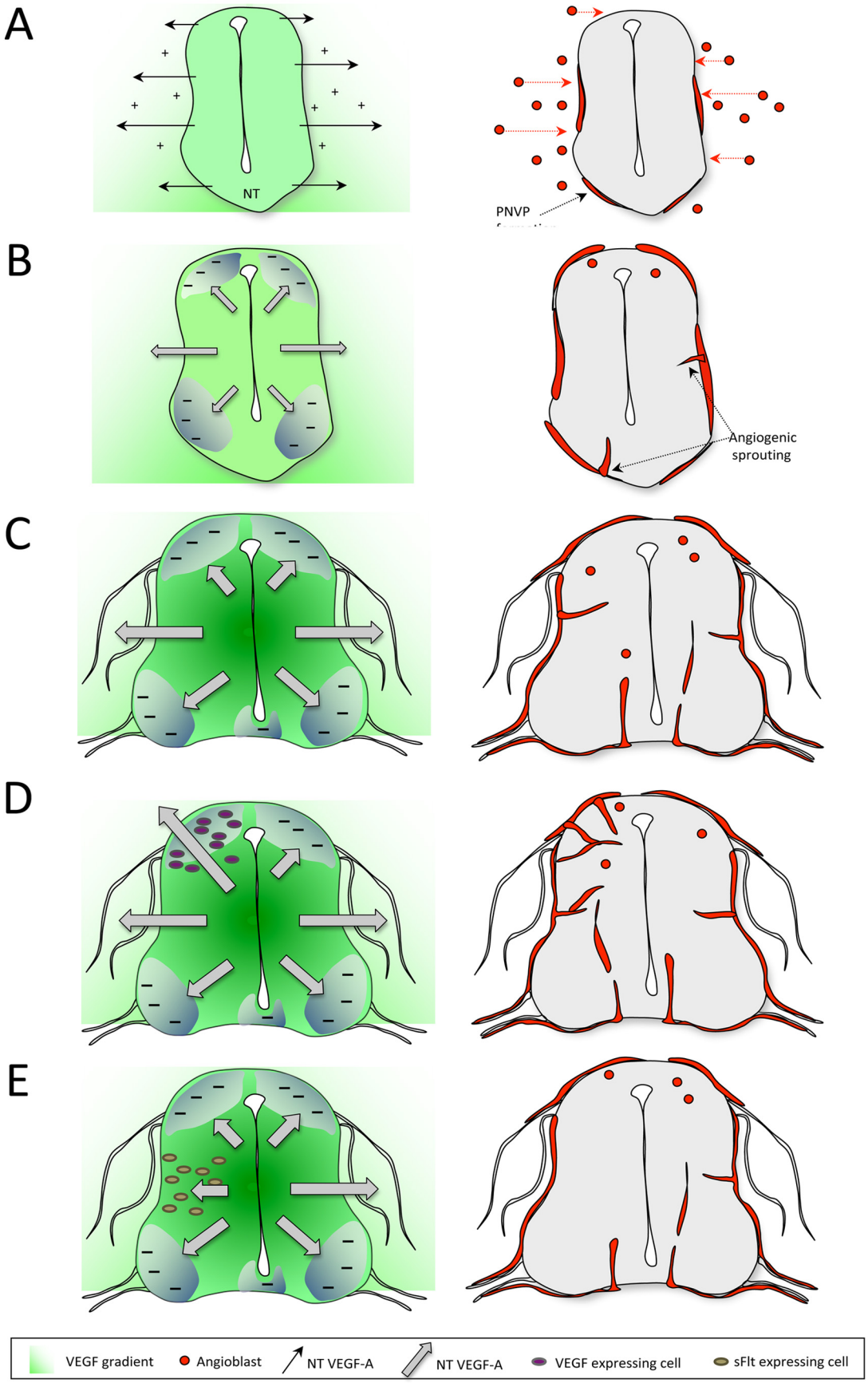

A
QH1
B
GFP
C
Merge
D
GFP
Pax7
E
GFP
Pax6
F
GFP
MNR2
G
GFP
Tuj1
H
Angle of vessel ingression
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
5
10
15
20
25
30
35
40
# of ingressing sprouts

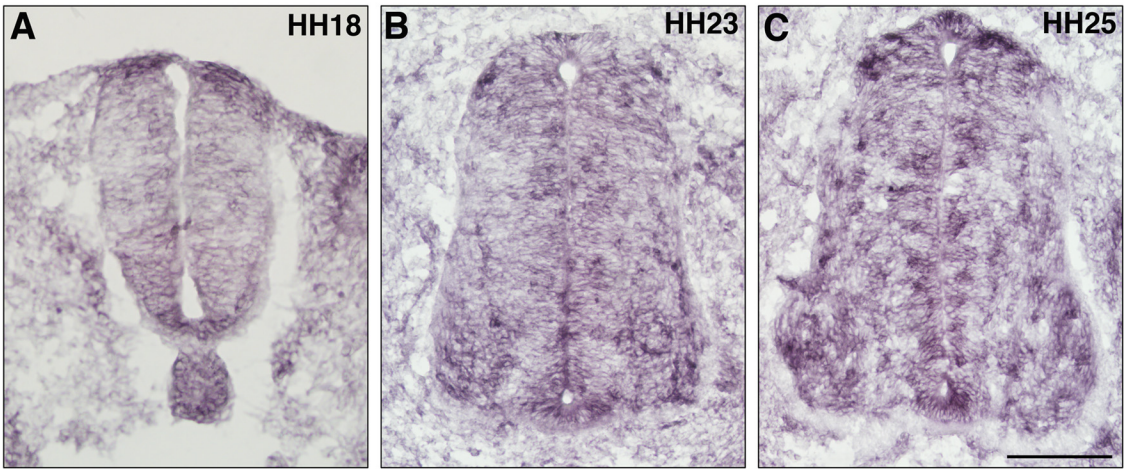
Figure 2.7: Model of blood vessel ingression into the developing neural tube.

The model covers events of neurovascular patterning between stages HH 16 and HH 26 in the avian embryo, and also shows the VEGF-A perturbations analyzed in this study. (**A**) At early stages (HH 16-18), VEGF-A isoforms (121, 165, 189) expressed by the developing neural tube set up a gradient that leads to angioblast migration from the lateral plate and presomitic mesoderm to form the PNVP. At this stage, blood vessel ingression does not occur because of insufficient levels of neural tube-derived VEGF-A. (**B**) At later stages (HH 22-25), increased levels of VEGF165 and VEGF189 are required for blood vessel ingression, but negative patterning cues that are co-expressed prevent ingression except at specific medial and ventral points. (**C**) By stage HH 26 there are obvious stereotypical blood vessel ingression points medially and ventrally, whereas angioblasts migrate in dorsally. (**D**) Neural tubes electroporated with VEGF165 or VEGF 189 show ectopic ingression in normally avascular dorsal areas on the electroporated side. (**E**) Neural tubes electroporated with sFlt-1 do not have ingression at the normal medial site on the electroporated side. For each set of panels, the left side demonstrates the signals and the right side demonstrates the vessel-patterning outcome. Symbols are described in the key below the figure.


A
NT
PNVP
B
Angiogenic
sprouting
C
D
E
VEGF gradient
Angioblast
NT VEGF-A
NT VEGF-A
VEGF expressing cell
sFlt expressing cell

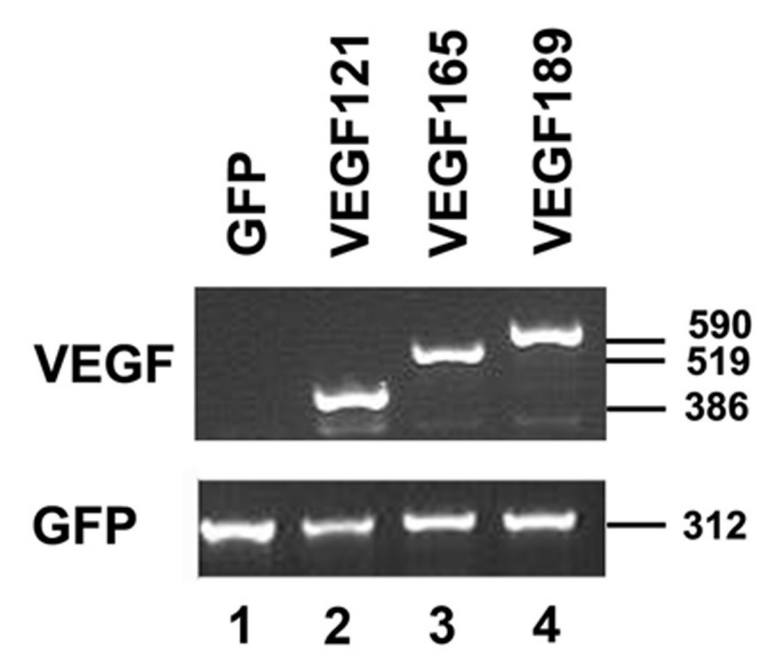
Supplementary Figure 2.1: Time course of VEGF-A expression in the quail neural tube at the limb (thoracic) level.

In situ hybridization of transverse cryostat sections of quail neural tubes with an antisense probe to VEGF-A. (**A**) At HH stage 18, when electroporations were performed, there was some VEGF-A reactivity, with a concentration in the floor plate and the roof plate. (**B**) At HH stage 23, VEGF-A reactivity was increased and seen prominently in the motoneuron area and the floor plate. (**C**) At HH stage 25, approximately when the analysis was done, the overall level of VEGF-A reactivity was increased, with some concentration of the signal in the floor plate and the roof plate. Scale bar: 100μm.


A
HH18
B
HH23
C
HH25

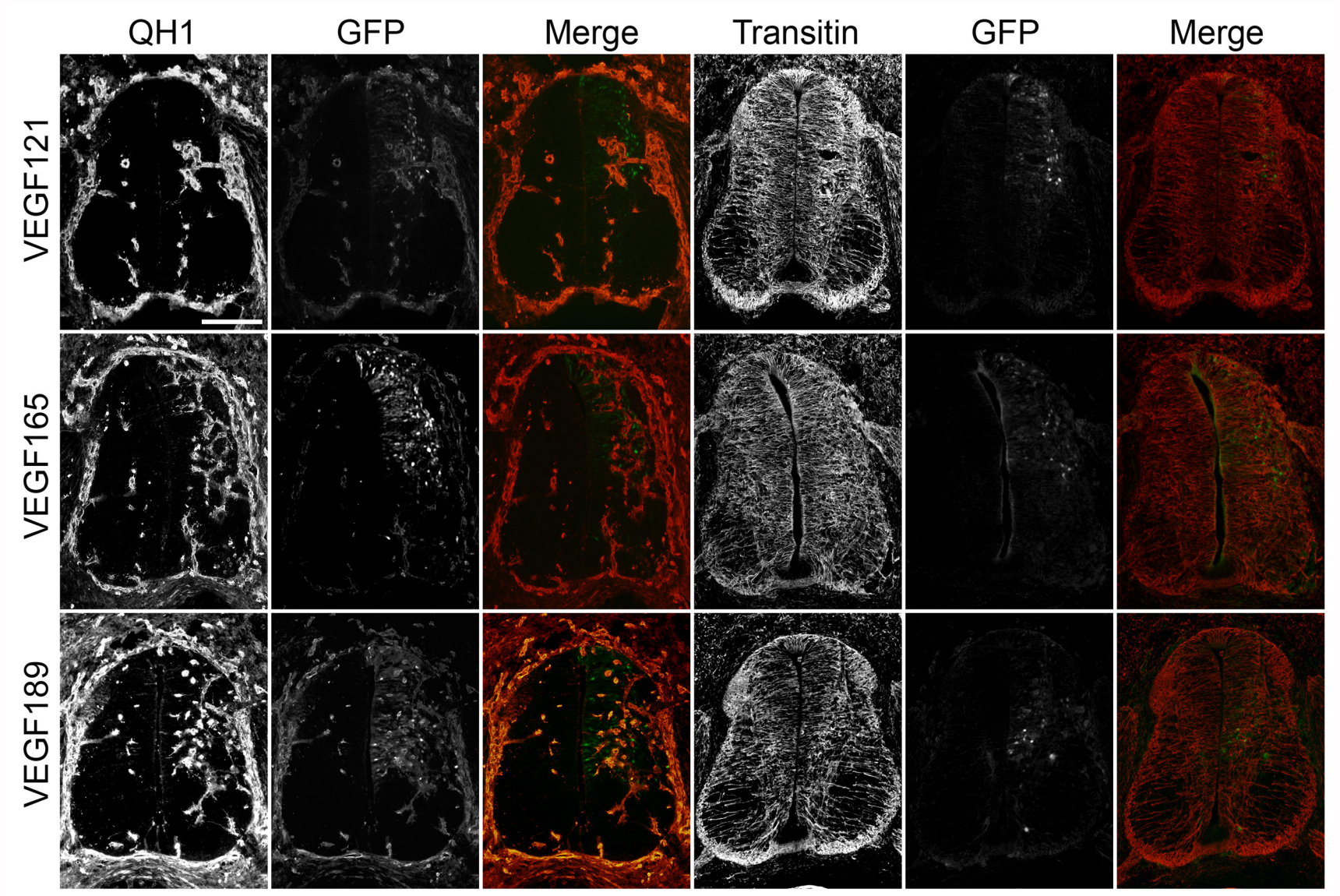
Supplementary Figure 2.2: Expression analysis of VEGF-A isoform transgenes in electroporated neural tubes.

Quail neural tubes were electroporated as described on day 3, and harvested on day 5. Total RNA was isolated from three to five pooled neural tubes and processed for RT-PCR using primers that recognize the hVEGF-A transgene (they span the alternative splice sites) or eGFP. Neural tubes were electroporated with: lane 1, eGFP only; lane 2, hVEGF121-GFP; lane 3, hVEGF165-GFP; lane 4, hVEGF189-GFP. The size of the amplification products is shown on the right.


GFP
VEGF121
VEGF165
VEGF189
VEGF
590
519
386
GFP
312
1
2
3
4

Supplementary Figure 2.3: Radial glia patterning is not perturbed in neural tubes that ectopically express VEGF-A isoforms.

(A-R) Quail neural tubes were electroporated with hVEGF121 (A-F), hVEGF165 (G-L) or hVEGF189 (M-R) on day 3 (HH 16-18) and harvested 48 hours later (HH 25-26). Neural tubes were sectioned and adjacent sections were stained with antibodies to: QH1 (red, A, G, M) to visualize vessels; and transitin (red, D, J, P) to visualize radial glia. (B, E, H, K, N, Q) eGFP expression (green) illustrates the neural tube side expressing ectopic VEGF-A isoforms (left) versus the control contralateral side (right) on adjacent sections; (C, F, I, L, O, R) merge of marker and eGFP channels for each section. Scale bar: 100μm.


QH1
GFP
Merge
Transitin
GFP
Merge
VEGF121
VEGF165
VEGF189

CHAPTER III

NEURAL TUBE DEVELOPMENT DIRECTS INTRANEURAL BLOOD VESSEL PATTERNING IN THE AVIAN EMBRYO

A. SUMMARY

Blood vessel sprouts form at a specific time and at highly stereotypical locations during neural tube development. Neural tube patterning is also spatially and temporally regulated. The work in this chapter explores how normal processes of neural tube development, such as programmed neurogenesis and dorsoventral (DV) patterning of neurons, influence the blood vessel pattern. We manipulated both the timing of neurogenesis and DV neuronal patterning via electroporation of genes known to regulate these processes. Here, we report that the specific time when neurons differentiate and migrate to the lateral edge of the neural tube is important for regulating the time that angiogenic sprouts enter the neural tube. Furthermore, we identified pro- and anti-angiogenic regions along the DV axis of the neural tube by precisely measuring where vessels ingress and where they do not, mapping these regions to particular neuronal subclasses in the quail neural tube. We found that blood vessels do not enter the region of the neural tube where motor neurons develop and hypothesized that motor neuron cell bodies found within this region were initially anti-angiogenic. This was confirmed when the block to angiogenesis was conferred to a normally permissive region of the neural tube by generating ectopic motor neurons there. This work demonstrates that normal processes of neural tube development and blood vessel patterning are linked, and that the neural tube directs both the timing and spatial distribution of ingressing blood vessels in quail.

B. INTRODUCTION

Blood vessels invade the avian neural tube at a specific time and at highly stereotypical locations during development, constituting a vascular pattern (Feeney and Watterson, 1946; Kurz et al., 1996). Specific aspects of neural tube development, such as programmed neural cell differentiation and dorsoventral (DV) neuronal cell fate specification, also have reproducible spatial and temporal components. These processes pattern the neural tube, forming distinct sub-populations of neural cells. On its medio-lateral axis, the differentiation and lateral migration of neurons segregates the neural tube into regions of progenitor cells (ventricular zone, or VZ) or differentiated neurons (marginal/mantle zone, or MZ). Along its DV axis, the neural tube further divides into specific subclasses of neurons, such as dorsal interneurons, ventral interneurons, and motor neurons, projecting axons toward different target tissues (Jessell, 2000; Lee and Jessell, 1999) (Figure 3.1 A). Within the avian neural tube, we can correlate the formation of these regions with the timing and spatial organization of specific blood vessel patterning events; however, little is known about how these sub-populations of neural cells communicate with ingressing blood vessels, or if certain regions can positively or negatively mediate intraneural blood vessel growth and patterning. In this chapter, we explore how both the maturation of the neural tube and the regional specification of neuronal subclasses influence the intraneural vessel pattern.

Endothelial cells (ECs) migrate and coalesce into a ring of blood vessels surrounding the neural tube known as the peri-neural vessel plexus (PNVP) during early stages of neural tube development (Ambler et al., 2001; Pardanaud et al., 1996; Wilms et

al., 1991; Wilting et al., 1995). During this time, the immature neural tube is comprised of a pseudo-stratified layer of rapidly proliferating progenitor cells maintaining contacts with both its luminal and pial surfaces (Huttner and Brand, 1997). These cells are initially known as neuroepithelial cells or neural stem cells, and they form the ventricular zone (VZ) of the neural tube at the onset of neurogenesis (reviewed in Gotz and Huttner, 2005). Certain genes regulate the progenitor state of these cells, such as members of the SOX family of transcription factors, including the SOXB1 sub-family members *Sox1*, *Sox2* and *Sox3* (Bylund et al., 2003; Graham et al., 2003; Kishi et al., 2000; Pevny et al., 1998). Notch1 signaling and its downstream mediator *Hes-1* also play roles in neural stem-cell maintenance (Gaiano and Fishell, 2002; Ishibashi et al., 1995).

Initially, neural progenitor divisions are symmetrical, producing two identical daughter progenitors acting to increase the size of the neural tube (McConnell, 1995; Rakic, 1995) (Fig. 3.1 B). As proliferative divisions proceed, opposing morphogen gradients of Sonic Hedgehog (Shh) from the floor plate and TGF-ß/BMP family members from the roof plate of the neural tube regulate expression of homeodomain transcription factors along its DV axis (reviewed in Briscoe and Ericson, 2001; Helms and Johnson, 2003). Overlapping expression of these proteins reveals a complex system of transcription factor coordination and cross-repression that delineates neuronal progenitor domains, specifying neuronal cell fate before the onset of neurogenesis. Ectopic expression of homeodomain transcription factors in chick neural tubes predictably changes the position of neural progenitors along the DV axis and also changes neuronal cell fates (Tanabe et al., 1998). Although vessels in the PNVP closely associate with the

neuroepithelium as these neuronal patterning events take place, blood vessels do not invade the neural tube at this time.

The regional fate restriction of neuronal progenitors by homeodomain transcription factors initiates programmed neurogenesis via the regulation of basic Helix-Loop-Helix (bHLH) transcription factor expression (Mizuguchi et al., 2001; Novitch et al., 2001; Roztocil et al., 1997). For example, Neurogenin-1, a bHLH transcription factor, coordinates cell cycle exit and neuronal differentiation (Sun et al., 2001). At the onset of neurogenesis, specified neural progenitor cells undergo a symmetrical division to form two neurons, or an asymmetrical division, forming one neuron and one radial glial cell (Fig. 3.1 B). Radial glial cells can also give rise to neurons in subsequent division cycles (Noctor et al., 2004). Changes in cell adhesion allow a differentiating neuron to leave the VZ and migrate laterally to the pial surface (marginal zone) of the neural tube where they begin to express markers of terminally differentiated neurons such as ß-tubulinIII and NeuN (Memberg and Hall, 1995; Sarnat et al., 1998). Neurogenesis occurs along the entire DV axis of the neural tube, and where a neuron is born along the DV axis determines what type of neuron it will become (reviewed in Jessell, 2000; Lee and Jessell, 1999).

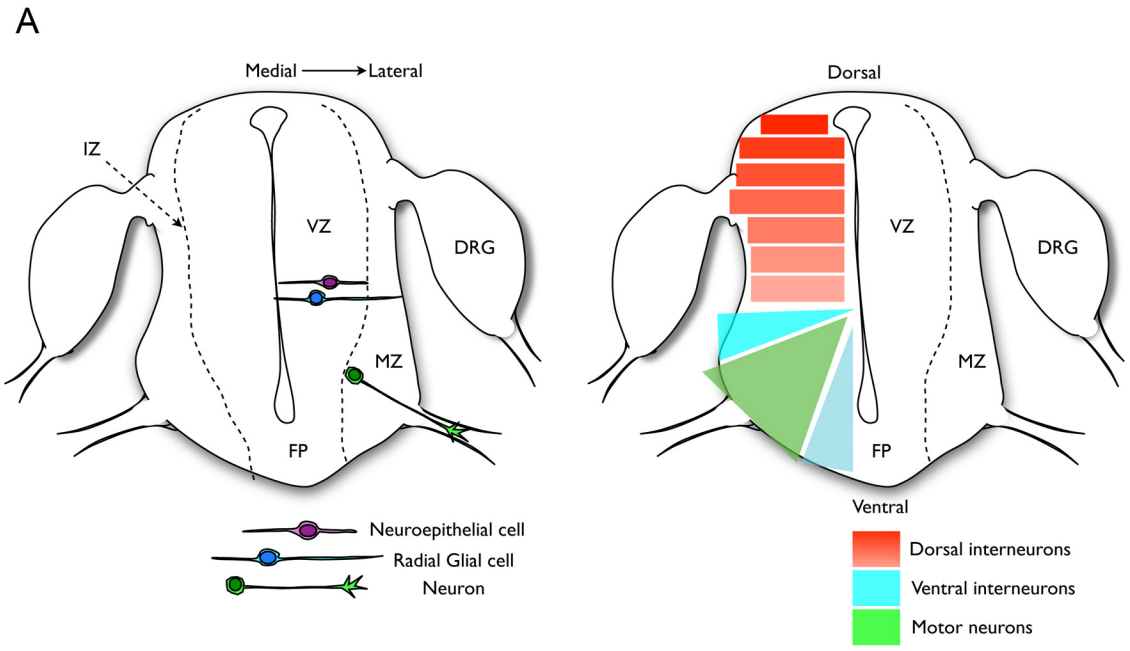
As terminally differentiated neurons begin to accumulate at the lateral edge of the neural tube, blood vessels of the PNVP invade the neuroepithelium (Fig. 3.2). They enter the avian neural tube in two stereotypical locations: adjacent to the floor plate in the ventral neural tube, and medio-laterally, between the motor neurons and the dorsal root entry zone—where the axons of neurons comprising the dorsal root ganglia invade the neural tube. Intriguingly, blood vessels in the PNVP wait for up to 24 hours before this

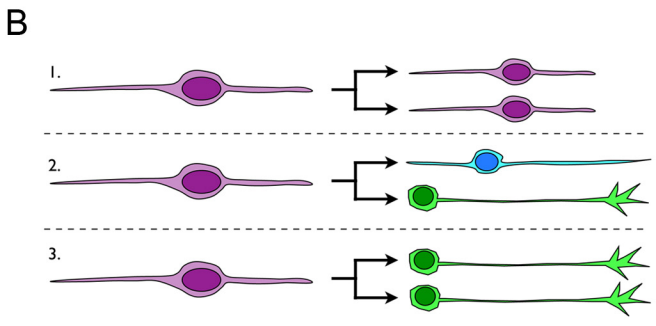
angiogenic sprouting occurs. Once an angiogenic sprout enters the neural tube, it branches laterally as it interfaces the VZ—closely associating with the progenitor cells, but never invading this zone (Kurz et al., 1996).

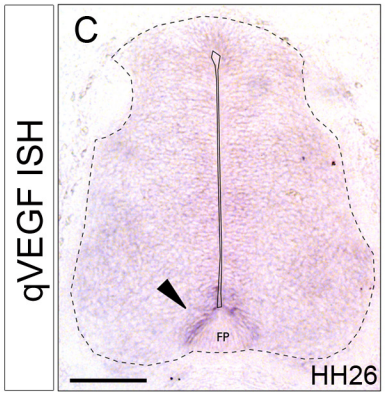
We previously showed that Vascular Endothelial Growth Factor-A (VEGF)-signaling from the neural tube is crucial for early blood vessel patterning events such as EC migration, PNVP formation (Ambler et al., 2003; Hogan et al., 2004), and more recently, that VEGF signaling is required for the first blood vessels to invade the neuroepithelium (James et al., 2009). VEGF mRNA levels in the neural tube increase over time. Additionally, VEGF is highly expressed around and within in the floor plate of the neural tube (Hogan et al., 2004; James et al., 2009; Nanka et al., 2006) (Fig. 3.1 C), suggesting that the time of ventral sprout ingression may be regulated by early and relatively high amounts of VEGF in this region. Less VEGF mRNA is detected in the medial neural tube, leading us to hypothesize that delayed medio-lateral ingression may be caused, in part, by insufficient VEGF levels at earlier stages. Furthermore, since medio-lateral blood vessel sprouts ingress only after the onset of neurogenesis, we were interested in understanding if the timing of neural cell differentiation also influenced the timing of blood vessel ingression. Due to the highly stereotypical nature of the blood vessel ingression pattern, we also hypothesized that certain sub-populations of neurons might promote angiogenesis while others may be anti-angiogeneic and block vessel sprouting from the PNVP. The data in this chapter addresses these hypotheses, and show that both the timing of neural tube differentiation and the spatial organization of the developing neural tube along the DV axis influence blood vessel ingression patterns.

Figure 3.1: Overview of neural development and VEGF expression in the neural tube.

(A) Division of the developing neural tube into progenitor and differentiated neurons along its medio-lateral axis (left), and specification of neuronal subclasses along the DV axis of the neural tube (right). Neuroepithelial cells (purple) form a pseudo-stratified layer comprising the VZ of the neural tube. Radial glial cells (blue) have processes spanning the entire width of the neural tube; however, their cell bodies remain in the VZ. As neurons (green) differentiate, their cell bodies leave the VZ, and they begin to project axons toward target tissues. Differentiated neurons are organized into subclasses along the DV axis of the neural tube before the onset of neurogenesis. Their position on the DV axis as progenitor cells determines what type of neuron they will become. Here, three broad neuronal subtypes are depicted: dorsal interneurons (red), ventral interneurons (blue), and motor neurons (green). **(B)** Neuroepithelial cells can undergo a proliferative division to give rise to two new neuroepithelial cells (1), or asymmetrical and symmetrical divisions to give rise to one radial glial cell and one neuron (2) or two neurons (3). Certain bHLH transcription factors, such as Neurogenin 1 and 2, promote neuronal differentiation (3) over asymmetrical divisions (2) during neurogenesis. **(C)** Quail VEGF ISH on a transverse, quail neural tube section at HH26. The ventral neural tube, including the floor plate, shows a stronger VEGF signal (arrowhead) than the medio-lateral neural tube. FP, floor plate; VZ, ventricular zone; IZ, intermediate zone; MZ, marginal zone; DRG, dorsal root ganglia. Scale bar: 100μm.


A
Medial
Lateral
IZ
VZ
DRG
MZ
FP
Dorsal
Ventral
Neuroepithelial cell
Radial Glial cell
Neuron
Dorsal interneurons
Ventral interneurons
Motor neurons


B
1.
2.
3.


qVEGF ISH
C
FP
HH26

C. MATERIALS AND METHODS

Expression constructs

The Sox2-IRES-eGFP vector was a gift from L. Pevny (Graham et al., 2003). Full length NeuroM cDNA (Roztocil et al., 1997) (a gift of J.M. Matter) was inserted into the *Eco*R1 site of the pCAGGS-IRES2-nucEGFP vector (pCIG) (Megason and McMahon, 2002) (gift of L. Pevny and A. McMahon), generating NeuroM-IRES-eGFP. The human VEGF165-IRES-eGFP was generated as previously described (James et al., 2009). Full length MNR2 cDNA (Tanabe et al., 1998) (a gift of T.M. Jessell) was inserted into the *Eco*R1 site of the pCIG vector, generating MNR2-IRES-eGFP.

In ovo electroporation

We performed quail, neural tube electroporations as previously described (James et al., 2009), with the following minor modifications: The Sox2 and NeuroM expression vectors were injected into HH14 quail neural tubes at a final DNA concentration of 2μg/μl. Embryos were incubated for an additional 2.5 (Sox2) or 1.5 (NeuroM) days post-electroporation before embryo dissection at HH25 or HH23, *respectively*. For VEGF timing experiments, the VEGF165-IRES-eGFP construct was injected at a final DNA concentration of 0.1μg/μl on HH14. Embryos were dissected approximately 1-1.5 days later at HH22-23. The MNR2 expression construct was injected on HH16-17 at a final DNA concentration of 1.5μg/μl. Embryos were incubated for 2 days before dissection at HH25.

Immunofluorescence

Monoclonal mouse antibodies to Islet1 and Nkx2.2 were used at a concentration of 1:500 (obtained from the Developmental Studies Hybridoma Bank developmed under the auspices of the NICHD and maintained by The University of Iowa Department of Biological Sciences, Iowa City, IA 52242). Antibody staining with QH1, Tuj1, and MNR2 was performed as previously described (James et al., 2009).

Analysis of ingression patterns/ neural tube measurements

We previously analyzed 14, QH1-stained sections per embryo and compiled the data from five embryos in our graphs (or 70 sections total) (James et al., 2009); however, we slightly modified our analysis method in this chapter. Here we analyzed *N* number of total sections in *X* number of embryos per construct electroporated, added the number of ingression events per 10˚ of arc from all sections analyzed, and divided these numbers by 25, to obtain the average number of sprouts per 25 sections. These numbers were plotted on the graphs. Other neural tube measurements, such as the upper and lower boundaries of the V3 interneuron domain, were obtained using the same method as the blood vessel sprouts. Overlayed images are from adjacent sections within the same embryo.

In situ hybridization

Quail, VEGF ISH was performed as previously described (James et al., 2009) on Sox2 and NeuroM-electroporated neural tube cryosections (courtesy of the UNC-ISH core facility and Megumi Aita).

D. RESULTS

The onset of neurogenesis correlates with the timing of neural tube angiogenesis

Migratory angioblasts begin to form the PNVP as early as HH14 (Fig. 3.2 A, G; arrowhead). We utilized the Tuj1 antibody, recognizing the neuron-specific microtubule component ß-tubulin III, to assess the progression of neurogenesis at key points in neural vessel development. At HH14, a few neurons have begun to terminally differentiate and migrate laterally to the pial surface of the neural tube (Fig. 3.2 D; arrowheads). Neurons continue to differentiate, migrate, and accumulate at the lateral edge of the neural tube until HH27, when developmental neurogenesis is largely complete in the spinal cord (reviewed in Gotz and Huttner, 2005) (data not shown). Medio-lateral sprout initiation occurs midway through neurogenesis (Fig. 3.2 B; arrowhead), between HH23 and HH24, *after* a significant number of differentiated neurons have migrated to the pial surface of the neural tube (Fig. 3.2 E, H). Ventral sprouts have already formed by this time (data not shown). Medio-lateral sprouts are first seen as filopodial extensions that extend into the neural tube from the PNVP (Kurz et al., 1996) (Fig. 3.2 B; arrowhead). As these angiogenic sprouts elongate within the neural tube, they stop and branch laterally within the intermediate zone (IZ) as they interface the VZ (Fig. 3.2 C, F, and I), but they never invade this region. These observations suggest that progenitor cells comprising the VZ may block angiogenic sprouts, while differentiated neurons within the marginal zone positively regulate intraneural angiogenesis. Therefore, it is possible that the temporally controlled generation of these neural sub-populations directs the timing of sprouting angiogenesis into the neural tube.

In order to understand how neural cell differentiation correlates with blood vessel patterning events within the neural tube, we developed a neuronal differentiation index to quantify the progression of neurogenesis at specific developmental time points. Here, we focus specifically on neurogenesis in the medial neural tube, as well as the medio-lateral blood vessel sprouting event, although the time of ventral sprout formation is also noted. We measured the area of Tuj1-positive cells (blue) in the medial neural tube between the dorsal boundary of motor neuron region and the dorsal horn and then divided this measurement by the total area in the medial region of the neural tube, to find the *percent* Tuj1-positive area as depicted (Fig. 3.2, J). These measurements were taken at various stages of quail neural tube development, ranging from HH22 through HH26, to create a neuronal differentiation index (Fig. 3.2 K). Few medio-lateral sprouts initiate between HH23 and HH24 (Figure 3.2 B, arrowhead); at this time the medial neural tube is between 25% and 30% tuj1-positive. Between HH24 and HH25 more sprouts enter the neural tube and existing sprouts elongate to reach the VZ, and medial sprouts begin to anastomose with ventral sprouts at this time (data not shown). We show an increased rate of neurogenesis between HH24 and HH25.

This data demonstrates that blood vessels begin to form the PNVP at HH14, and they wait for approximately 1.5 days before sprouting medio-laterally at HH23. Why the delay? We wanted to explore whether or not the normal program of neurogenesis influenced the timing of neural blood vessel ingression, or determine if the two are independent of one another. To begin to answer these questions, we perturbed the timing of neurogenesis by electroporation of specific transcription factors that either delayed

neural progenitor cell differentiation or induced premature neuronal differentiation, and assessed whether or not the blood vessels followed suit.

The timing of neurogenesis influences the timing of blood vessel ingression

If neurogenesis directs the timing of neural tube angiogenesis, we expect to see a delay in medio-lateral blood vessel sprout formation in neural tubes where neurogenesis is delayed. To test this, we electroporated a Sox2-cDNA construct (Graham et al., 2003) into the neural tubes of HH14 (Day2.5) quail embryos and allowed them to develop until HH25 (Day 5)—a stage when medio-lateral sprouting has occurred in unperturbed embryos. *Sox2* is expressed in neural progenitor cells, and it is normally down-regulated before the onset of neurogenesis (Bylund et al., 2003). *Sox2* maintains neural progenitor identity when constitutively expressed in neural progenitor cells within chick neural tubes, essentially blocking neuronal differentiation (Graham et al., 2003). We utilized this gene as a tool to prevent neuronal differentiation in quail neural tubes. The stages chosen for electroporation and embryo dissection are significant because we wanted to electroporate the embryos early enough to maximize the number of progenitor cells receiving the construct, thereby reducing the number of differentiated cells migrating to the lateral edge, and dissect late enough to assess defects in medio-lateral blood vessel sprouting by ending the experiment at a stage when medio-lateral sprouts would normally be present in unperturbed embryos.

Sox2 electroporation delayed neuronal differentiation in quail neural tubes and this perturbation significantly reduced the number of medio-lateral angiogenic sprouts invading the neuroepithelum (Fig. 3.3). Qh1 immunostaining of Sox2-electroporated

embryos showed normal blood vessel ingression on the control side of the embryo (Fig. 3.3 C; arrow), whereas no vessel sprouting occurred on the electroporated side. Though angiogenic sprouting is blocked on the Sox2-electroporated side, angioblast immigration still occurred (Fig. 3.3 C; arrowheads). Furthermore, the PNVP is unperturbed in these embryos, indicating that a delay in neuronal differentiation does not influence PNVP formation. Analysis of blood vessel ingression in Sox2-electroporated embryos revealed a reduction in blood vessel sprouts on the electroporated side of embryo when compared to the contralateral control analysis (Fig. 3.3 D). Tuj1 immunostaining confirms that Sox2 electroporation restricts neuronal differentiation in quail (Fig. 3.3 E-G). We see a significantly reduced Tuj1-positive area on the Sox2-electroporated side of the neural tube when compared to the contralateral control side and unperturbed HH25 control neural tubes (Fig. 3.3 H). We compared the left and right sides of an unperturbed HH25 embro to show that no significant differences normally exist between each half of the neural tube (Fig. 3.3 H; grey bars). Tuj1 analysis indicates that Sox2-electroporated embryos are developmentally delayed and fall between HH22 and HH23 on the neuronal differentiation index (Fig 3.2 K). Blood vessels do not normally invade the medio-lateral neural tube at these stages. These data strongly suggest that while proliferating progenitor cells positively direct PNVP formation, they negatively regulate angiogenic sprouting into the neural tube.

Since delayed neuronal differentiation blocked neural tube angiogenesis, it seemed feasible that premature neuronal differentiation might induce premature angiogenic sprouting. To test this, we electroporated a NeuroM-cDNA construct

(Roztocil et al., 1997) into HH14 neural tubes and allowed them to develop until HH23-HH24 (Day 4). NeuroM is a bHLH transcription factor expressed during a transitional stage in neurogenesis when neurons become post-mitotic but have not yet begun to migrate laterally to the pial surface of the neural tube. NeuroM is expressed within the intermediate zone, along the entire DV axis of the neural tube as early as HH10 (E1.5). Studies have indirectly shown that NeuroM, and its mouse homolog Math3, can induce premature neurogenesis, though not as robustly as *Neurogenin-1 or Neurogenin-2* (Bylund et al., 2003; Lee and Pfaff, 2003). We used NeuroM as a tool to subtly promote premature neuronal differentiation in the medial neural tube. We electroporated at HH14 to maximize the number of progenitor cells receiving the construct, thereby maximally increasing the potential for more cells to differentiate and migrate to the lateral edge of the neural tube. The electroporated embryos were dissected between HH23-HH24, a developmental time point when medio-lateral sprouting is just commencing, in order to assess whether or not premature vessel sprouts formed.

NeuroM electroporation induced premature neuronal differentiation, and this perturbation resulted in premature and (or) supernumerary angiogenic sprout ingression (Fig. 3.4). QH1 immunostaining on NeuroM-electroporated neural tube sections showed that medio-lateral blood vessel sprouts formed on the NeuroM-electroporated side of the neural tube (Fig. 3.4 C; arrowhead) while no medio-lateral sprouts were seen on the contralateral control side at this stage. Analysis of blood vessel ingression reveals that medio-lateral sprouting occurred on both the NeuroM-electroporated and contralateral control sides of the neural tube; however, fewer medio-lateral sprouts have formed on the control side of the embryo (Fig. 3.4, D). Based on these results, it is likely that additional

sprouts entering the neural tube at slightly later stages (between HH24 and HH25) may be entering prematurely, thus accounting for the supernumerary ingression at earlier stages of development. Furthermore, medio-lateral sprouts forming on the control side of the embryo appear to be less mature than sprouts on the NeuroM-electroprated side and consist mainly of filopodial extenstions (Fig. 3.4, E and G); these sprouts were included in the ingression analysis. More mature sprouts on the NeuroM-electroporated side of the embryo ingressed farther (Fig. 3.4, F), and are beginning to anastomose with ventral sprouts (Fig. 3.4 H; arrow). This suggests that angiogenic sprouts began to form on the NeuroM-electroporated side of the neural tube *before* they formed on the contralateral control side.

Tuj1 immunostaining confirmed that the NeuroM perturbation increases the Tuj1-positive area in the medial neural tube, with a concomitant reduction in VZ area when compared to the contralateral control side (Fig. 3.4, I-L). Tuj1 analysis indicates that NeuroM-electroporated embryos are developmentally advanced and are similar to HH24-HH25-stage embryos (41% Tuj1-positive area) on the neuronal differentiation index—a time frame when medio-lateral neural tube angiogenesis occurs, while the control side is more comparable to HH24-stage embryo (29% Tuj1-positive area). These results strongly suggest that the timing of blood vessel ingression is influenced by the progression of neurogenesis.

We previously showed that VEGF regulates angiogenic sprouting into the neural tube (James et al., 2009). Levels of VEGF increase within the neural tube over time, indicating that developmentally delayed Sox2-electroporated neural tubes may have a

reduction in VEGF expression at HH25. Reduced VEGF levels could account for the delay in blood vessel ingression. Conversely, NeuroM electroporated neural tubes may display increased VEGF expression at a younger age, leading to premature blood vessel ingression. To test this, we performed quail VEGF (qVEGF) *in situ* hybridizations (ISH) on Sox2 and NeuroM-electroporated neural tubes. We found that there were no detectable changes in VEGF expression on the electroporated side of either the Sox2 or NeuroM-electroporated embryos when compared to the contralateral control side (Fig. 3.5, A and B) or unperturbed control embryos (Fig. 3.1, C). These results indicate that the amount of VEGF present at HH24 is sufficient to allow heightened angiogenesis *if* more differentiated neurons form prematurely at the lateral edge of the neural tube. Conversely, although VEGF is present in Sox2-electroporated embryos, it is not sufficient to overcome the angiogenic block accompanying delayed neurogenesis.

We showed VEGF overexpression in the neural tube induces ectopic and supernumerary blood vessel sprouting by HH25 (James et al., 2009); however, we did not know if *earlier* VEGF overexpression could induce premature vessel ingression in younger neural tubes. To test this, we electroporated HH14 (Day 2.5) neural tubes with VEGF165 and let them incubate for one day, before dissecting between HH22 and HH23 (Day 3.5-4). QH1 immunostaining of embryo sections showed that early VEGF165 overexpression induced premature blood vessel ingression (Fig. 3.6 A-C). VEGF overexpression in the ventral neural tube induced premature sprouting; however, this was not the case for VEGF overexpression in the dorsal and medial neural tube. VEGF overexpression in these areas did not result in premature vessel sprouts. Instead, ectopic

vessels with large lumens formed outside of the dorsal and medio-lateral neural tube (Fig. 3.6, D and E; arrowhead). Blood vessel analysis of VEGF-electroporated embryos confirmed these observations. No medio-lateral sprouts ingressed on the control side of the embryo, while sprouts were found on the VEGF-electroporated side (Fig. 3.6, F). Ventral sprouts formed on both sides of the neural tube at this stage.

Intriguingly, premature sprouts were also *ectopic*. Blood vessels do not initially sprout into the region of the ventral neural tube where motor neurons develop (Fig. 3.7); however, when VEGF was overexpressed early in this region of the neural tube, sprouts were able to ingress. Premature vessels invaded the neural tube in the region where the motor neuron marker, MNR2, was expressed (Fig. 3.6, G and H). The motor neurons normally differentiate earlier, in greater numbers than other neuronal subtypes (Ericson et al., 1992; Hollyday and Hamburger, 1977), as evidenced by the thickened Tuj1-positive area in the ventral neural tube (Fig. 3.6 I; bracket). When VEGF-electroporated neural tubes were allowed to develop until HH24, the vascular phenotype became reminiscent of later-stage VEGF perturbations, exhibiting ectopic and supernumerary ingression into the dorsal and medio-lateral neural tube (Fig. 3.6, J and K; arrowhead). These results indicate that early VEGF overexpression can induce premature vessel ingression, but only if a sufficient number of differentiated neurons are also present in the area. This suggests a requirement for *both* sufficient VEGF and differentiated neurons to be present at the lateral edge of normally developing neural tubes *before* sprouts can invade the neuroepithelium.

Blood vessels ingress at stereotypical locations along the DV axis of the neural tube

Differentiated neurons form along the entire DV axis of the neural tube, and VEGF mRNA is distributed evenly throughout, so why don't vessel sprouts invade all regions of the neural tube? We hypothesized that neuronal subclasses forming in specific locations along the DV axis may either *positively* or *negatively* regulate angiogenesis into the regions of the neural tube where they differentiate. To begin to address this hypothesis, we first needed to quantify exactly where blood vessels ingress relative to specific sub-populations of neural cells along the DV axis of the neural tube.

We observed that initial blood vessel sprouts do not ingress into the somatic motor neuron (sMN) domain, the area within the ventral neural tube where differentiated motor neurons reside. The neurons in this region can be identified with the MNR2 antibody, which labels motor neuron progenitors in their last mitotic cycle in early neural tubes, and also cross-reacts with the HB9 protein (a transcription factor expressed by a sub-type of differentiated motor neurons) in older neural tubes (HH24-25) (Fig. 3.7 A). Instead, blood vessels enter the neural tube both dorsal and ventral to this region (Fig. 3.7 B and C; *overlay*). Ventrally, blood vessels ingress *between* the floor plate and the sMN domain. The neurons in this region comprise the V3 interneuron subclass and can be identified with an antibody against the homeodomain transcription factor Nkx2.2 (Fig. 3.7, D). Adjacent, Nkx2.2 and Qh1-stained sections show that blood vessels positively associate with Nkk2.2-labeled cells (Fig. 3.7, E and F; *overlay*). Blood vessels ingressing into the medio-lateral neural tube do so between the dorsal boundary of the sMN domain and the dorsal root entry zone—where DRG axons invade the neural tube (Fig. 3.7, C;

arrow). The medio-lateral region of the neural tube contains many different types of differentiated neurons, including the ventral interneuron subclasses V2, V1, and V0 (Briscoe and Ericson, 2001), as well as the dorsal interneuron domains dI4, dI5 and dI6 (Helms and Johnson, 2003). Specific marker genes, usually bHLH transcription factors, are used to identify differentiated neuronal subclasses in the medio-lateral region of the neural tube. For example, the V0 interneurons express Evx1, while the V1 neurons express En-1 (Engrailed-1) (Diez del Corral and Storey, 2001). We used antibodies directed against some of these markers on neural tube sections and superimposed Qh1-labeled, adjacent sections to determine whether or not blood vessels invaded the neural tube where specific neuronal subclasses were found within the medio-lateral neural tube; however, we were unable to establish a pattern. We saw blood vessels associating with all of the neuronal subclasses we labeled in this region (data not shown). These findings suggested that the medio-lateral neural tube was generally pro-angiogenic, or *permissive* to angiogenic sprouts.

In order to quantify these observations, we labeled HH25 neural tube sections with Nkx2.2, MNR2/HB9, or Qh1, then took a series of measurements on adjacent sections to determine where blood vessels enter the neural tube relative to the specific regions labeled (Fig. 3.7 G). We first analyzed the angle of blood vessel ingression for 25 medio-lateral sprouts and 25 ventral sprouts from HH25 neural tubes. For each ventral sprout we measured the angle from zero degrees to the lateral boundary of the floor plate (violet), the upper and lower boundary of the Nkx2.2-positive region (blue), and the lower boundary of the MNR2/HB9-positive sMN domain (green) on sections adjacent to the sprouting event. For each medio-lateral sprout, we measured the upper

and lower boundary of the dorsal root entry zone (orange) on the same section as the sprout, in addition to the upper boundary of the MNR2/HB9-positive sMN domain (green) on adjacent sections. Areas falling between the upper boundary of the sMN domain and the lower boundary of the dorsal root entry zone are considered the medial neural tube (yellow). The dorsal region of the neural tube falls between the upper boundary of the dorsal root entry zone and 180 degrees (red).

We plotted all of the measurements on a graph (Fig. 3.7 H). We found that 100% of the ventral sprouts entered the Nkx2.2-positive, V3 interneuron domain (blue), and 96% of the medio-lateral sprouts entered the medial neural tube (yellow). Only one medial sprout entered the sMN domain, close to the upper boundary of MNR2/HB9 expression (medial sprout #2). In total, 98% of the sprouts analyzed completely avoided the motor neurons. This suggests that motor neurons may be anti-angiogenic, while other neuronal subtypes, such as the V3 interneurons, are pro-angiogenic.

Ectopic motor neurons block medio-lateral angiogenic sprouting into the neural tube

To begin to test the hypothesis that motor neurons negatively regulate angiogenesis, we electroporated an MNR2-cDNA expression construct into HH16-17 (Day2.5-3) quail neural tubes (Fig. 3.8 A-C). Ectopic MNR2 expression in neural progenitor cells is sufficient to drive the acquisition of motor neuron properties in post-mitotic neural progenitor cells (Tanabe et al., 1998). We utilized MNR2 as a tool to generate ectopic motor neurons in the medio-lateral neural tube—and then asked whether or not *ectopic* motor neurons could confer an angiogenic block to this normally permissive region of the neural tube.

MNR2 electroporation blocked medio-lateral angiogenic sprout formation. QH1-staining of MNR2-electroporated neural tube sections showed that blood vessels ingress normally on the contralateral control side of the embryo, while no medio-lateral sprouts are seen on the electroporated side (Fig. 3.8 D-F). Blood vessel analysis of MNR2-electroporated embryos reveals almost a complete block in medio-lateral angiogenesis when compared to the contralateral controls (Fig. 3.8 G).

We were unable to assess motor neuron differentiation utilizing the MNR2 antibody because it labels both motor neuron progenitor cells and HB9-positive, differentiated motor neurons. Additionally, MNR2 was present in all electroporated cells, making it impossible to distinguish progenitor cells from differentiated neurons. Instead, we utilized another marker for differentiated motor neurons, *Islet1*. Islet1 is a homeobox transcription factor expressed by differentiated motor neurons (Ericson et al., 1992). A monoclonal antibody directed against this protein labels motor neurons, dorsal root ganglia, as well as medio-lateral and dorsal populations of interneurons within the neural tube. We stained MNR2-electroporated neural tube sections with the Islet1 antibody (Fig. 3.8 H-J) and counted positive cells in both the medio-lateral and dorsal regions of the neural tube (Fig. 3.8 K). We saw a significant increase in Islet1-positve cells in the medio-lateral region on the MNR2-electroporated side of the neural tube when compared to the contralateral control side. There was only a slight difference between dorsal populations. This shows that MNR2 electroporation is able to induce ectopic motor neuron generation in the medio-lateral neural tube, resulting in a block to vessel ingression in that area. This suggests that the motor neurons negatively regulate angiogenesis into the neural tube. Furthermore, this work demonstrates that the normal

process of neuronal subclass specification along the DV axis of the neural tube *is* important for proper blood vessel ingression and intraneural blood vessel patterning.

E. DISCUSSION

Blood vessels entering the neural tube do so at a specific time, and at highly stereotypical locations. These observations prompted us to explore the relationship between blood vessel ingression patterns and the mechanisms that pattern the neural tube. The work presented in this chapter demonstrates that two major processes of neural development, programmed neurogenesis and neuronal cell fate specification, direct intraneural blood vessel patterning.

Programmed neurogenesis

Blood vessels begin to form the PNVP at HH14 (Day 2.5), which coincides with the onset of neurogenesis and the appearance of Tuj1 positive neurons within the neural tube; however, medio-lateral blood vessel sprouts do not initiate until HH23-HH24 (Day 3.5-4), after neurogenesis is well underway. We did not know if the neuroepithelium, which is known to positively interact with angioblasts and ECs during PNVP formation, could actually *negatively* regulate angiogenic sprouting into the neural tube. Or, conversely, it was possible that blood vessels forming the PNVP were not competent to ingress until a particular developmental stage. To begin to address these hypotheses, we manipulated the timing of neurogenesis by altering the expression of genes known to either prevent neural progentitor differentiation or induce premature neurogenesis.

Intriguingly, we found that both manipulations predictably perturbed neural tube angiogenesis. Constitutive Sox2 expression prevented neuronal differentiation and blocked blood vessel ingression, while NeuroM expression increased Tuj1-positive

neurons at the lateral surface of the neural tube in younger embryos—a perturbation resulting in premature blood vessel ingression. These experiments suggest that blood vessels are competent to enter the neural tube earlier than they actually do. These results also indicate that the early neuroepithelium negatively regulates blood vessel ingression, while differentiated neurons allow vessels to enter the neural tube.

It is unclear whether differentiated neurons or radial glial cells are positively regulating intraneural angiogenesis. It is unlikely that these cell types are instructive to the blood vessels, but rather they may break down the barrier established by the progenitor cells—making the neural tube permissive to angiogenic invasion. Blood vessels positively associate with radial glial cell processes in the brain (Virgintino et al., 1998). Just as neurons utilize radial glial cells as a scaffold for migration, blood vessels entering the neural tube may also require these cells for support; however, the role of radial glial cells in the neural tube during neurogenesis is poorly understood. Studies show that neural progenitors within the neural tube begin to acquire radial glial properties after neurogenesis is largely complete, and instead maintained neuroepithelial characteristics during the time blood vessels ingress (reviewed in, (Gotz and Huttner, 2005)). Electroporation of a *Neurogenenin-1* or *Neurogenin-2* expression construct into early neural tubes may begin to address these questions. Neurogenin expression directly induces progenitor cell cycle exit and promotes neuronal differentiation at the expense of radial glial cell formation (Mizuguchi et al., 2001; Sun et al., 2001). Would blood vessels ingress in a situation where radial glial processes were largely absent while differentiated neurons still formed?

Refining the role of VEGF in intraneural angiogenesis

In addition to adequate support, blood vessels must also receive the right signals to promote angiogenesis. We know the early neural tube expresses VEGF, and that this VEGF is required for PNVP formation; however, it was possible that levels of VEGF present at early stages of neural tube development were insufficient to direct blood vessel ingression. As a result, the immature neural tube could not support angiogenic sprouting until VEGF expression increased over time. We found that this was unlikely to be the case. First of all, VEGF levels were not perturbed in Sox2 or NeuroM-electroporated embryos, even though the timing of vessel ingression was altered. This suggested that the timing of blood vessel ingression was partially independent of VEGF-signaling. Secondly, VEGF165 overexpression was unable to induce premature medio-lateral sprouts at early stages of neural tube development. Premature sprouts formed only when VEGF was expressed in areas where the most differentiated neurons were found—the region of motor neuron development in the ventral neural tube. In Chapter II, we showed that regional depletion of VEGF in the neural tube, via ectopic S-Flt expression, prevents blood vessel ingression—even though neurons differentiated normally in these embryos (Fig. 2.6 G). Taken together, these results indicate that *both* sufficient levels of VEGF *and* the proper number of differentiated neurons must be present at the lateral edge of the neural tube before vessel ingression can occur.

Additionally, *matrix-binding* VEGF must be present in order for angiogenic sprouts to invade the neural tube at the proper time. Analysis of mouse neural tubes only expressing the non-matrix-binding VEGF120 isoform displayed a delay in neural tube angiogenesis (data not shown). These data further refine the role of VEGF in regulating

neural tube angiogenesis. Matrix-binding VEGF must work in coordination with differentiated neurons to promote proper neural tube angiogenesis. Perhaps matrix-binding VEGF becomes localized to the extracellular matrix secreted by differentiated neurons, creating depots in the marginal zone that guide angiogenic sprouts. Perhaps progenitor cells do not produce the proper matrix to trap VEGF, and at younger neural tube stages, most of the VEGF produced in the neural tube escapes to the surrounding mesodermal tissue. Unfortunately, it is difficult (if not impossible) to visualize where this small protein is localized in the extracellular spaces of the neural tube.

Neuronal cell fate specification along the DV axis of the neural tube

The coordinated activity of VEGF and differentiated neurons is not sufficient to explain the intricacies of the blood vessel pattern. Though both are required for regulation of the timing of blood vessel ingression, they do little to explain why blood vessels enter the neural tube in such highly stereotypical locations along the DV axis. We hypothesized that certain neuronal subtypes positively associated with blood vessel sprouts (or were permissive to angiogenesis), while others negatively regulated angiogenesis.

In order to determine if neural patterning along the DV axis of the neural tube could influence the blood vessel pattern, we manipulated the range of motor neuron differentiation via MNR2 expression. Essentially, we extended the domain of motor neuron differentiation to the medio-lateral neural tube—an area normally permissive to blood vessel ingression. Intriguingly, ectopic motor neurons were able to confer the angiogenic block in this region. This data provides strong evidence that regional

specification of neuronal subtypes does influence the pattern of vessel ingression. Exactly how the motor neurons negatively regulate angiogenesis remains unclear. Fortunately, mechanisms regulating motor neuron cell fate determination are among the most well-characterized processes in neural tube development. In Chapter IV, we begin to explore how the motor neurons may negatively regulate angiogenesis, and where in the motor neuron differentiation process the block actually occurs. We also identified a population of cells that may positively regulate neural tube angiogenesis: the Nkx2.2-positve, V3 interneurons. 100% of ventral sprouts enter the V3 interneuron domain. Based on this data, we predict that ectopic V3 interneurons would induce ectopic or supernumerary vessel sprouts.

The work in this chapter demonstrates that normal processes of neural development regulate neural tube angiogenesis. We have demonstrated that different regions of the neural tube interact differently with developing vessels. Only when we understand the relationship between each neural cell type—progenitor cell, differentiated cell, dorsal interneuron, ventral interneuron, or motor neuron—and the vasculature, can we get a complete picture of how vessel patterning is regulated by the neural tube.

Figure 3.2: The onset of neurogenesis correlates with the timing of blood vessel ingression.

(A-I) Comparison of blood vessel development and the progression of neurogenesis at early stages of neural tube development in quail. **(A-C)** QH1-stained neural tube sections at HH14, HH23, and HH25. PNVP formation initiates at HH14 when the first ECs are seen adjacent to the neural tube (A; arrowhead). Sprout initiation occurs at HH23 (B; arrowhead), and ingressing vessels branch laterally as they interface the VZ (C). **(D-F)** Tuj1-stained neural tube sections at HH14, HH23, and HH25. At HH14, few Tuj1 positive cells are beginning to migrate to the pial surface of the neural tube (D; arrowheads). The Tuj1-positive area at the lateral edge of the neural tube increases during later stages of development (E, F). **(G-I)** Models depicting blood vessel patterning events and the progression of neurogenesis at HH14, HH23, and HH25. Progenitor cells in the VZ (purple) become increasingly restricted over time as neurons (blue) differentiate and migrate to the lateral edge of the neural tube. Blood vessels enter the neural tube only after neurons differentiate. **(J)** % Tuj1-area analysis scheme. The Tuj1-positive area (blue) is divided by the total, medial area on one side of the neural tube to obtain the % Tuj1-positive region in the medio-lateral neural tube. **(K)** Neuronal differentiation index. % Tuj1-positive area measurements from HH22, 23, 24, 25, and 26-stage embryos were plotted on a graph to illustrate the progression of neurogenesis over time. Asterisk: developmental stage when medio-lateral ingression occurs.

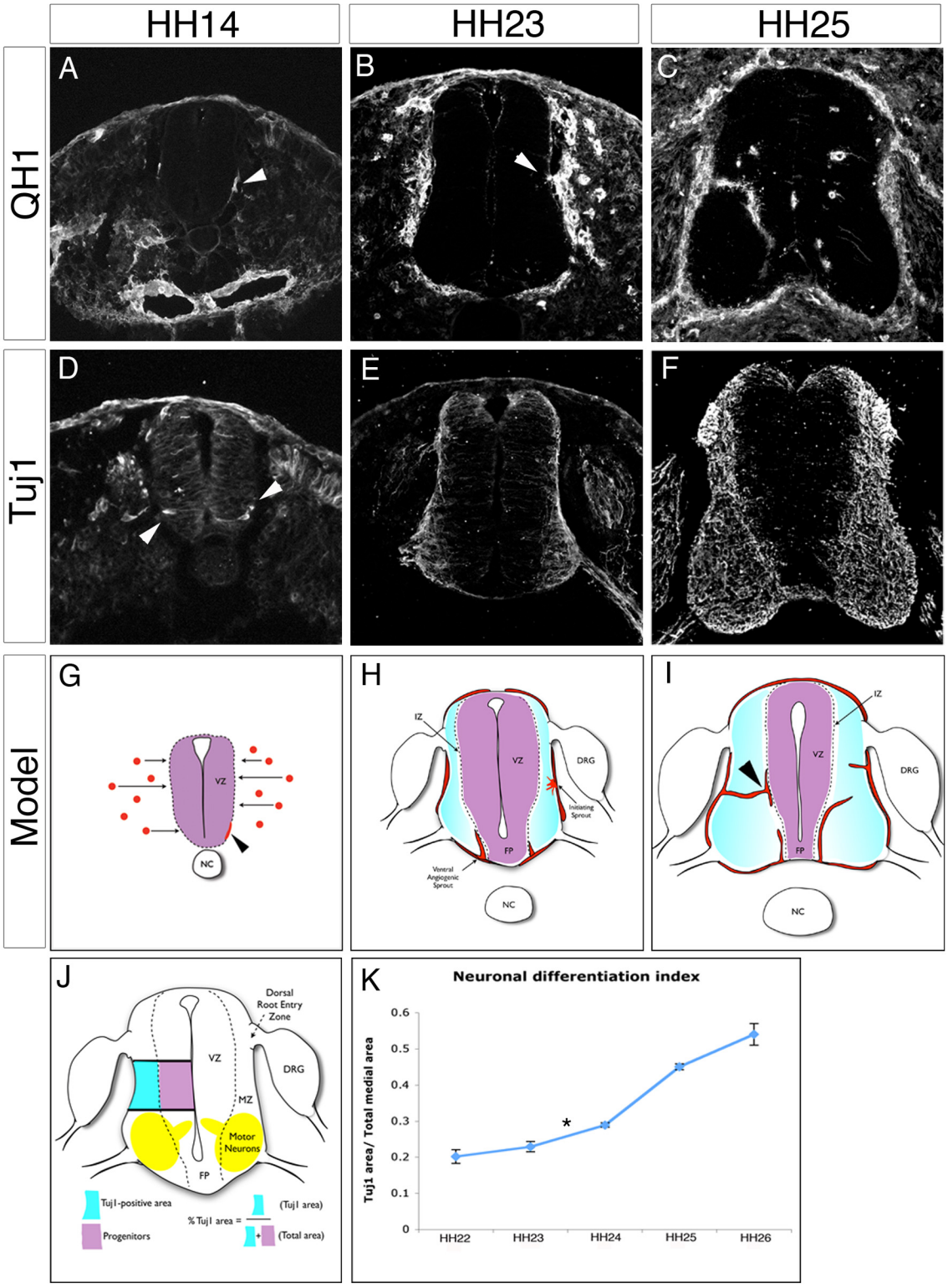

HH14
HH23
HH25
QH1
Tuj1
Model
A
B
C
D
E
F
G
H
I
J
K
VZ
NC
IZ
DRG
Initiating Sprout
FP
Ventral Angiogenic Sprout
MZ
Motor Neurons
Dorsal Root Entry Zone
Tuj1-positive area
Progenitors
% Tuj1 area =
(Tuj1 area)
(Total area)
Neuronal differentiation index
Tuj1 area/ Total medial area
0.6
0.5
0.4
0.3
0.2
0.1
0
HH22
HH23
HH24
HH25
HH26
*

Figure 3.3: Delayed neuronal differentiation blocks neural tube angiogenesis.

Quail neural tubes were electroporated with a Sox2-IRES-eGFP construct at HH14 (Day 2.5) and harvested approximately two days later at HH25 (Day 4.5-5). **(A-C)** QH1 immunostaining shows a blood vessel sprout ingressing into the medio-lateral region of the neural tube on the control side (C; arrow), while no sprouts are seen on the Sox2-electroporated side. Migratory angioblasts are able to invade the neuroepithelium on the Sox2-electroporated side (C; arrowheads). **(D)** Analysis of blood vessel ingression on the Sox2 (green) or contralateral control (black) sides of electroporated embryos (n=103 sections). **(E-G)** Sox2 expression prevents neuronal differentiation in quail. Tuj1 immunostaining is drastically reduced within the medio-lateral region of the neural tube on the Sox2-electroporated side when compared to the control side and unperturbed. These observations are quantified in the Tuj1 area analysis (n=17 electroporated sections) **(H)**. Bars indicate that Tuj1 area is significantly reduced on the Sox2-electroporated side of the neural tube (green) when compared to the contralateral control side (black) and unperturbed, HH25 control neural tubes (grey).

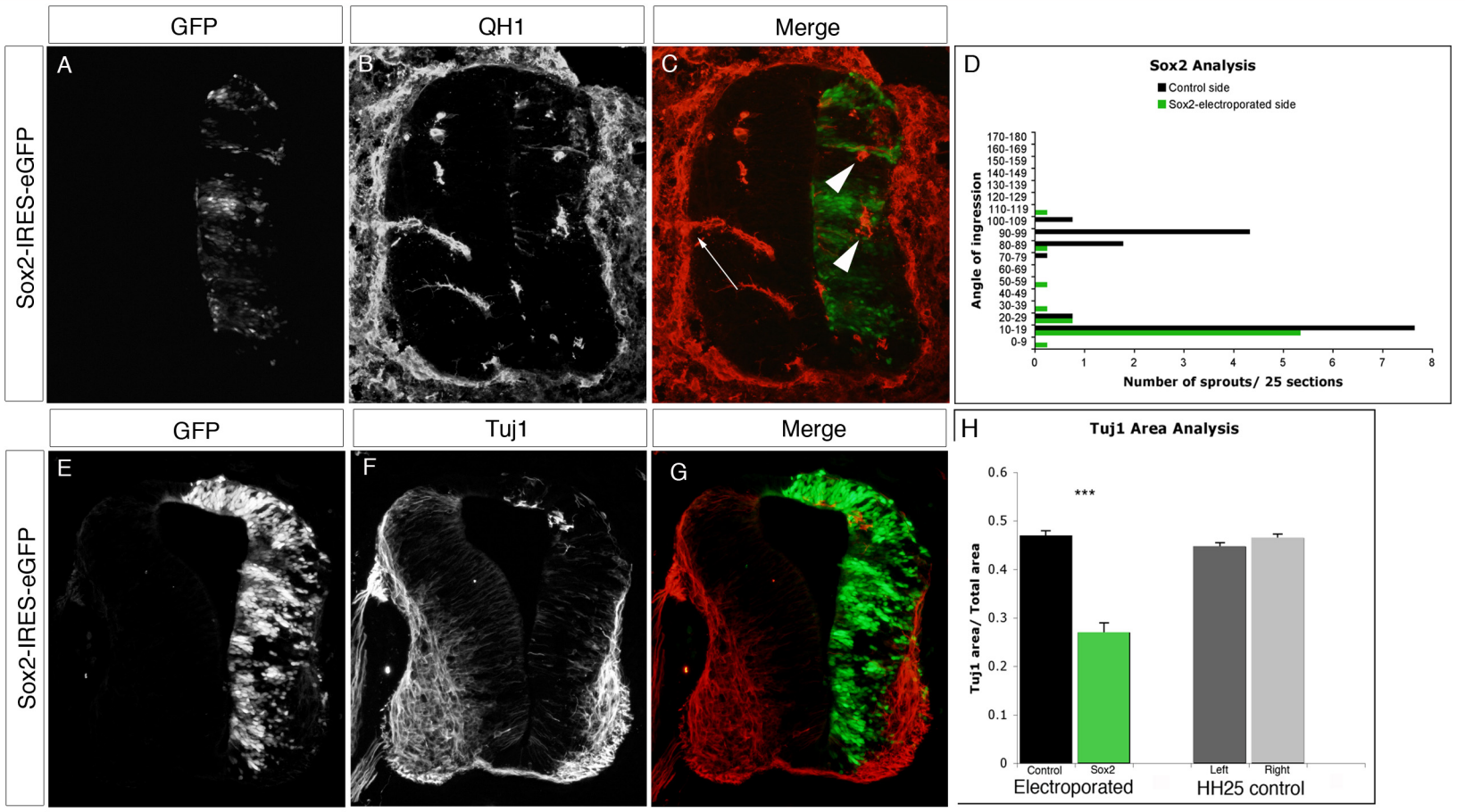

GFP
QH1
Merge
Sox2-IRES-eGFP
A
B
C
D
Sox2 Analysis
Control side
Sox2-electroporated side
Angle of ingression
170-180
160-169
150-159
140-149
130-139
120-129
110-119
100-109
90-99
80-89
70-79
60-69
50-59
40-49
30-39
20-29
10-19
0-9
0
1
2
3
4
5
6
7
8
Number of sprouts/ 25 sections
GFP
Tuj1
Merge
Sox2-IRES-eGFP
E
F
G
H
Tuj1 Area Analysis
Tuj1 area/ Total area
0.6
0.5
0.4
0.3
0.2
0.1
0
***
Control
Sox2
Left
Right
Electroporated
HH25 control

Figure 3.4: Premature neural tube differentiation promotes early vessel ingression.

Quail neural tubes were electroporated with a NeuroM-eGFP construct at HH14 (Day 2.5) and harvested approximately 1-1.5 days later at HH23. **(A-C)** QH1 immunostaining shows that a premature blood vessel sprout ingressed into the medio-lateral region of the neural tube (C, arrowhead) and is beginning to anastomose with a ventral sprout. **(D)** Analysis of blood vessel ingression on the electroporated (green) and control sides (black) of NeuroM-electroporated neural tubes. More sprouts have formed on the NeuroM-electroporated side; however, vessel sprout distribution along the DV axis is not perturbed in these embryos (n=85 sections). **(E-H)** QH1 staining of vessel sprouts on control and electroporated sides of NeuroM-electroporated neural tubes depicts emerging sprouts on the control side of the neural tube that are either small (E) or consist of mainly filopodial extensions (G), while sprouts on the NeuroM-electroporated have ingressed farther (F), or are beginning to anastomose with ventrally ingressing vessels (H, arrow). **(I-K)** Tuj1 immunostaining shows increased thickness of differentiated neurons on the NeuroM-electroporated side of the neural tube when compared to the contralateral control side. **(L)** % Tuj1 area analysis shows a significant difference between NeuroM and contralateral control sides of the neural tube (n=25 electroporated sections).

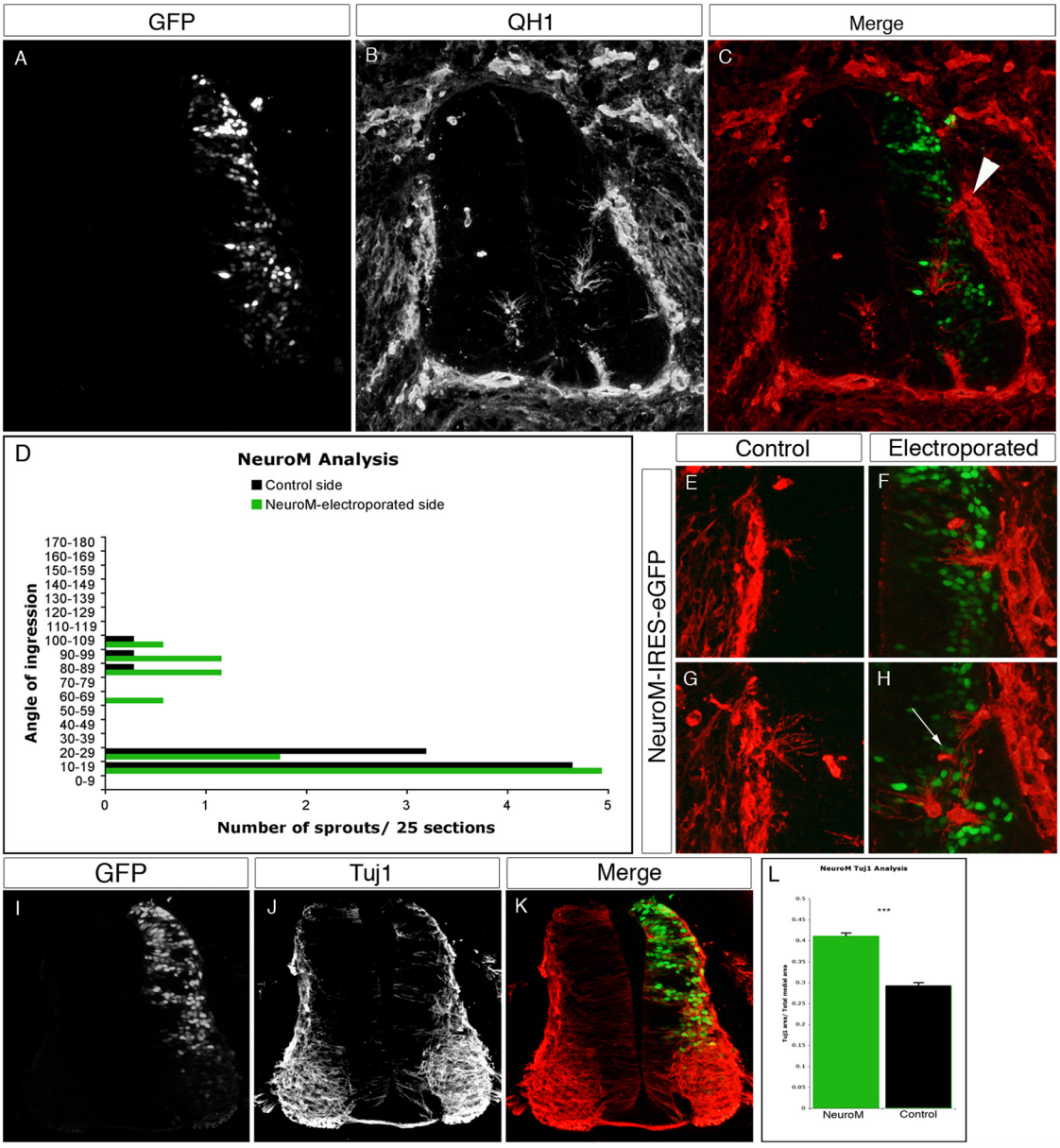

GFP
QH1
Merge
A
B
C
D
NeuroM Analysis
Control side
NeuroM-electroporated side
Angle of ingression
170-180
160-169
150-159
140-149
130-139
120-129
110-119
100-109
90-99
80-89
70-79
60-69
50-59
40-49
30-39
20-29
10-19
0-9
0
1
2
3
4
5
Number of sprouts/ 25 sections
Control
Electroporated
NeuroM-IRES-eGFP
E
F
G
H
GFP
Tuj1
Merge
I
J
K
L
NeuroM Tuj1 Analysis
***
NeuroM
Control

Figure 3.5: Altered temporal regulation of neurogenesis does not perturb VEGF expression in the neural tube.

In situ hybridization of Sox2 and NeuroM-electroporated quail neural tubes with an anti-sense probe to quail VEGF-A. **(A)** Sox2-electroporated neural tubes show equivalent VEGF-A reactivity on the Sox2-electroporated and contralateral control sides of the neural tube at HH25. **(B)** NeuroM-electroporated neural tubes display no differences in VEGF-A expression between the electroporated and control sides of the neural tube at HH23. Green bars indicate electroporated side of each neural tube section. Scale bar: 100μM.

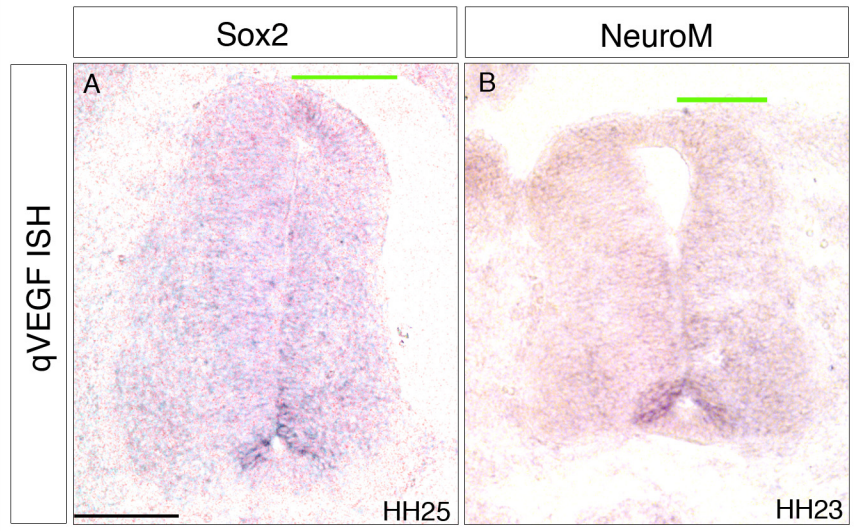

Sox2
NeuroM
qVEGF ISH
A
B
HH25
HH23

Figure 3.6: Early VEGF overexpression induces premature, ectopic blood vessel ingression into the neural tube.

Quail neural tubes were electroporated with the hVEGF165-eGFP construct at HH14 (Day 2.5) and harvested 24 hours later, between HH22-23. **(A-C)** QH1 immunostaining shows that a premature blood vessel sprout ingressed into the ventro-lateral region of the neural tube (C; arrow) in the vicinity of VEGF over-expression. **(D, E)** VEGF165 overexpression in the medio-lateral and dorsal neural tube did not result in premature blood vessel sprouts. Instead, vessels with large lumens sometimes formed outside of the neural tube, adjacent to regions of VEGF165 overexpression (E; arrowhead). **(F)** Vessel ingression analysis on VEGF-electroporated (green), or contralateral control (black) sides of the neural tube (n=45 sections). **(G, H)** Premature blood vessel sprouts ingress into the motor neuron domain in the ventral neural tube. Adjacent neural tube sections were stained with either QH1 (red) or MNR2 (yellow) to label blood vessels or motor neurons. These images were superimposed to show that blood vessel sprouts co-localize with motor neurons in the ventral neural tube (G and H; arrowheads). Green bars indicate the VEGF165-electroporated side of the neural tube. **(I)** Tuj1 immuno-staining shows that more differentiated neurons have formed in the ventral neural tube than in the medio-lateral or dorsal areas at HH22-23 (bracket). **(J, K)** At HH24, VEGF165 overexpression in the dorsal and medio-lateral neural tube initiates ectopic and supernumerary blood vessel sprouts (K; arrowhead).

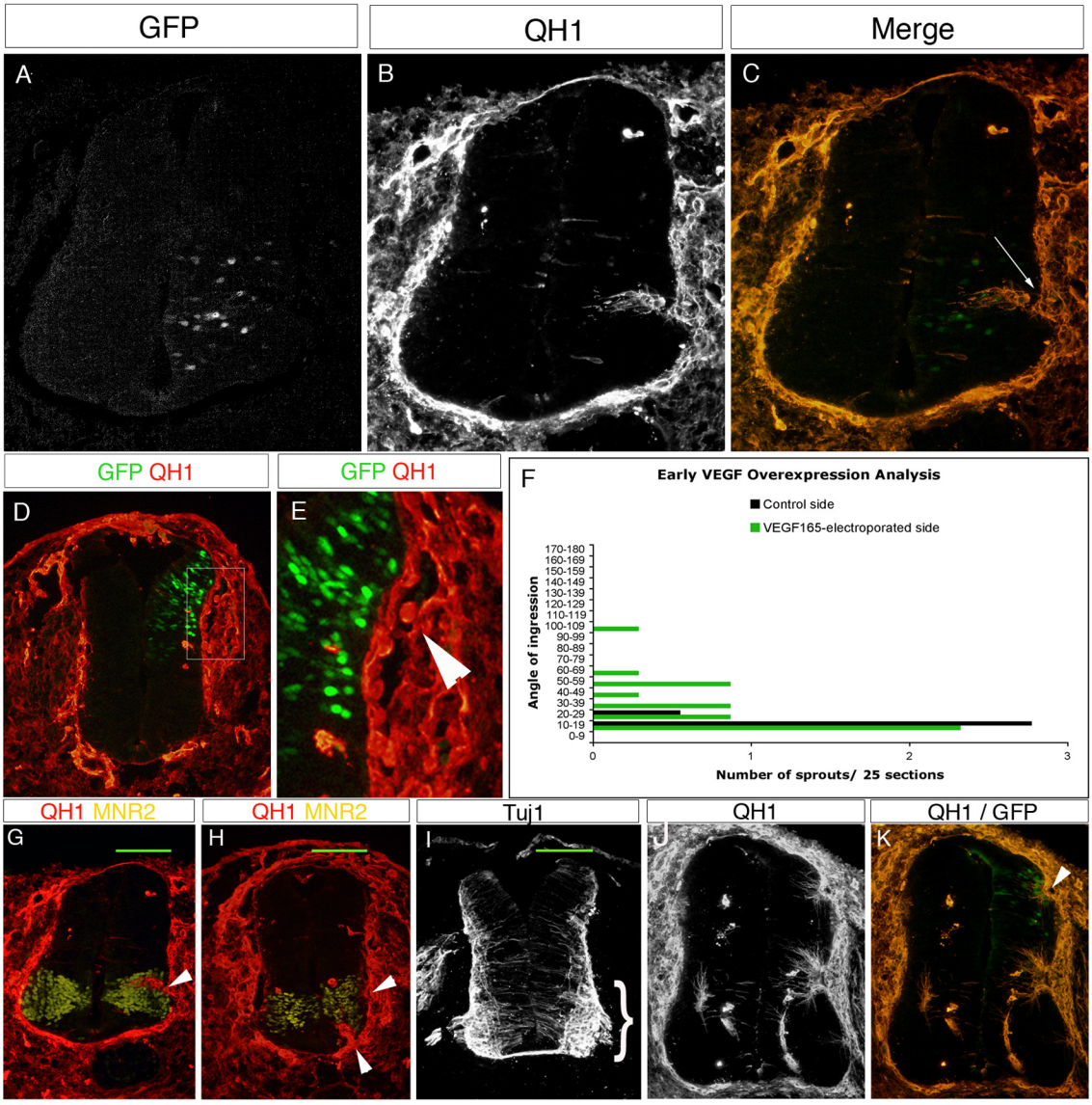

GFP
QH1
Merge
A
B
C
GFP QH1
GFP QH1
D
E
F
Early VEGF Overexpression Analysis
Control side
VEGF165-electroporated side
Angle of ingression
170-180
160-169
150-159
140-149
130-139
120-129
110-119
100-109
90-99
80-89
70-79
60-69
50-59
40-49
30-39
20-29
10-19
0-9
0
1
2
3
Number of sprouts/ 25 sections
QH1 MNR2
QH1 MNR2
Tuj1
QH1
QH1 / GFP
G
H
I
J
K

Figure 3.7: Blood vessels ingress into stereotypical regions of the neural tube.

(A-C) Vessel ingression relative to MNR2/HB9 expression at HH25. MNR2/HB9 (A) and QH1-immunostained (B) sections were superimposed to show that ingressing vessels (C; arrow) avoid the sMN domain (green) in the ventral neural tube. **(D-F)** Vessel ingression relative to Nkx2.2 expression at HH25. Nkx2.2 expression in the ventral neural tube marks the V3 interneuron domain (D). Ventral blood vessel sprouts (E) appear to ingress into this region in the QH1/Nkx2.2 overlay (F). Scale bar: 100μm. **(G)** Analysis of vessel ingression relative to DV neuronal patterning. Measurements (arrows) were taken at the lateral boundary of the floor plate (purple), the Nkx2.2 boundaries (blue), the ventral and dorsal boundary of the sMN domain (green) and at the ventral and dorsal boundary of the dorsal root entry zone (DREZ) (orange). The medial neural tube (yellow) is defined by the dorsal boundary of the sMN domain and the ventral boundary of the DREZ. The dorsal neural tube (red) is defined by the dorsal boundary of the DREZ and 180°. **(H)** Ingression analysis by neural tube region. 25 ventral and 25 medio-lateral sprouts were analyzed on HH25 neural tube sections, and each point was plotted on a graph relative to measurements taken on adjacent sections for each sprout. Black dots indicate vessel spouts. 100% of ventral sprouts analyzed ingressed ino the Nkx2.2, V3 interneuron domain, while 96% of medio-lateral sprouts ingressed into the medial neural tube.

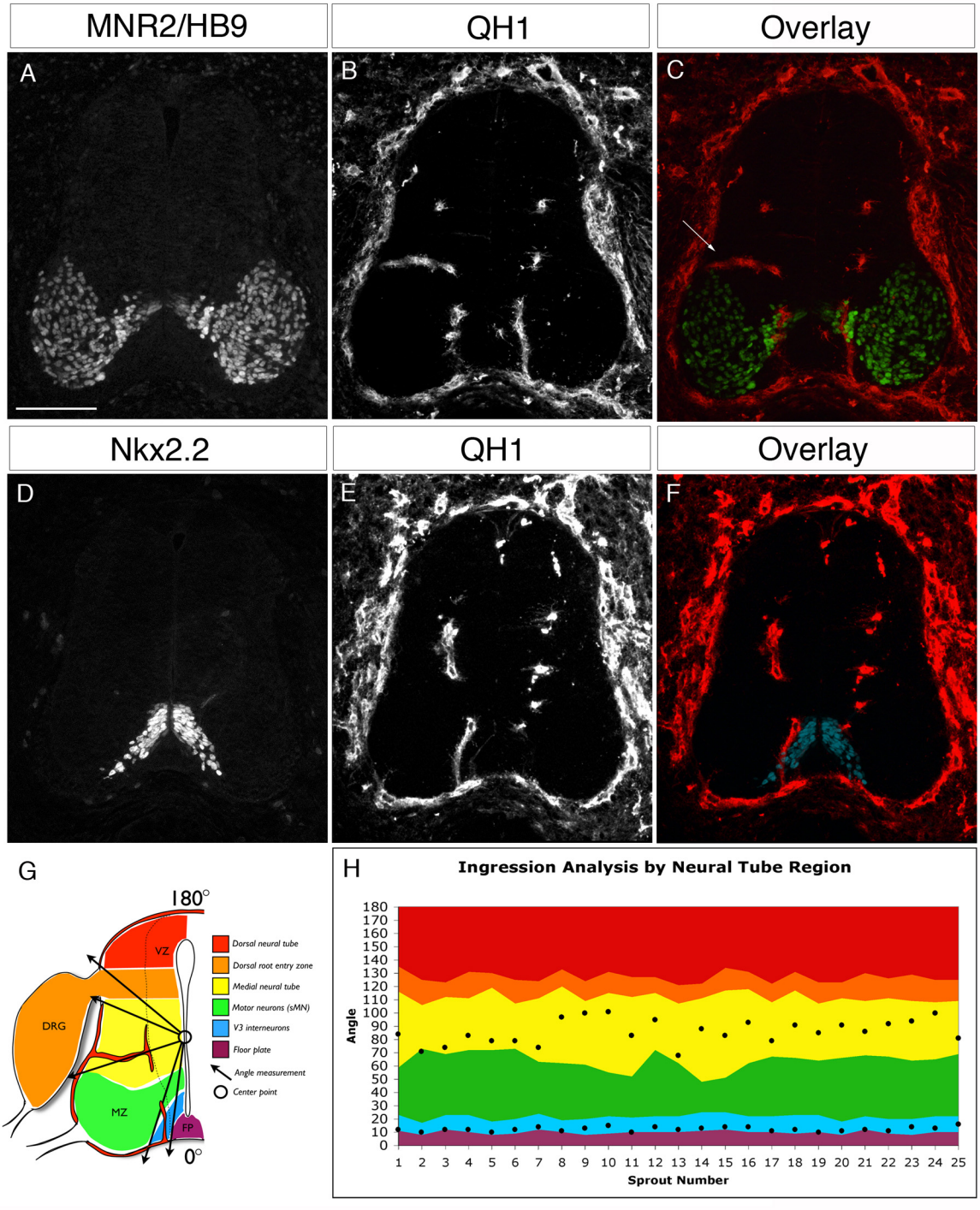
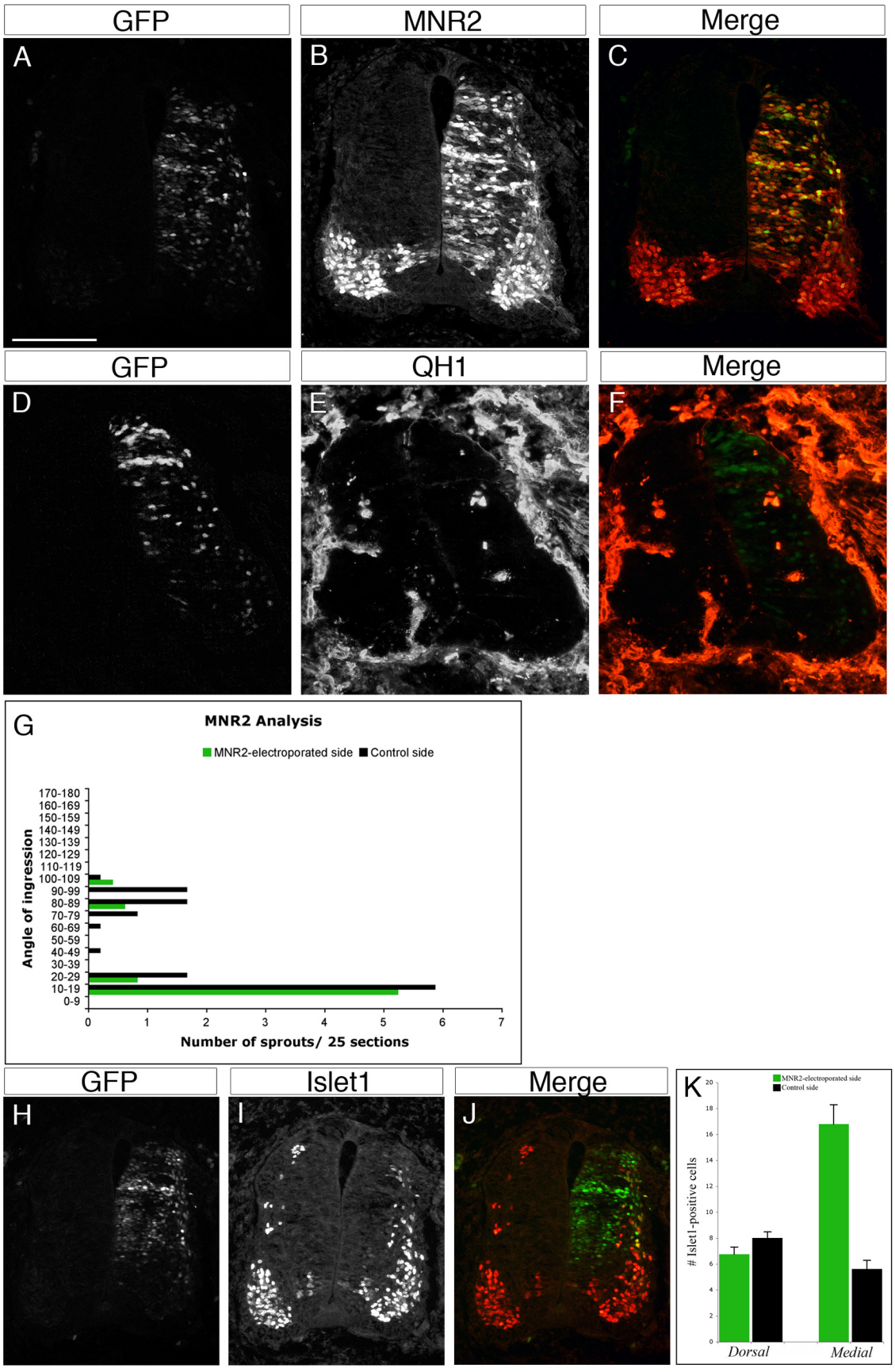

MNR2/HB9
QH1
Overlay
A
B
C
Nkx2.2
QH1
Overlay
D
E
F
G
180°
VZ
DRG
MZ
FP
0°
Dorsal neural tube
Dorsal root entry zone
Medial neural tube
Motor neurons (sMN)
V3 interneurons
Floor plate
Angle measurement
Center point
H
Ingression Analysis by Neural Tube Region
Angle
Sprout Number
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

Figure 3.8: Motor neurons negatively regulate neural tube angiogenesis.

Quail neural tubes were electroporated with an MNR2-IRES-eGFP construct at HH16-17 and were dissected at HH25. **(A-C)** MNR2 expression in electroporated neural tubes. **(D-F)** QH1 immunostaining on neural tube sections depicts blood vessel ingression on the control, but not MNR2-electroporated, side of the neural tube. **(G)** Blood vessel ingression analysis shows that blood vessel ingression is stereotypical on the control side of the neural tube (black) and drastically decreased on the MNR2-electroporated side (green) (n=119 sections). **(H-J)** Islet1 expression in MNR2-electroporated neural tubes shows that Islet1 cells are increased on the electroporated side of the neural tube. **(K)** Islet1 cell counts in the dorsal and medial neural tube shows that medial Islet1 cells are significantly higher on the MNR2-electroporated side (green) when compared to the contralateral control side (black) indicating that ectopic motor neurons form in MNR2-electroporated embryos. Dorsal Islet1 cell numbers do not drastically change (n=25 sections).


GFP
MNR2
Merge
A
B
C
GFP
QH1
Merge
D
E
F
G
MNR2 Analysis
MNR2-electroporated side
Control side
Angle of ingression
170-180
160-169
150-159
140-149
130-139
120-129
110-119
100-109
90-99
80-89
70-79
60-69
50-59
40-49
30-39
20-29
10-19
0-9
0
1
2
3
4
5
6
7
Number of sprouts/ 25 sections
GFP
Islet1
Merge
H
I
J
K
MNR2-electroporated side
Control side
Islet1-positive cells
0
2
4
6
8
10
12
14
16
18
20
Dorsal
Medial

CHAPTER IV

DIFFERENTIATED MOTOR NEURONS, BUT NOT MOTOR NEURON PROGENITORS, NEGATIVELY REGULATE ANGIOGENESIS.

A. INTRODUCTION

Blood vessels invade the developing avian neural tube in highly stereotypical locations along its DV axis (Feeney and Watterson, 1946; James et al., 2009; Kurz et al., 1996). Perhaps as striking as the regions where vessels invade the neural tube, are the places where they don't. We show in Chapter III that ingressing blood vessels initially avoid the region of the ventral neural tube where motor neurons differentiate. We also show that ectopically generated motor neurons block blood vessel ingression. Our work demonstrates that this neuronal sub-type is anti-angiogenic. We are interested in understanding *how* the motor neurons block blood vessel ingression, and at which stage of motor neuron development the block occurs. In order to do this, it is important to understand which genes regulate motor neuron development, and when they are expressed relative to the timing of blood vessel ingression.

The first step in motor neuron development occurs at Hamburger and Hamilton (HH) stage 9-10 (Hamburger and Hamilton, 1992), as opposing gradients of Sonic Hedgehog (Shh) from the notocord and floor plate and Transforming Growth Factor-ß (TGF-ß) and Bone Morphogenic Proteins (BMPs) from the roof plate of the neural tube are established along its dorsoventral (DV) axis (Fig. 4.1 A). Graded Shh signaling is critical for specifying five neuronal progenitor domains in the ventral neural tube—p3, pMN, p2, p1, and p0 (reviewed in (Briscoe and Ericson, 2001)) (Fig. 4.1 B). The pMN domain contains motor neuron progenitors (Fig. 4.1 B, asterisk) that differentiate to form the somatic motor neurons (sMNs). Additionally, FGF and Retinoic Acid-signaling

contribute to the specification of ventral neuronal progenitor domains (Diez del Corral et al., 2003; Diez del Corral and Storey, 2004).

Each of the five ventral progenitor domains is specified by a subset of homeodomain transcription factors, regulated by Shh signaling, that direct neuronal sub-type identity at the onset of neurogenesis (Briscoe and Ericson, 2001; Briscoe et al., 2000). There are three key homeodomain transcription factors expressed by cells in the pMN domain, regulating motor neuron specification in the chick neural tube: Pax6, Nkx6.1, and Nkx6.2 ((Ericson et al., 1997; Sander et al., 2000; Vallstedt et al., 2001). Nkx6.1 and Nkx6.2 (Nkx6) are upregulated by intermediate and high levels of Shh, while Pax6 is repressed by the highest levels of Shh in the ventral-most neural tube. Essential genes specifying motor neuron identity are depicted in Figure 4.1 C (reviewed by (Briscoe and Novitch, 2008)). As these events are taking place, blood vessels have not yet begun to form the PNVP.

Once the progenitor cells in the pMN domain have been regionally restricted by homeodomain transcription factors, they begin to coordinate their exit from the cell cycle by upregulating the basic Helix-Loop-Helix (bHLH) transcription factor Olig2 at HH10 (Novitch et al., 2001). Olig2 expression expands in the ventral neural tube until HH15, the developmental stage when motor neuron differentiation occurs (Ericson et al., 1992; Hollyday and Hamburger, 1977), and becomes downregulated in post-mitotic motor neurons. The first ECs begin to form the PNVP just prior to this stage, at HH14. Olig2 expression persists in motor neuron progenitor cells until HH25, when motor neuron generation is largely complete (Novitch et al., 2001). The unique role of Olig2 is to synchronize the acquisition of motor neuron sub-type identity by upregulating MNR2,

with cell cycle exit (i.e. motor neuron differentiation) by regulating Neurogenin2 (Ngn2) expression (Fig. 4.1 C). MNR2 is a homeodomain transcription factor expressed by motor neuron progenitors as they enter their last mitotic cycle, and its expression is sufficient to drive specific aspects of post-mitotic motor neuron differentiation; however this gene, like other homeodomain transcription factors, cannot induce motor neuron exit from the cell cycle (Tanabe et al., 1998). Ngn2 is a bHLH transcription factor that promotes neuronal differentiation at the expense of radial glial cell formation (Sun et al., 2001). Furthermore, bHLH proteins have been shown to induce upregulation of pan-neuronal markers such as B-tubIII and neurofilament (Lee et al., 1995). Coordinated expression of these transcription factors ensures that motor neuron progenitors differentiate into motor neurons at a specific time and in a certain place within the developing neural tube. As motor neurons begin to differentiate, they express another cascade of transcription factors, largely coordinated by MNR2 expression (Fig. 4.1 C, D). MNR2 becomes down-regulated in post-mitotic motor neurons which initiates an upregulation in Islet1 expression. Islet1, in part, drives the expression of Islet2 and HB9, transcription factors specifying motor neuron sub-type identity (Lee and Pfaff, 2003; Tanabe et al., 1998). Islet1 expression persists until later stages of development (Ericson et al., 1992). Blood vessels eventually do enter the sMN domain (Feeney and Watterson, 1946); however, they do so at HH27-HH28, *after* motor neuron differentiation is largely complete.

Establishing sub-type identity is only the first step in neuronal organization. Differentiated neurons project axons that must reach their correct targets in order for the central and peripheral nervous systems to function properly. The axonal growth cone is

an extremely tactile structure, sending out filopodia to interact with multiple signals in its environment. Signals are used to determine which way to migrate, based on a fine balance of positive and negative axonal guidance cues. Numerous studies show that molecules regulating axonal guidance and patterning also regulate blood vessel patterning, and the tip cell of a growing vessel acts much like an axonal growth cone. There are four main classes of guidance molecules that have roles in both axonal and blood vessel patterning: Semaphorins, Slits, Netrins, and Ephrins (reviewed by Carmeliet and Tessier-Lavigne, 2005). The Semaphorins and Slits are of particular interest to us because of their motor neuron-restricted patterns (Holmes and Niswander, 2001; Luo et al., 1995). A family of Semaphorin molecules, the Semaphorin III's (Sema3), negatively patterns axons when expressed outside of the neural tube, placing motor axons on the correct trajectory toward muscle targets, while Sema3-sigaling within the neural tube sets axon sensitivity to this peripheral Sema expression by regulating Nrp1 expression (Moret et al., 2007). Certain Sema3 family members also negatively regulate angiogenesis and EC migration (Gu et al., 2005; Serini et al., 2003; Torres-Vazquez et al., 2004). Studies suggest that Slit2-signaling through the Robo4 receptor on ECs, also negatively regulates EC migration (Jones et al., 2008; Park et al., 2003). Slit2 is highly expressed in the motor neurons, and presumably Robo4 is expressed on neural vessels; however, this has not been explored. These studies highlight possible mechanisms for the negative regulation of blood vessel ingression by the motor neurons demonstrated in Chapter III.

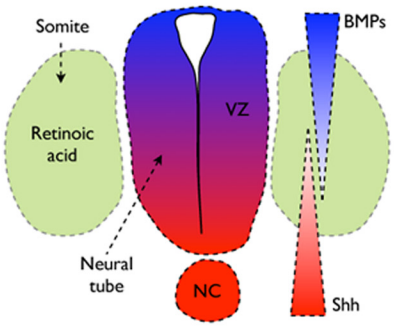
There is little known regarding the role of motor neuron (or motor neuron progenitor)-blood vessel interactions *within* the walls of the neural tube during early embryonic stages. We have shown that intraneural blood vessel patterning is influenced

by motor neuron development. Although blood vessels do not initially enter the sMN domain, the close association of blood vessels with motor neuron cell bodies is likely to be important for motor neuron survival. Studies of motor neuron development comprise the most complete story of how sub-populations of neural cells become specified in the early neural tube. Unlike most neuronal subclasses, transcription factors and signaling pathways required to make a motor neuron are now known. We have a unique opportunity to use this information to begin to answer questions about intraneural blood vessel patterning. Specifically, we want to understand at which stage of development motor neurons acquire/lose their anti-angiogenic properties. We also seek to identify negative signals that make this region anti-angiogenic. We begin to answer these questions in this chapter; this work is ongoing.

Figure 4.1: Motor neuron specification in the ventral neural tube.

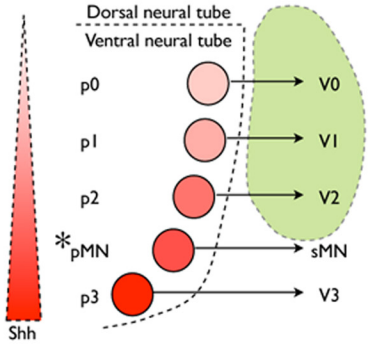
(A) Opposing gradients of Shh-signaling (red) from the floor plate, BMP-signaling (blue) from the roof plate, and Retinoic Acid-signaling from the somites (green) patterns the early neural tube before the onset of neurogenesis. This is the first step in motor neuron specification in the ventral neural tube. **(B)** Graded Shh from the floor plate is required for the specification of the five ventral progenitor domains (p3-p0), including the motor neuron progenitors in the pMN domain (asterisk). Motor neuron progenitors within the pMN domain differentiate and form the sMN domain where the somatic motor neurons reside. **(C)** Major genes in motor neuron specification. The pMN domain is first specified by combinatorial expression of three homeodomain transcription factors: Nkx6.1, Nkx6.2, and Pax6. These genes coordinate the expression of Olig2, which then upregulates MNR2 and Ngn2 to coordinate motor neuron identity and cell cycle exit (or differentiation) by HH15. Isl1, in part, coordinates the expression of Isl2 and HB9, while Ngn2 expression upregulates NeuroM expression in post-mitotic neurons. Asterisk: stage in motor neuron development where blood vessels enter the neural tube in stereotypical locations, avoiding the sMN domain. **(D)** Schematic representation of motor neuron specification in the ventral neural tube depicting when and where important genes are expressed. MNR2 labels progenior cells, while Isl1, Isl2, and HB9 label differentiated motor neurons. V2 neurons, but not motor neurons, express Lim3, and a dorsal population of interneurons (D2 interneurons) also expresses Isl1.

A



Adapted from Briscoe and Novitch
Phil. Trans. R. Soc. B (2008)

B



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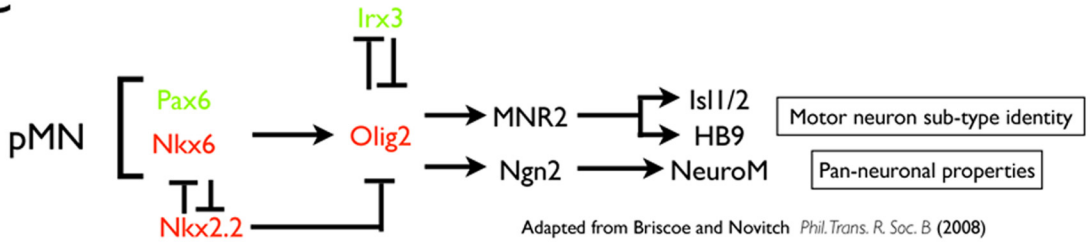
C



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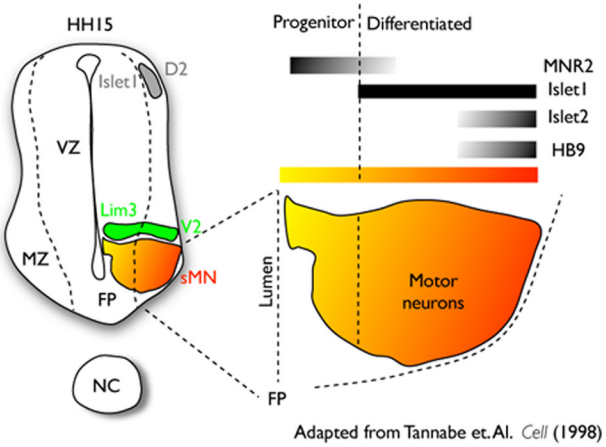
D



Adapted from Tannabe et. Al. *Cell* (1998)

B. MATERIALS AND METHODS

Expression constructs

The MNR2-IRES-eGFP construct was generated as previously described in Chapter III. The Sox2-ER-IRES-eGFP vector was a gift from L. Pevny (Graham et al., 2003). The S-Flt1 expression vector was generated by inserting full-length mouse s-Flt cDNA (Kappas et al., 2008) into the *Eco*R1 sites within the pCAGGS-IRES-Tomato (pCIT) vector (a gift from T. Maynard). Sema3a-IRES-eGFP and Sema3c-IRES-eGFP vectors were gifts of V. Castellani (Moret et al., 2007).

In ovo electroporation

We performed quail, neural tube electroporations as previously described (James et al., 2009), with the following minor modifications: The Sox2-ER and MNR2 expression vectors were injected into HH16-17 quail neural tubes at a final DNA concentration of 2µg/µl. Sema3a and Sema3c expression vectors were injected into HH16-17 quail neural tubes at a final DNA concentration of 1.5µg/µl. All embryos were incubated for an additional 2 days post-electroporation before embryo dissection at HH25.

Immunofluorescence

Antibody staining with QH1, Tuj1, and MNR2, Pax6, Pax7, and Islet1 was performed as previously described ((James et al., 2009) and Chapter III).

Analysis of ingression patterns/ neural tube measurements

Blood vessel ingression analyses, image overlays, and Islet1 cell counts were performed as described in Chapter III.

In situ hybridization

Quail, VEGF ISH was performed as previously described (James et al., 2009) on MNR2 over-expressing neural tube cryosections (courtesy of the UNC-ISH core facility and Yonquin Wu).

C. RESULTS

Motor neuron progenitors are pro-angiogenic

We show in Chapter III that blood vessels avoid the ventral neural tube where motor neurons develop (Fig. 3.7). Ectopic motor neurons generated in the medio-lateral neural tube via MNR2 electroporation were also anti-angiogenic (Fig. 3.8). This experiment was performed by "mis-expressing" MNR2 along the DV axis of the neural tube in order to achieve motor neuron *differentiation*—as evidenced by increased Islet1 expression in the medio-lateral neural tube (Fig. 3.8). We performed a related experiment where we electroporated a higher concentration of MNR2 (Fig. 4.2 A-C) into quail neural tubes in an attempt to generate *more* ectopic motor neurons; however, we did not achieve the desired result with this manipulation. MNR2 "over-expression" along the DV axis of the neural tube results in ectopic angiogenic sprouts that closely associate with MNR2-positive cells (Fig. 4.2 D-I). The blood vessel ingression analysis shows that ectopic sprouts ingress along the entire length of the DV axis of the neural tube in MNR2 over-expressing embryos (Fig. 4.2 F and I; arrowheads), while the contralateral control side of the neural tube maintained a stereotypical ingression pattern (Fig. 4.2 J).

These results contrasted the MNR2 mis-expression phenotype in chapter III, prompting us to examine whether or not highly MNR2-positive cells differentiate properly and migrate to the lateral edge of the neural tube as motor neurons. Islet1 immunostaining shows that high MNR2 expression does not result in ectopic motor neuron differentiation (Fig. 4.3 A-C) and that Islet1 cell populations are reduced on the electroporated side of the neural tube in both the medial and dorsal regions (Fig. 4.3 B;

arrows). Islet1 cell counts in the medial and dorsal neural tube confirmed these observations (Fig. 4.3 D). There was a drastic decrease in dorsal Islet1 cell populations on the electroporated side of the neural tube when compared to the contralateral control side; however, the medial population of Islet1-positive cells remained more stable, with only a slight decrease in cell number on the MNR2-electroporated side.

In addition to electroporating high concentrations of MNR2, we were able to induce MNR2 over-expressing cells via Sox2-ER electroporation. The Sox2-ER construct consists of the Sox2 gene fused to the Engrailed Repressor (ER) domain (Graham et al., 2003). Sox2 normally acts as a transcriptional activator; however, when fused to the ER domain, it acts as a transcriptional repressor—or, essentially, a dominant negative form of Sox2. We initially electroporated this gene to induce premature neuronal differentiation in quail neural tubes; however, Sox2-ER-expressing cells did not prematurely differentiate. Instead, Sox2-ER-expressing cells induced both a differentiation and DV patterning defect where clumps of motor neuron progenitor cells (highly MNR2-positive cells) formed all along the DV axis of the neural tube but never fully differentiated (Fig. 4.4). This perturbation resulted in ectopic blood vessel sprouts (Fig. 4.4 A-C and J). GFP-positive, Sox2-ER-expressing cells only co-localized with MNR2 (Fig. 4.4 D-F), and not other neural progenitor markers such as Pax6 or Pax7, no matter where these cells were found along the DV axis of the neural tube (Fig. 4.4 G and H). Furthermore, Sox2-ER-expressing cells did not co-localize with Tuj1, indicating that they do not terminally differentiate (Fig. 4.4 I). These experiments suggest that motor neuron progenitor cells are highly pro-angiogenic. These results may also explain why

ventral sprouts are able to pass through MNR2-postive cells as they migrate dorsally along the VZ in unperturbed embryos (Fig. 4.4 K; arrowhead).

VEGF-signaling from ectopic motor neuron progenitors induces ectopic angiogenic sprouts

Ectopic sprouting events occurring within MNR2-overexpressing neural tubes were reminiscent of matrix-binding VEGF perturbations, where localized matrix-binding VEGF expression induced blood vessel ingression into the dorsal neural tube (Fig. 2.3). We were interested in understanding if VEGF-signaling was mediating ectopic ingression events in MNR2 over-expressing neural tubes. To test this, we performed quail VEGF *in situ* hybridizations on tissue sections from MNR2-electroporated embryos displaying ectopic angiogenesis. We found that cells highly expressing GFP co-localized with regions of increased VEGF expression on adjacent neural tube sections (Fig. 4.5 A-C). Ectopic angiogenesis in MNR2 overexpressing embryos could be rescued by co-electroporation with s-Flt1 (Fig. 4.5 D-G). Ingression analysis of high MNR2/s-Flt1 co-electroporated embryos revealed reduced medio-lateral sprouting and a complete rescue of ectopic, dorsal sprouts on the electroporated side of the embryo when compared to the contralateral control side (Fig. 4.5 I) and neural tubes electroporated with high MNR2 alone (Fig. 4.5 H). These results show that ectopic, highly MNR2-positive cells induce angiogenesis by upregulating VEGF. Furthermore, these results, in combination with the results in the previous sections, demonstrate that motor neurons become anti-angiogenic as they differentiate—or they acquire anti-angiogenic properties over time.

Semaphorin III ligands negatively regulate neural tube angiogenesis.

Since MNR2-positive progenitor cells positively interact with blood vessels, perhaps via VEGF high VEGF expression, we now want to understand how *differentiated* motor neurons *negatively* regulate angiogenesis. One possibility is that differentiated motor neurons secrete anti-angiogenic signals that prevent blood vessel ingression. Three secreted Sema3 ligands: Sema3A, Sema3C, and Sema3E, are highly expressed in motor neurons (Cohen et al., 2005; Moret et al., 2007). These molecules have been shown to negatively regulate angiogenesis; however, their role in shaping the intraneural blood vessel pattern has not been characterized.

We electroporated Sema3A and Sema3C expression constructs into HH16-17 quail neural tubes and dissected the embryos at HH25. Ectopic Sema3A and Sema3C expression in neural tubes blocked angiogenesis (Fig. 4.6). QH1 immunostaining showed in Sema3A-electroporated sections showed that that blood vessel sprouts did not form in the medio-lateral region of the neural tube (Fig. 4.6 A-C), and regions of the PNVP were decimated (Fig. 4.6 C; arrowheads). The ingression analysis showed that Sema3A expression almost completely blocked medio-lateral sprouting (Fig. 4.6 D). QH1 immunostaining showed that ectopic Sema3C also negatively regulated vessel ingression; however, the results produced a less severe phenotype than the Sema3A perturbation. The PNVP was not interrupted in these embryos (Fig. 4.6 E-G). Blood vessel ingression analysis in Sema3C-electroporated embryos revealed a reduction in medio-lateral sprouts (Fig. 4.6 H); however, blood vessels did enter the neural tube more frequently where Sema3C was ectopically expressed than in regions where Sema3A was ectopically expressed (compare to Fig. 4.6 D). These results suggest that molecules expressed by the

motor neurons have the ability to negatively regulate neural tube angiogenesis when ectopically expressed in the medio-lateral neural tube. While not conclusive, these results suggest that Semaphorin signaling in the motor neuron region may contribute to the block to angiogenesis we see there. We have not yet looked at the effects of Sema3E on blood vessel ingression. We predict that ectopic Sema3E expression will also block medio-lateral blood vessel ingression, as it has recently been shown to have drastic, negative effects on angiogenesis when ectopically overexpressed outside of the neural tube (Gu et al., 2005).

D. DISCUSSION

The beauty of science is that mistakes can sometimes be more telling than a perfectly planned and executed experiment. This statement lends itself well to the *in ovo* electroporation technique. In an attempt to generate *more* ectopic motor neurons in the medio-lateral neural tube we found that motor neurons aren't always anti-angiogenic. We show in this chapter that neural cells highly expressing MNR2 not only fail to differentiate, but they upregulate VEGF and induce ectopic and supernumerary blood vessel sprouts. This is in stark contrast to what we show in Chapter III, where neural cells moderately expressing MNR2 upregulate Islet1 as they form ectopic motor neurons in the medio-lateral neural tube—conferring a block to angiogenesis there. Intriguingly, these data suggest that there is something about the motor neuron *differentiation* process that makes these cells anti-angiogenic, while motor neuron progenitors maintain pro-angiogenic properties.

When do motor neurons become anti-angiogenic?

Many of the steps in motor neuron differentiation are now known. We illustrate the major players needed to drive motor neuron development in the neural tube in Figure 4.1. Under normal physiological conditions, MNR2 is upregulated in motor neuron progenitors as they undergo their final mitotic cycle, and then MNR2 becomes rapidly downregulated at the onset of neurogenesis (Tanabe et al., 1998). Though not tested in the context of neural development, it is possible that constitutively high levels of MNR2 prohibit neuronal differentiation, maintaining a progenitor-like state. Only a down-

regulation in MNR2 expression (or perhaps moderate to low MNR2 expression in electroporated cells) triggers differentiation and upregulation of the Islet1 transcription factor. This seems to be the case for an upstream modulator of MNR2 expression as well, the homeodomain transcription factor Olig2. Constitutive overexpression of Olig2 also prevents motor neuron differentiation, whereas physiological Olig2 results in motor neuron generation (Briscoe and Novitch, 2008). These results may explain why MNR2 overexpressing cells do not differentiate, allowing us to determine that motor neuron progenitors are pro-angiogenic.

If motor neuron progenitors are pro-angiogenic, then why don't vessels normally enter this region of the ventral neural tube during early stages of neural tube development? We showed in Chapter III that differentiated neurons are important for regulating the timing of blood vessel ingression. Perhaps blood vessels would enter the motor neuron progenitor region if there were sufficient Tuj1-positive neurons present at very early stages. Motor neurons form in one major wave of differentiation (Novitch et al., 2001), meaning that there are many progenitors (and relatively few differentiated cells) until the time when they all differentiate. It is possible that blood vessels cannot enter the pMN domain due to insufficient Tuj1-positive neurons at the lateral edge of the neural tube, and they cannot enter the sMN domain upon neurogenesis because negative regulators of angiogenesis are expressed at that time.

Perhaps the block occurs at the first stage of motor neuron differentiation, when Islet1 is upregulated in differentiating motor neurons. We are interested in Islet1 due to the correlation of increased Islet1 expression in neural tubes mis-expressing MNR2 and the vessel ingression block in these embryos. Furthermore, Islet1 is expressed in the

dorsal root ganglia and in a group of dorsal interneurons, both regions are also initially devoid of angiogenic sprouts. It is possible that the stage of motor neuron development when Islet1 is expressed marks the time when motor neurons become anti-angiogenic.

Based on our experiments with MNR2, showing that VEGF expression is drastically increased in highly MNR2-positive cells, it is reasonable to assume that other transcription factors may regulate expression of signaling molecules (such as Sema3) that pattern blood vessels and axons. We do not know what regulates Sema3 expression in the neural tube; however, it is likely that factors promoting motor neuron specification also regulate Sema3 expression in these cells.

We have shown that ectopic Sema3 expression can block blood vessel ingression in the medial neural tube—a region normally permissive to angiogenic sprouts. This highlights a possible mechanism for how motor neurons block ingressing vessels. It is imperative to understand if ectopic motor neurons express Sema3's. We are currently trying to address this issue. This work is ongoing.

Figure 4.2: MNR2 over-expression results in ectopic blood vessel ingression.

Quail neural tubes were electroporated with an MNR2-IRES-eGFP construct at HH16-17 and were dissected at HH25. **(A-C)** MNR2 immunostaining in quail neural tube sections shows that MNR2 in electroporated cells is higher than endogenous MNR2 expression (B; arrow). **(D-I)** QH1 immunostaining on MNR2-electroporated neural tube sections depicts ectopic blood vessel ingression in both the dorsal (F; arrowhead) and ventral (I; arrowhead) regions of the neural tube. **(J)** Blood vessel ingression analysis shows that blood vessel patterning is stereotypical on the control side of the neural tube (black) and randomized on the MNR2-electroporated side (green) (n=70 sections).

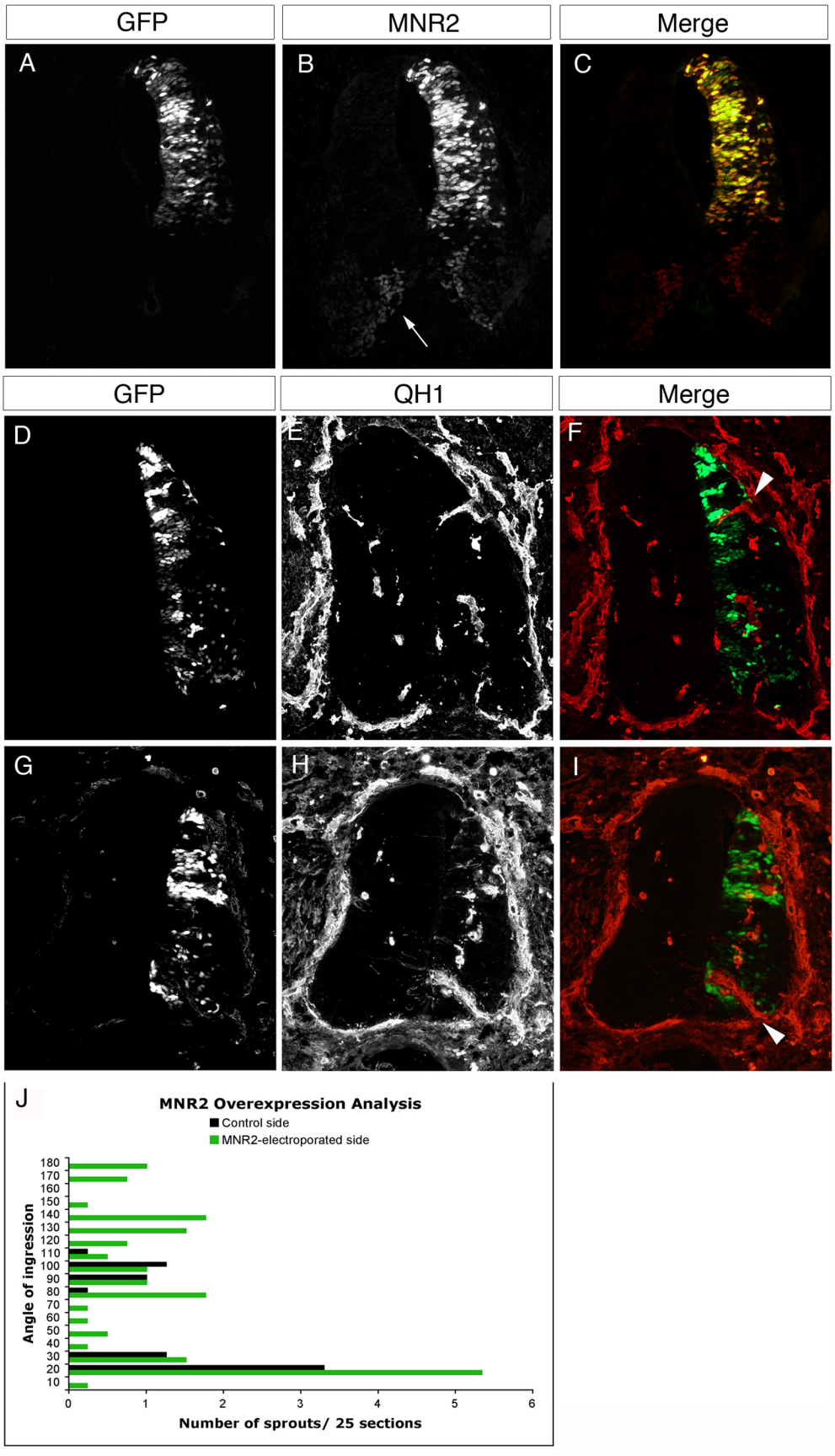

GFP
MNR2
Merge
A
B
C
GFP
QH1
Merge
D
E
F
G
H
I
J
MNR2 Overexpression Analysis
Control side
MNR2-electroporated side
Angle of ingression
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
Number of sprouts/ 25 sections

Figure 4.3: MNR2 over-expression prevents motor neuron differentiation.

Quail neural tubes were electroporated with an MNR2-IRES-eGFP construct at HH16-17 and were dissected at HH25. **(A-C)** Islet1 immunostaining on MNR2-electroporated neural tube sections. Dorsal and medial Islet1-positive cells are reduced in number when compared to the contralateral control populations (Fig. 4.3 B; arrows). **(D)** Quantification of Islet1-positive cells in the dorsal and medial neural tube on the control (black) and MNR2-electroporated (green) sides of MNR2-electroporated neural tube sections (n=23 sections).

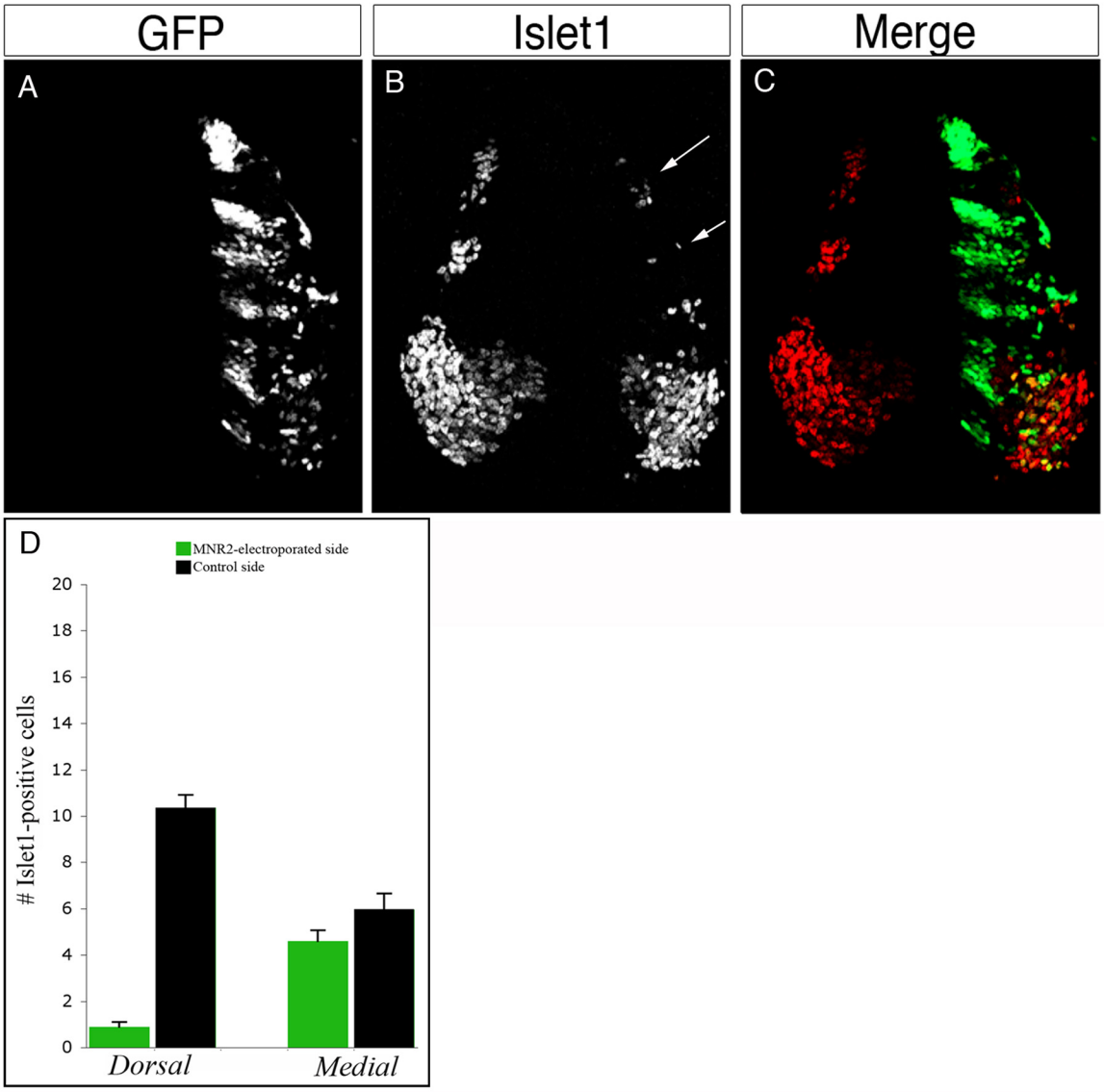

GFP
Islet1
Merge
A
B
C
D
MNR2-electroporated side
Control side
Islet1-positive cells
0
2
4
6
8
10
12
14
16
18
20
Dorsal
Medial

Figure 4.4: Sox2-ER electroporation induces ectopic blood vessel ingression.

Quail neural tubes were electroporated with a Sox2ER-IRES-eGFP construct at HH16-17 and were dissected at HH25. **(A-C)** QH1 immunostaining of Sox2ER-electroporated quail neural tube sections shows that ectopic and supernumerary blood vessel sprouts form in close proximity to GFP-positive cells. The vessel ingression pattern is analyzed in **J**. Blood vessel ingression is randomized on the Sox2ER-electroporated side of the neural tube (green) when compared to the contralateral control side (black) (n=28 sections). **(D-F)** MNR2 immunostaining shows that GFP-positive cells are also highly expressing MNR2, while Pax6 **(G)** and Pax7 **(H)** expression does not co-localize with GFP, even though Sox2ER-expressing cells are found along the entire DV axis of the electroporated neural tube. **(I)** Sox2ER-expressing cells do not co-localize with regions of Tuj1 expression. **(K)** MNR2/QH1 overlay of an adjacent, unperturbed HH25 neural tube section depicting the association of MNR2-positive motor neuron progenitors with a ventral sprout (arrowhead).

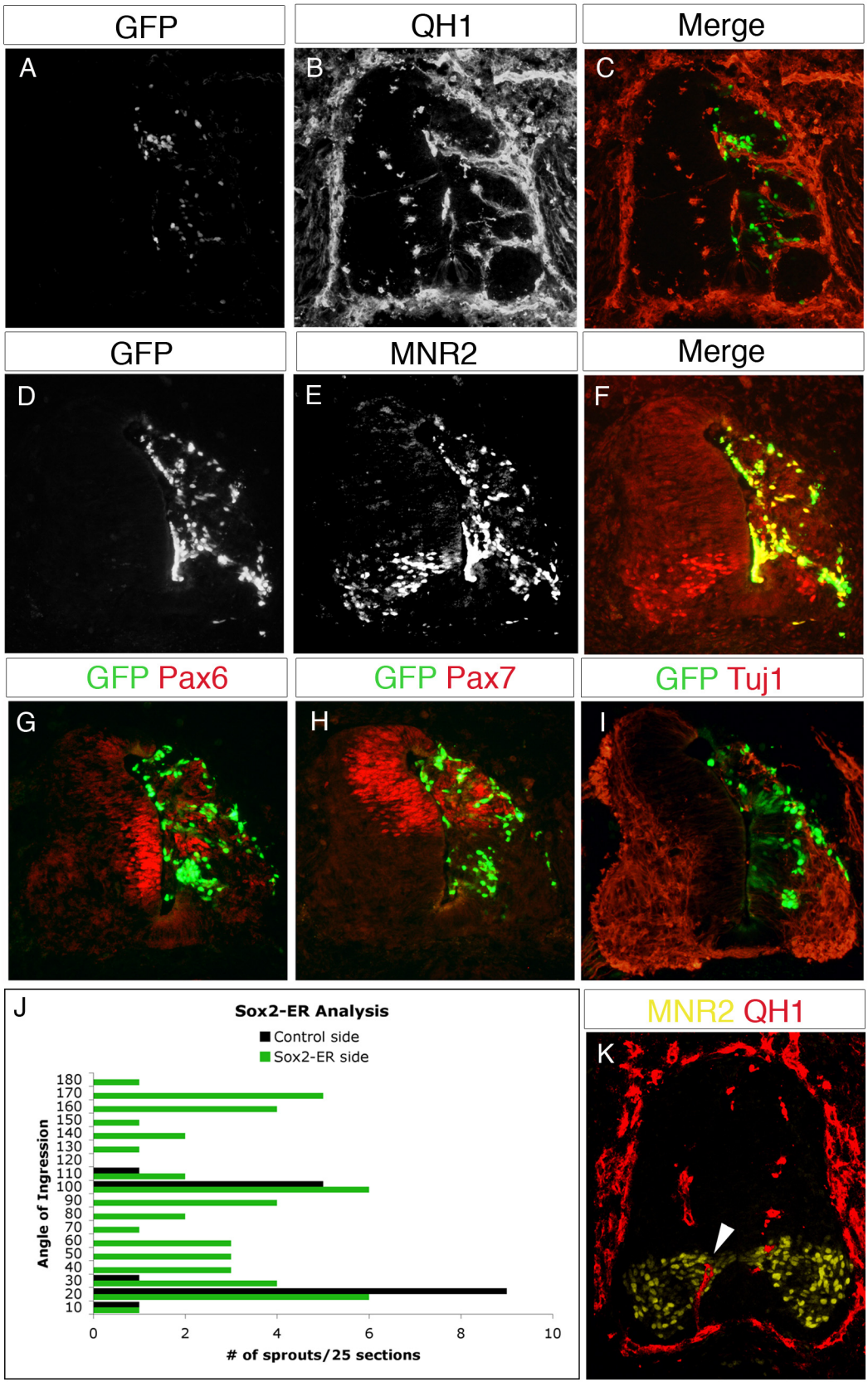

GFP
QH1
Merge
A
B
C
GFP
MNR2
Merge
D
E
F
GFP Pax6
GFP Pax7
GFP Tuj1
G
H
I
J
Sox2-ER Analysis
Control side
Sox2-ER side
Angle of Ingression
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
# of sprouts/25 sections
MNR2 QH1
K

Figure 4.5: MNR2-overexpressing cells induce ectopic ingression via VEGF upregulation.

Quail neural tubes were electroporated with an MNR2-IRES-eGFP construct or co-electroporated with MNR2-IRES-eGFP and s-Flt1-IRES-Tomato constructs at HH16-17, and were dissected at HH25. **(A-C)** *In situ* hybridization using a quail VEGF probe was performed on MNR2-electroporated quail neural tube sections (B), revealing increased VEGF signal in regions corresponding to the highest levels of GFP-expression on adjacent sections (C, *overlay*). **(D-I)** MNR2/s-Flt coexpression rescues ectopic vessel sprouting. (D) MNR2 (GFP) expression; (E) s-Flt1 (Tomato) expression; (F) QH1 expression in co-electroporated neural tubes. There are no ectopic sprouts in these sections (F and G; *overlay*). (H) Electroporation of high MNR2 alone induces ectopic blood vessel ingression (n=28 sections); however, co-electroporation of high VEGF and s-Flt1 (I) rescues these effects, and also reduces sprout number in the medio-lateral neural tube on the electroporated side (green) when compared to contralateral controls (black) (n=56 sections).

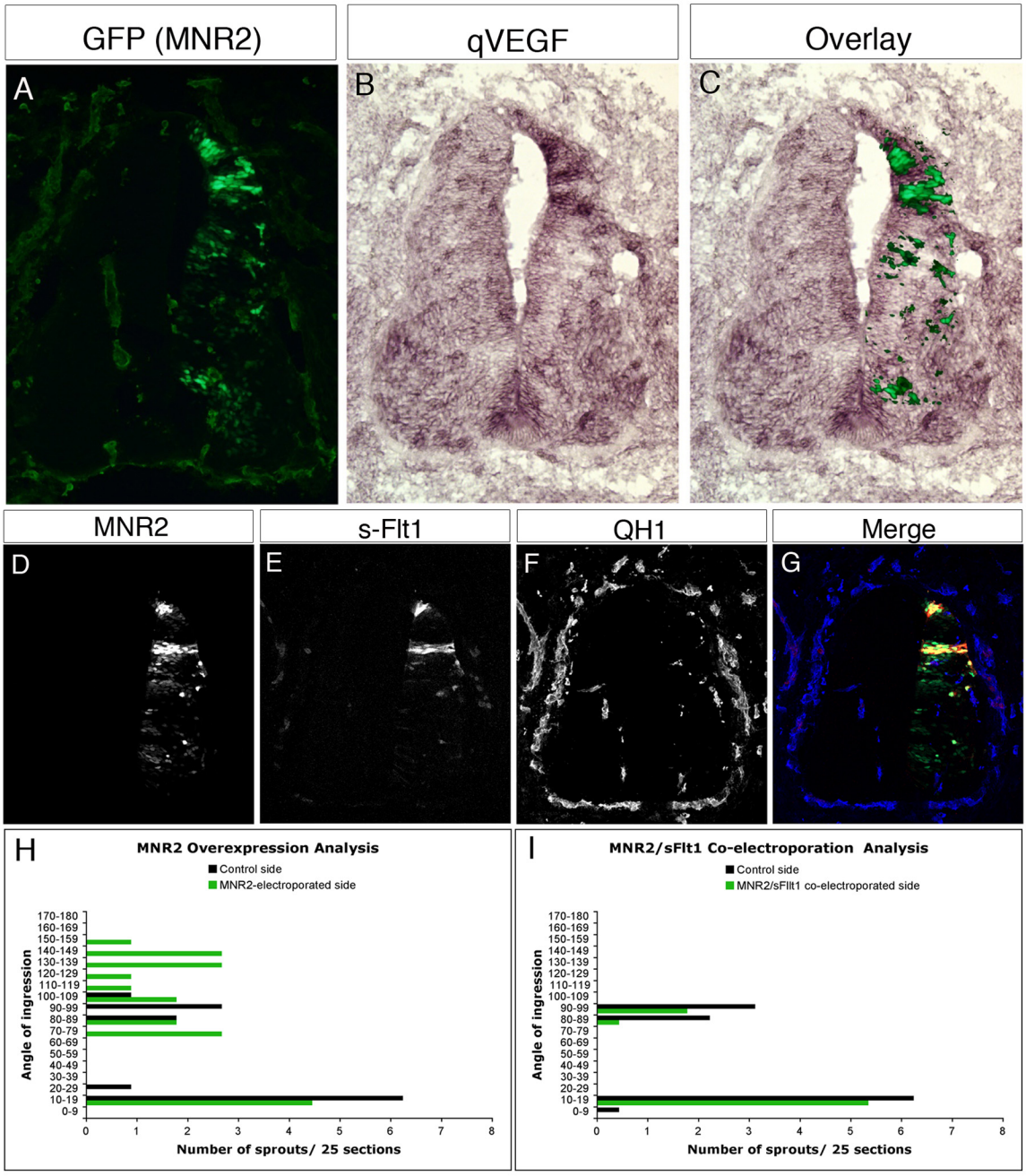
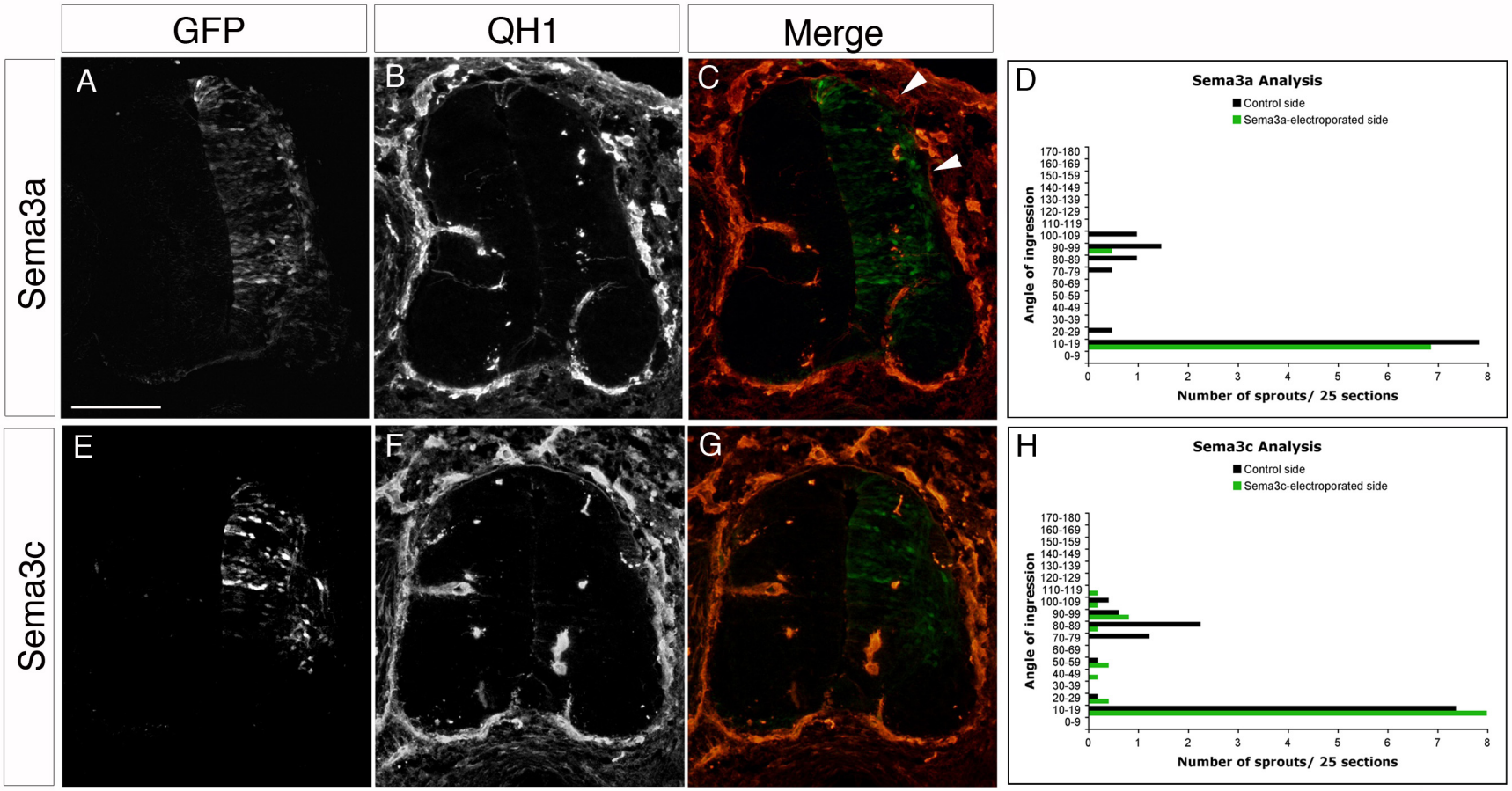

GFP (MNR2)
qVEGF
Overlay
A
B
C
MNR2
s-Flt1
QH1
Merge
D
E
F
G
H
MNR2 Overexpression Analysis
Control side
MNR2-electroporated side
Angle of ingression
170-180
160-169
150-159
140-149
130-139
120-129
110-119
100-109
90-99
80-89
70-79
60-69
50-59
40-49
30-39
20-29
10-19
0-9
0
1
2
3
4
5
6
7
8
Number of sprouts/ 25 sections
I
MNR2/sFlt1 Co-electroporation Analysis
Control side
MNR2/sFlt1 co-electroporated side
Angle of ingression
Number of sprouts/ 25 sections

Figure 4.6: Sema3 expression in the medio-lateral neural tube blocks blood vessel ingression.

Quail neural tubes were electroporated with either Sema3A-IRES-eGFP or Sema3C-IRES-eGFP constructs at HH16-17 and were dissected at HH25. **(A-C)** QH1 immunostaining of Sema3A-electroporated sections depicts normal ingression on the control side of the embryo, while no sprouts are apparent on the electroporated side. PNVP formation is perturbed in these embryos. Arrows indicate sections of the PNVP that are missing (C). **(D)** Blood vessel ingression analysis shows that sprouting is almost completely blocked on the Sema3A-electroporated side of the neural tube (green) while ingression is normal on the contralateral control side (black) (n=51 sections). **(E-G)** QH1 immunostaining on Sema3C-electroporated sections depicts normal ingression on the control side of the embryo while no sprouts are apparent on the electroporated side. PNVP formation is not perturbed in these embryos. **(D)** Blood vessel ingression analysis shows that sprouting is partially blocked on the Sema3C-electroporated side of the neural tube (green) while ingression is normal on the contralateral control side (black) (n=122 sections).


GFP
QH1
Merge
Sema3a
Sema3c
A
B
C
D
E
F
G
H
Sema3a Analysis
Control side
Sema3a-electroporated side
Angle of ingression
170-180
160-169
150-159
140-149
130-139
120-129
110-119
100-109
90-99
80-89
70-79
60-69
50-59
40-49
30-39
20-29
10-19
0-9
0
1
2
3
4
5
6
7
8
Number of sprouts/ 25 sections
Sema3c Analysis
Control side
Sema3c-electroporated side
Angle of ingression
Number of sprouts/ 25 sections

CHAPTER V

GENERAL DISCUSSION

The CNS requires more metabolic support than any other organ system in our bodies (Tracey and Metz, 2007). A constant supply of nutrients and oxygen are required for neuronal survival, and blood vessels are responsible for delivering this support. It is therefore imperative that the developing CNS establishes contact with the blood vessels early in development to ensure that neural cells receive adequate nourishment— and to allow for further CNS growth and development. In the present study, we are interested in understanding how a specific region of the CNS, the neural tube, establishes contact with the blood vessels, resulting in the formation of a reproducible vessel pattern.

Intriguingly, blood vessels entering the neural tube do so at a precise time and in highly stereotypical locations—suggesting that the neural tube orchestrates key blood vessel patterning events. Previous studies have shown that the neural tube produces signals that are required for the formation of the PNVP (Ambler et al., 2003; Hogan et al., 2004), a ring of vessels that surrounds the early neural tube; however, much less is known about how the neural tube directs the next step of CNS vascularization, as angiogenic sprouts from the PNVP invade the neuroepithelium. In this thesis, we present three novel findings that begin to address this important question: 1) Neural tube-derived VEGF is required for blood vessel ingression. 2) Programmed neurogenesis regulates the timing of blood vessel ingression, and 3) Specific subclasses of differentiated neurons play distinct roles in patterning blood vessels along the DV axis of the neural tube.

Neural tube-derived VEGF is required for blood vessel ingression.

In Chapter II, we show that regional depletion of VEGF in the neural tube locally blocks blood vessel ingression. This finding demonstrates not only that VEGF is absolutely necessary for blood vessel ingression, but also that *local* VEGF present at the ingression site is needed to induce sprout formation. Ectopic expression of matrix-binding and non-matrix-binding VEGF isoforms supports this model. Matrix-binding VEGF isofoms have the ability to induce ectopic sprouts while non-matrix-binding isoforms do not. By virtue of their ability to become localized, or stuck in the extracellular spaces, matrix-binding VEGF isoforms relay stable patterning information to the blood vessels and act as guideposts for ingressing sprouts. We also demonstrate that soluble VEGF cannot provide this information to PNVP vessels.

These results also show that the blood vessel pattern is not intrinsic to the vessels themselves. Instead, vessels respond to cues generated by the neural tube to guide them to appropriate ingression locations. This indicates that even though blood vessels are competent to enter specific regions of the neural tube (such as the dorsal region), they do not receive the appropriate signals to do so. Unfortunately, we are limited by our inability to visualize VEGF *protein* localized at the sites of blood vessel ingression, and instead have to rely on VEGF mRNA distribution to get a general sense of where VEGF protein is expressed in the neural tube. Quail VEGF *in situ* hybridization revealed a higher signal in the ventral neural tube within, and just lateral to, the floor plate. This expression maps to the ventral blood vessel ingression point—the earliest and most frequent vessel to invade the neural tube. This is strong correlative evidence that local deposits of VEGF determine where vessels ingress.

With the exception of the ventral-most region, VEGF mRNA is uniformly distributed throughout the rest of neural tube—lending little insight into why medio-lateral sprouts enter in such highly stereotypical locations. It is possible that VEGF protein is *not* uniformly distributed in the neural tube. Perhaps the medio-lateral neural tube traps VEGF in some way, concentrating it to promote blood vessel ingression in specific locations. If this is true, it may be interesting to drive expression of a quail VEGF siRNA construct in different regions of the neural tube to see if the effects mimic the s-Flt1 electroporation experiments. Our results indicate that s-Flt1 binds *local* VEGF, no matter where the VEGF originated. Regional VEGF mRNA knockdown would allow us to determine if VEGF required for medio-lateral or ventral sprouting is expressed by cells at those locations or if VEGF is upregulated in other locations and becomes localized to sites of ingression. This type of experiment is possible in the avian system by controlling the electroporated gene from murine enhancer elements, localizing the knockdown to a specifc region of the neural tube (Timmer et al., 2001).

Levels of VEGF are also important for proper blood vessel patterning in the CNS (Haigh et al., 2003) and may influence the timing of vessel ingression. Low levels of VEGF are expressed in the early neural tube and are sufficient to induce PNVP formation (Aitkenhead et al., 1998; Miquerol et al., 2000); however, it is entirely possible that not *enough* VEGF is produced in the medio-lateral neural tube to induce ingression there, until a later developmental time point. In Chapter III we overexpressed VEGF *earlier* in development to see if we could induce pre-mature ingression. We found that high VEGF could induce pre-mature ingression, but only when the VEGF overexpression occurred in regions of the neural tube where differentiated neurons were also present. This

experiment suggests that VEGF levels are not the only regulator of blood vessel ingression, and that the timing of neurogenesis and the formation of differentiated neurons may also play a role in regulating the timing of angiogenic sprouting into the neural tube. These results are discussed further in the next section.

Programmed neurogenesis regulates the timing of blood vessel ingression.

Even though VEGF is expressed early in neural tube development and is sufficient to direct PNVP formation by HH14 (Day 2), medio-lateral sprouts do not enter the neural tube until much later (Day 4-4.5). We observed that vessel sprouts were only seen in the neural tube *after* differentiated neurons migrated to the pial side of the neural tube, prompting us to explore whether or not the timing of neurogenesis played a role in the timing of medio-lateral sprout ingression. In Chapter III we demonstrate that angiogenic sprouting is blocked by neural progenitor cells in the VZ and positively regulated by differentiated motor neurons (and perhaps radial glia) in the marginal zone. These results demonstrate that blood vessels are competent to invade the neuroepithelium *earlier* than they actually do, providing evidence that the neural tube actively blocks blood vessel ingression until an appropriate developmental stage—or that the timing of neurogenesis does influence the timing of blood vessel ingression.

Preliminarily, we show that the block to ingression conferred by progenitor cells cannot be overcome by increased VEGF at early stages. Early VEGF overexpression induced premature blood vessel ingression, but *only* in regions of the neural tube where differentiated neurons were present, such as the sMN domain in the ventral neural tube.

This suggested that *both* VEGF and differentiated neurons must be present in order for ingression to occur.

How do blood vessels interact with differentiated neurons and radial glial cells in comparison to neuroepithelial cells? Extremely limited information is available for blood vessel interaction with *spinal* neurons and radial glial cells in embryos. Most information comes from studies of the brain. In the brain, radial glial cells form as neurogenesis commences, and they act as scaffolds for neuronal migration to outer cortical layers (Rakic, 1972). The brain is structured differently than the spinal cord. As neurogenesis proceeds in the brain, new neurons migrate past older neuronal layers toward the outer surface of the cortex, forming an "inside out" configuration. Radial glial cells are an integral part of this process, maintaining contacts with the pial surface of the brain throughout neurogenesis. Blood vessels in the brain have been shown to migrate along radial glial processes (Virgintino et al., 1998). Brain and hindbrain vessels are radially oriented, branching laterally when they interface the VZ (Gerhardt et al., 2004). The role of radial glial cell-blood vessel interactions in the spinal cord is less clear. Unlike the brain, radial glial precursors are not seen in the mouse spinal cord until E11, *after* blood vessel ingression has occurred. Spinal cord progenitor cells remain neuroepithelial-like throughout neurogenesis, and progenitor cells only take on radial glial properties after neurogenesis is largely complete (reviewed in, (Gotz and Huttner, 2005)). This finding, in combination with our results, suggests that blood vessels are positively patterned by *neurons* and negatively patterned by *neuroepithelial* cells in the spinal cord at early stages; however, transitin staining in the quail neural tube indicates that radial glial

processes do exist at early stages. Further studies are needed to assess the role of these cell types in neural tube angiogenesis.

While the early neural tube blocks *angiogenesis*, it does not prevent *migration* of angioblasts and ECs. This highlights the dualistic properties of the early avian neural tube: *pro*-migratory vs *anti*-angiogenic. Interestingly, the early neural tube does not block angioblast and EC *immigration* into the neural tube (Kurz et al., 1996). How, and why, do single angioblasts invade the neuroepithelium while vessel sprouts initially do not enter? While VEGF plays a major role in both the formation of the PNVP and sprouting angiogenesis, it is unclear if VEGF also induces angioblast immigration or if other signals within the neural tube cause this effect. Angioblast immigration does not occur in mouse, instead the neural tube is vascularized solely via angiogenic sprouting from PNVP vessels (Bar, 1980); Risau, 1993). In any case, it is intriguing that the neural tube can produce signals to attract ECs and angioblasts, then keep these cells out (with the exception of the few that squeeze in), and then produce signals to let sprouts in. Perhaps the neural tube should be named *Simon*, as in Simon says, "migrate," Simon says, "stop," and Simon says, "go."

Specific subclasses of differentiated neurons play distinct roles in patterning blood vessels along the DV axis of the neural tube.

If blood vessels are positively patterned by neurons, then why don't they "go" into all regions of the neural tube where differentiated neurons are found? In Chapter II, we proposed a model where positive signals produced in the neural tube were counteracted by negative signals in certain regions—blocking angiogenesis in those

places. To test this model, we first mapped blood vessel ingression points to regions where particular neuronal subclasses are located along the DV axis of the neural tube. We found that 98% of medio-lateral and ventral vessels analyzed do not ingress into the sMN domain—the region of the ventral neural tube where motor neurons develop. To test whether or not these cells actively block angiogenesis, we generated ectopic motor neurons in the medio-lateral neural tube. Ectopic motor neurons also block angiogenesis. This work demonstrated, for the first time, that the process of neuronal cell fate specification along the DV axis of the neural tube played a role in intraneural blood vessel patterning.

What do the motor neurons express that make them anti-angiogenic? Recent studies suggest that signals, such as Sema3, patterning motor neuron axons may also negatively pattern the vessels (reviewed by, (Carmeliet and Tessier-Lavigne, 2005)) (V. Castellani, personal communication). We demonstrate that ectopic Sema3 expression is sufficient to block medio-lateral blood vessel ingression; however, is endogenous Sema3 responsible for making motor neurons anti-angiogenic? This is a complex question. There are three Sema3 family members expressed in the sMN domain that have been independently shown to have anti-angiogenic effects on vessels (Gu et al., 2005). Perhaps the combinatorial effects of all three Sema3's convey a strong anti-angiogenic message. No studies have been conducted to specifically address this possibility, and we provide the first data that ectopic Sema3 expression can block neural tube angiogenesis. We are currently trying to determine whether or not ectopic motor neurons express Semaphorins, as well as determine if knockdown of Sema3's in the sMN domain will remove the block to vessel ingression. There will be caveats to these experiments:

Sema3s induce motor axon fasciculation (or bundling) and promote axon egression from the neural tube—knockdown of Sema3s have been shown to affect these aspects of neural patterning in the avian system (Moret et al., 2007). The inability to segregate neuronal and vascular defects may complicate results.

All four of the major axonal guidance pathways (Semas, Netrins, Ephrins, and Slits) have distinct intraneural expression patterns, ensuring axons within the neural tube make appropriate connections; however, virtually no studies have been conducted to show how these genes are regulated. We know that VEGF is regulated (at least in part) by hypoxia; however, upregulation of VEGF by motor neuron progenitors suggests that other mechanisms may regulate VEGF expression. Some VEGF may be induced via transcription factors directing neuronal cell fate specification (high MNR2). Are transcription factors driving motor neuron differentiation also regulating Sema3 expression in the neural tube? It may take years of research just to answer this one question.

Conclusions.

Taken together, the work in this thesis demonstrates that neurovascular communication is complex. Blood vessels, like axons, receive multiple patterning signals that they must simultaneously interpret in order to make the appropriate connections with target tissues. In the neural tube, blood vessels are patterned by signals secreted by neural cells that are utilizing the same signals to pattern themselves, yet the vessel pattern is not a by-product of neural development. Our central nervous system exists because blood vessels acquired the ability to respond to neural patterning signals through

evolution. These changes allowed vessels to coordinate with the CNS—allowing the CNS to expand because it now had a means of metabolic support.

Here we show that normal processes of neural development are linked to the intraneural blood vessel pattern. Manipulations in the timing of neurogenesis result in temporal blood vessel ingression defects. Perturbations in DV neuronal patterning predictably perturb vessel ingression patterns. We also provide novel insight into the role of VEGF in neurovascular communication.

The work in this thesis demonstrates that the quail neural tube is a beautiful model system in which to study neurovascular interactions. Extensive studies of neural development have been performed in chick, while studies of vascular development have been performed in quail; however, this is the first time that the two have been combined *within* the quail neural tube.

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