THE ROLE OF FGF SIGNALING IN NEURAL CREST DEVELOPMENT

by

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A dissertation submitted in partial fulfillment of the requirements for the degree

of

Doctor of Philosophy

in

Neuroscience

MONTANA STATE UNIVERSITY
Bozeman, Montana

July 2014
DEDICATION

This dissertation is dedicated to my parents, Ronald and Kayl Arthun. They gave me the greatest opportunities and encouraged me to pursue my education.
ACKNOWLEDGEMENTS

First and foremost I would like to thank my advisor, Professor Frances Lefcort. She is the most enthusiastic scientist I have ever met and had the privilege to study under. Dr. Lefcort allowed me the freedom to pursue independent scientific thought with the flexibility to also be a mother. I would not have finished this dissertation without her continued support.

I would like to thank my committee members, Professors: Valerie Copie, Lynn George, Roger Bradley, and Michael Babcock. Thank you for your time and scientific input. I am especially indebted to Professor Roger Bradley for discussions about the cellular mechanisms of my project. Also, I am thankful for the funding and support of the MBS Program and the direction from Stephanie Cunningham.

I could not have pursued this dream without the members of the Lefcort Lab. I consider it an honor to have worked with Lynn George, for her expertise in live imaging and data analysis; she was really a second advisor to me. Martha Chaverra, the sunshine of the lab, helped me in critical dissections, troubleshooting experiments, and life’s wisdom. Finally, Sarah Ohlen, for her refreshing friendship and the shared experience of being a graduate student.

To conclude I would like to thank my family. I would not have kept my sanity without my loving husband James. I am incredibly appreciative of my sister Kirsten for reading my dissertation and supporting me throughout the years. I owe my deepest gratitude to my mother who selflessly and lovingly cared for my sons Wyatt and Waylon while I was away exploring research.
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ABSTRACT

The mechanisms that stimulate Neural Crest Cell (NCC) migration and cessation into discrete sympathetic ganglia (SG) and dorsal root ganglia (DRG) are incompletely understood. In this study we investigated the role of Endothelial Cells (ECs) and the shared growth factors of the nervous and vascular system: fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) in the development of the peripheral nervous system. We hypothesized that ECs impact neural crest patterning. Using live time-lapse microscopy, we found that NCCs and ECs interact extensively during neural crest migration and DRG development, including stimulating proliferation of Pax3+ neural progenitors. These studies also revealed that ECs of the intersomitic vessel perhaps act as a substrate for migrating NCCs while the perisomitic vessel acted as a boundary keeping NCCs in the forming DRG. In order to determine the role of ECs in PNS development we focally eliminated them with FGF receptor (FGFR) blocker Su5402. Su5402 focally reduced ECs and caused NCCs to remain dorsal. Given that FGFs effects could be direct or indirect on NCC, we sought to reduce FGF signaling solely in NCC. To this end we overexpressed a dominant negative FGFR1 (DNFGFR1) and FGFR3 in ovo in NCCs. Our studies demonstrated that while blocking FGFR3 signaling did not impair NCC migration, NCCs expressing the DNFGFR1 behaved similarly as did the Su4502 treated NCCs: they stayed dorsally at the expense of the SG and DRG and exhibited distinct morphologies. Stimulating FGFR signaling with the ligand FGF8 promoted migration in vitro and increased ventral migration of NCCs in vivo. To determine if the alterations in DNFGFR1+ NCCs was due to changes in cell adhesion molecule function, we transfected NCC with DNFGFR1 and gain and loss of function N-cadherin constructs. Surprisingly the fate of DNFGFR1+ NCCs was rescued by increasing expression of N-cadherin, while double transfections of DNFGFR1 and DN-N-cadherin further impaired migration. While it is possible that N-cadherin and FGFR1 directly interact, the exact interactions of FGFR1 and N-cadherin need to be further investigated. FGFR3 was not found to function in NCC migration, but preliminary findings show it is required for TrkA+ neural differentiation.
INTRODUCTION

Development of the nervous and vascular systems have been studied as separate entities for decades; however, neither of these systems arises independently. Each is formed by the migration and path-finding of dynamic leading cells that traverse a diverse environment interacting with multiple cell types and extracellular molecules. In this study we sought to understand the development of these systems in relation to one another as well as shared signaling molecules and receptors between the two.

Why is this research important? The term neurovascular refers to the simultaneous development and crosstalk of neurons and blood vessels. These systems are dependent on each other as well as many other cell types. Neurons require blood vessels to bring oxygen and nutrients, while blood vessels require neurons to vasoconstrict and dilate, controlling blood flow. It is important to research the development of theses systems and their interactions with one another because of the implications for neurovascular diseases. Neurovascular diseases include Alzheimers, Amyloid Lateral Sclerosis (ALS), stroke, trauma, and others. In order to treat these diseases, the role of both systems needs to be addressed.

The two cell types that were focused on in this research were endothelial cells (ECs) and neural crest cells (NCCs). Both cells types extend dynamic filopodia extensions—cellular protrusions filled with cytoskeletal actin; that can respond to environmental cues. Tip cells are navigating ECs of the vascular system. They respond to hypoxic cues from their surrounding environment during development.
and invade those oxygen deficient regions forming new blood vessels. Similar to the
dynamic migrating endothelial cells; the peripheral nervous system is formed from
highly motile and pluripotent NCCs (LeDourian and Kalcheim, 1999). NCCs
emigrate from the dorsal neural tube, migrate, and differentiate into an assortment
of derivatives that range from neurons of the autonomic nervous system to
melanocytes—pigment cells in the skin.

NCCs and ECs share common signaling molecules. The two molecules that
were focused on in this research were Vascular Endothelial Growth Factor (VEGF)
and Fibroblast Growth Factor (FGF). Previously, each molecule has been shown to
be instrumental to the development of the nervous system and vasculature. The
specific roles of these growth factors and their influence on NCCs behavior was
previously unknown and a major focus of this study.
NEURAL CREST

Mechanisms of Neural Crest Migration

NCCs are a multipotent group of cells that reside and emerge from the dorsal portion of the neural tube (García-Castro & Bronner-Fraser, 1999). These cells, like the rest of the nervous system are derived from the developing ectoderm (García-Castro & Bronner-Fraser, 1999). NCCs undergo an epithelial-to-mesenchymal transition (EMT) during development and migrate out of the neural tube ((Akitaya & Bronner-Fraser, 1992; Nakagawa & Takeichi, 1998)). Essentially they transform from a stationary adhered phenotype into motile cells. The first NCCs migrate in a wave ventrally through the sclerotome of the somite while the later emigrating NCCs migrate dorsolaterally, dorsal to the dermomyotome into the epidermis and differentiate into melanocytes (Servedzija, Fraser, & Bronner-Fraser, 1990) (Bronner-Fraser, 1985; Bronner-Fraser & Cohen, 1980; Fraser & Bronner-Fraser, 1991) (Schwarz, Maden, Davidson, & Ruhrberg, 2009). A subset of ventral migrating NCCs migrate as far as the dorsal aorta and differentiate into the sympathetic neurons and glia of the autonomic nervous system (Bronner-Fraser & Fraser, 1989; Servedzija et al., 1990). Other ventral migrating neural crest cells stop adjacent to the neural tube to form the neurons and glia of the dorsal root ganglia (DRG) (Loring & Erickson, 1987).

During migration NCCs are guided along distinct paths in response to both repulsive and attractive cues. NCCs are repelled from the posterior somite resulting
in a reiterative pattern of a chain of discreet chain of DRG in the anterior somite (Bronner-Fraser, 1985; Rickmann, Fawcett, & Keynes, 1985; Servedzija et al., 1990). These repulsive cues sculpt the migratory pattern of NCCs. NCCs express Eph receptors while the posterior somite cells express ephrin ligands (Krull et al., 1997; Wang & Anderson, 1997). Specifically Ephrin B1 is expressed in the caudal half with complementary expression of EphB3 receptor on the migrating NCCs. EphB3-Ephrin B1 interactions result in filopodial collapse, thus repelling NCCs from the posterior somite. Strong experiment evidence for a repulsive effect of EphrinB1 on NCCs came from culturing ECs in vitro on dishes that had been coated with stripes of EphrinB1: NCCs migrated in regions without the Ephrin-b1 ligand in defined stripes (Krull et al., 1997). Additional repulsive molecules expressed in the posterior somite include molecules known as semaphorins. Sema3A, also called collapsin, expressed in the posterior somite, specifically binds the NCC receptor Neuropilin2 (Nrp2) and inhibits migration (Gammill, 2006). In an experiment performed in mice, null mutations in Sema3A or Nrp2 resulted in NCCs migrating in a continuous sheet and a loss of segmental distribution of NCCs (Gammill, 2006).

Repulsive molecules also prevent premature migration into the epidermis. Ephrin B ligands expressed dorsolaterally inhibit the early migrating NCCs from traversing into the epidermis, which occurs almost 24 hours later (Erickson, et al 1992) (Santiago & Erickson, 2002). When Ephrin B in the dorsolateral pathway was inhibited using blocking antibodies, early migrating NCCs migrated erroneously into the epidermis. However, Eph/EphrinB interactions of later migrating NCCs that are
destined to become melanocytes, actually promote migration into the epidermis via activation of the Cdc42/Rac GTPases (Santiago & Erickson, 2002). Thus Eph-Ephrin interactions have dual purposes; they can promote migration or repel depending on the specific receptor-ligand interactions and circumstances. Another repulsive molecule that resides in the dermomyotome preventing early NCC migration is Slit. NCCs express Slit receptors Robo-1 and Robo-2. The function of Slit/Robo was determined by using a dominant negative Robo-1, which resulted in increased numbers of early NCCs migrating dorsolaterally (Jia, Cheng, & Raper, 2005).

In addition to negative repulsive signals, NCCs also respond to permissive factors. In the anterior, permissive, region of the somite a chemotactic molecule known as SDF-1 is expressed by cells surrounding the dorsal aorta—the stopping point for SG formation. A subset of NCCs express CXCR4 that bind to SDF-1 and promotes migration (Kasemeier-Kulesa, McLennan, Romine, Kulesa, & Lefcort, 2010; Saito, Takase, Murai, & Takahashi, 2012) (Rehimi et al., 2007). Thus, NCC destined for the SG chemotax towards the target they will eventually regulate. They will innervate smooth muscle cells leading to vasoconstriction and vasodilation, regulating autonomic function.

Other permissive factors are found in the extracellular matrix (ECM). NCCs have to traverse a diverse ECM that is composed of fibronectin, laminin, proteoglycans, and collagen (Hay, 1989). NCCs are able to bind and respond to the ECM via integrin receptors. There are a plethora of integrin receptors that have been found on NCCs (Delannet et al., 1994; Sanes, 1989). Specific integrin receptors
that influence migration include alpha v Beta 3, which binds fibronectin, vitronectin, and laminin (Delannet et al., 1994; Desban & Duband, 1997; Testaz, Delannet, & Duband, 1999). Previous research using blocking antibodies to the integrin receptor, alph4, impaired NCC migration. The number of NCCs that emigrated from the NT was reduced as well as the overall distance that NCCs migrated (Kil, Krull, Cann, Clegg, & Bronner-Fraser, 1998). Other experiments blocking integrin receptors have not been as successful at elucidating their specific function because of the redundancy of these receptors.

Cells respond to growth factors and other ECM molecules in their environment by transducing signals intracellularly. These intracellular signals facilitate migration by actively changing cytoskeletal components. Polarized, migrating cells extend broad actin-rich lamellipodia and filopodia from their leading cellular edge. The lamellipodia and filopodia membranes are filled with receptors that respond to environmental cues and transduce those extracellular signals into distinct cytoplasmic responses. These signals can promote the binding of actin monomers, or polymerization, in a specific direction, or result in collapse of actin—steering a cell away from a region. An experiment blocking actin with cytochalasin resulted in migration impairment of NCCs in vitro (Haendel, Bollinger, & Baas, 1996) (Lefcort, O'Conner, & Kulesa, 2007).

Not only do NCCs interact with their environment, but they also communicate with one another. NCCs in a stream differ morphologically depending upon their position as leader or trailing cells. The leading cells extend multiple
filopodia with increased length, while the trailing NCCs are more polarized, contacting neighboring cells with thin filopodial extensions (Wynn, Rupp, Trainor, Schnell, & Kulesa, 2013). These distinct phenotypes of leader cells vs. follower cells result from the different environment these cells are exposed to. Leading cells have to “feel” their way through the ECM while the trailing cells simply get to “follow”. These cellular extensions not only allow NCC and other cells to sense their environment, but they also can interact with other cells. A recent experiment demonstrated the communication of cellular position by observing NCCs exchanging small blebs of cytoplasmic material (McKinney, Stark, Teddy, & Kulesa, 2011). These connections between cells are critical for directed migration. Time-lapse imaging of cells that lose contact within their migratory stream demonstrated that these cells do not navigate to their targets (Kasemeier-Kulesa, Kulesa, & Lefcort, 2005). NCCs can also communicate and connect through Cell Adhesion Molecules (CAMs).

**Cell Adhesion Molecules that Influence Neural Crest**

CAMs homotypically bind each other through their extracellular domain. These molecules form bridges and connections between neighboring cells and the ECM (reviewed in (Wheelock & Johnson, 2003). Binding of CAMs also results in intracellular changes. Specifically CAMs known as cadherins bind to alph and beta catenins intracellularly and can directly alter the actin cytoskeleton of the cell. The quantity of CAMs expressed and the type of CAM determine the degree of binding
and can influence/affect cell migration, binding, and tissue sorting (Reviewed in (Jessell, 1988; Pla et al., 2001)). Tighter binding usually occurs in type 1 Cadherins like E-cadherin resulting in less movement, “anchoring” cells to each other. Alternatively loose or transient CAM binding, such as Cadherin-7 may function more in collective cell migration (Hansen, Berezin, & Bock, 2008; Pla et al., 2001).

CAMs play a role in cancer biology, in particular in cancer metastasis. CAMs have implication in cancer metastasis. When epithelial cancer cells switch from expressing E-cadherin to N-cadherin they become more mesenchymal or motile (Reviewed in (Christofori, 2003; Kerosuo & Bronner-Fraser, 2012; Wheelock, Shintani, Maeda, Fukumoto, & Johnson, 2008)). This allows the cancerous cells to invade new territories or metastasize. Thus, the type of CAM expressed regulates adhesion or motility.

Because CAMs promote adhesion they are important in tissue sorting. Before NCCs emigrate from the chick neural tube, they express Neural Cell Adhesion Molecule (NCAM), N-cadherin, and Cadherin6B (Thiery, 1992) (Nakagawa & Takeichi, 1998). Interestingly a metalloprotease (MMP) called ADAM10 cleaves the intracellular domain of N-cadherin. This cleavage results in a free cytoplasmic tail (CTF2) that increases the cytoplasmic concentration of b-catenin, which is transported to the nucleus and regulates gene expression. When ADAM10 is blocked, NCCs fail to emigrate from the neural tube. This phenotype was rescued by adding CTF2. Therefore, ADAM10 is required to cleave N-cadherin and promote EMT of NCCs from the NT(Reiss et al., 2005). Upon the down-regulation of these
CAMs NCCs become more migratory and leave the neural tube. Migratory NCCs express Cadherin 7 and some researchers argue (Nakagawa & Takeichi, 1995) Cadherin 11 and N-cadherin as well (Coles, Taneyhill, & Bronner-Fraser, 2007), (Bronner-Fraser, Wolf, & Murray, 1992; Monier-Gavelle & Duband, 1995). N-cadherin is shown to be expressed on migrating NCCs in vitro (Monier-Gavelle & Duband, 1995) but not on early trunk migrating NCCs in vivo (Bronner-Fraser et al., 1992). Recent research using immuno staining and RT-PCR have shown cadherin 11 expressed in early migrating chick trunk NCCs (Chalpe, Prasad, Henke, & Paulson, 2010). However, cadherin-11 is strongly expressed in the mesenchyme surrounding NCCs so it is difficult to differentiate the NCCs from their surrounding tissue. Moreover, Cadherin 11 is definitively expressed by Xenopus migrating NCCs (Vallins, 1998).

Later in their migration, NCCs up-regulate expression of N-cadherin and NCAM, which allows them to adhere to one another and sort into discrete SG and DRG (Bronner-Fraser et al., 1992; Kasemeier-Kulesa, Bradley, Pasquale, Lefcort, & Kulesa, 2006). Experiments increasing the expression of N-cadherin resulted in more compact SG while blocking N-cadherin had the opposite effect (Kasemeier-Kulesa et al., 2006). Recently protocadherins have been found to be important in DRG localization. Specifically chicken protocadherin-1 (cpdcdh-1) functions to localize and keep NCC in the DRG. Overexpressing cpdcdh-1 resulted in an abundance of NCC in the DRG at the expense of the SG. Alternatively, decreasing the
expression resulted in more NCC migrating to the SG (Bononi, Cole, Tewson, Schumacher, & Bradley, 2008).

**Differentiation of the Neural Crest**

Cranial NCCs have the ability to differentiate into neurons, glia, chondrocytes (formation of bone and cartilage of the face), smooth muscle cells, and melanocytes. Trunk NCCs have the ability to differentiate into all of these derivatives except chondrocytes (Abzhanov, 2003). Upon cessation of migration, NCCs differentiate into these various derivatives. In the DRG NCCs differentiate into Schwann cells (support cells), mechanoreceptors, proprioceptors, nociceptors, and thermoreceptors. A subset of NCCs that migrate ventrally become neurons and support cells of the SG. Interestingly, by time-lapse imaging, it was shown that formation of discrete SG is a two step process in which migration to the dorsal aorta is mediated by distinct cues that then induce the segregation of a stream of NCCs into condensed SG—with the later dependent on EphrinB2 and N-cadherin (Kasemeier-Kulesa et al., 2006) ((Kasemeier-Kulesa, Lefcort, & Kulesa, 2007). The later migrating cells that migrate dorsolaterally express Wnt-1 and Wnt-3 and differentiate into melanocytes—pigment cells in the skin (Dorsky, Moon, & Raible, 1998).

In the DRG each NCC differentiates and the derived neurons specifically binds neurotrophic factors that are critical for survival (Ernsberger & Rohrer, 1988). Developing neurons compete for these limited survival factors that are
produced by their targets. Without these neurotrophic factors cells undergo programmed cell death. This intriguing developmental phenomenon regulates the quantity of neurons (Nikoletopoulou et al., 2010). Researchers found that the amount of neurons correlated to the target area of innervation. Studies that experimentally reduced the amount of target tissue by ablating limbs resulted in fewer neurons while the opposite experiments in which target tissue was ectopically increased led to an increase in naturally occurring neurons (reviewed in (Barde, 1989)). There is an overabundance of neurons made during development and only those that are appropriately exposed to neurotrophins survive. Early experiments blocking neurotrophin NGF resulted in fewer nociceptors and thermoreceptors with no difference in mechanoreceptors or proprioceptors. Later NGF was found to bind trkA receptors and promote their survival (review in (Snider, 1994)). Similar to NGF other family members were later discovered. Mechanoreceptors express trkB and bind BDNF (brain derived neurotrophic factor). Finally proprioceptors express trkC and bind neurotrophin-3 (NT-3) (Lefcort, Clary, Rusoff, & Reichardt, 1996). In the DRG, neurogenesis occurs in two distinct waves. During the first wave trkC and trkB, large diameter neurons are born. This wave is followed by a second wave during which the majority of small diameter, trkA neurons are born (Rifkin, Todd, Anderson, & Lefcort, 2000) (Barde, 1989). Progenitor cells for the first wave and second wave of neurogenesis can be distinguished by the transcription factors they express. The first wave progenitors express the basic helix-loop-helix transcription factor neurogenin 2 (Ngn2), while
the later wave expresses Ngn1 (Ma, Fode, Guillemot, & Anderson, 1999). A unique subset of migrating NCCs traverse contralateral and are destined for the perimeter and dorsal pole of the DRG. These progenitor cells become progenitor cells of the DRG and differentiate into TrkA neurons later in development (George, Kasemeier-Kulesa, Nelson, Koyano-Nakagawa, & Lefcort, 2010) (George, Chaverra, Todd, Lansford, & Lefcort, 2007).

**Theories about Neural Crest Fate**

There are opposing theories about how NCC fate is established. Some researchers favor the theory that NCCs are a heterogenous population of cells with intrinsic or predetermined fates (N. M. Le Douarin & Dupin, 2003; Lefcort & George, 2007a). In this theory the NCC fate potential is limited and partially determined prior to emigration from the neural tube. Alternatively, others favor the theory that NCCs are a homogenous pluripotent population of cells that respond to extrinsic or environmental cues (Harris & Erickson, 2007; Lefcort & George, 2007b). As part of this cell theory, cell-cell contacts influence NCC fate. Each cell’s fate is determined by environment and surrounding cells (Ulmer et al., 2013). There is experimental support for each claim. For instance, early experiments in chick using fluorescent dextran lineage tracing of NCCs showed that the progeny of a single cell could develop into sensory neurons, melanocytes, and glia. Further evidence is the expression of enzymes to synthesize acetylcholine and norepinephrine by thoracic and trunk NCC. Thoracic neural crest that were placed into the neck were able to
develop into cholinergic neurons (N. M. Le Douarin, Renaud, Teillet, & Le Douarin, 1975). Alternatively there are multiple examples for the heterogeneous theory. As described, a discrete subset of migrating NCCs expresses CXCR4 receptors and those cells migrate toward SDF-1 and become SG (Kasemeier-Kulesa et al., 2010). Other NCCs expressing Wnt-1 and Wnt-3 are committed toward a dorsolateral pathway and become melanocytes (Reedy & Erickson, 2006). Recent research has focused on the different genes expressed in leading migrating cells vs their trailing partners. These cells express distinct genes depending on their position within the stream. To test this theory researchers removed the leading cells and found that the trailing cells started expressing genes expressed by leading cells and phenotypically began resembling the leading cells. Specifically the leading NCCs express more matrix metalloproteases compared to the trailing NCCs allowing them to degrade the ECM that lies in their path (McKinney et al., 2011; Wynn et al., 2013; Wynn, Kulesa, & Schnell, 2012). However, this experiment could be interpreted with either theory. NCCs are multipotent and have the ability to change into a “leader” cell phenotype, or alternatively the different exposure to environmental ECM causes a change in cell morphology. NCC fate determination is most likely controlled by both intrinsic and extrinsic factors.
ENDOTHELIAL CELLS

Angiogenesis and Vasculogenesis

The formation of new blood vessels involves both the migration of endothelial cells and their formation of multicellular hollow tubes and results from two distinct processes. Vasculogenesis is a term used to describe the formation of new blood vessels from the differentiation of mesoderm cells into ECs. Alternatively, the budding off of ECs from existing blood vessels and migrating to other regions is referred to as angiogenesis (reviewed in (Eichmann, Le Noble, Autiero, & Carmeliet, 2005)). Both processes occur throughout development while angiogenesis continues post development during wound healing and tumor vascularization. The expression of the receptor tyrosine kinase, Vascular Endothelial Growth Factor 2 (VEGF2) has been found to promote angioblast differentiation and is required for angiogenesis and vasculogenesis (Nimmagadda, Loganathan, Wilting, Christ, & Huang, 2004; Yamaguchi, Dumont, Conlon, Breitman, & Rossant, 1996). Both of these processes rely extensively on EC pathfinding through a diverse ECM communicating with one another and with other cell types.

Migration of Endothelial Cells

Similar to NCCs, ECs are able to migrate and respond to their environment via actin rich filopodial extensions of navigating tip cells. These tip cells are similar to the leading cells of migratory NCC chains or waves. Following the tip cells are
endothelial cells that form the lumen of each vessel, referred to as stalk cells 
(reviewed in (Ruhrberg & Bautch, 2013)). ECs coordinate proliferation and 3-D juxtapositioning to form vessels within the ECM (Auerbach, Lewis, Shinners, Kubai, 
& Akhtar, 2003). Tip cells and stalk cells communicate while forming new blood vessels through VEGF signaling. Tip cells express VEGFR2, Neuropilin-1 (NRP-1), and Neuropilin-2 (NRP-2) that can bind and respond to extracellular VEGF (Bates et al., 2003; Eichmann et al., 2005) (Fantin et al., 2013). High levels of VEGF are expressed in regions of hypoxia, directing ECs towards the area to support vessel formation. Overexpressing VEGF in tissue results in aberrant angiogenic sprouting toward the source (Ruhrberg & Bautch, 2013). However, studies have shown that ECs still form distinct and regularly patterned intersomitic vessels in the absence of certain hypoxic cues, supporting the postulate that ECs are wired to form certain vessels without environmental cues (Eichmann et al., 2005).

Analogous to NCCs, ECs are also repulsed by Sema3A activating through NRP-1 receptors (Bates et al., 2003; Eichmann et al., 2005). Placing Sema3A soaked beads in the limbs of developing chicks resulted in decreased vasculature in the region and a decrease in neural innervation (Bates et al., 2003). An additional repulsive molecule netrin-1, regulates EC migration through activation of the Unc5 receptor. Unc5 null mice display abnormal extensive vessel branching and networks (Lu et al., 2004).

Ectopic expression of VEGFR2 (Flk) in mesodermal stem cells can induce them to differentiate into ECs, pericytes, or vascular smooth muscle. The expression
of VEGF directs these cells towards an EC fate, alternatively platelet derived growth factor (PDGF) steers these cells toward a pericyte fate (Yamashita et al., 2000). When blood vessels are forming it is important for arteries and veins to be separate. This separation is mediated by differential expression of Ephrin B1 on arteries, while veins express EphB4, the same family of repulsive molecules that NCCs use (Adams & Klein, 2000; Santiago & Erickson, 2002)(Wang, 1998). Mouse models that are mutant for Ephrin B2 or EphB4 show that these molecules are important for migration of ECs branching during angiogenesis, regulation of vessel size, and distinctions between arteries and veins (Gerety, Wang, Chen, & Anderson, 1999) (Y. H. Kim et al., 2008). Understanding the molecular mechanisms that mediate angiogenesis is essential because of the implications in cancer and stroke. Tumors that have developed their own blood supply can grow more rapidly and spread to other regions of the body readily. Therefore it is important to understand angiogenesis from a developmental perspective because of the implications for cancer research. Identifying factors that decrease angiogenesis is necessary to stop vessel growth in tumors. Alternatively, in stroke or other traumas it is imperative to stimulate the production of new blood vessels to regenerate damaged tissue regions.

**Cell Adhesion Molecules that Mediate Endothelial Cell Behavior**

There are two main CAMs that ECs express: N-cadherin and VE-cadherin. VE-cadherin mediates homotypic adhesion, forming the tight junctions of blood
vessels, cell polarity, sprouting inhibition, and lumen formation (Reviewed in (Giannotta, Trani, & Dejana, 2013)). An important function of EC tip cells is their ability to recognize other cells and form tight junctions and create functional anastomosis. When VE-cadherin is blocked, ECs no longer recognize one another to form these tight junctions and continue to extend filopodia in alternate directions (Lenard, 2013). N-cadherin's role in ECs remains more elusive. Intriguingly, N-cadherin has been shown to promote motility in ECs in a Fibroblast Growth Factor Receptor 1 (FGFR1)-dependent manner most likely by maintaining FGFR1 stability (Giampietro et al., 2012). Conversely, VE-cadherin also binds FGFR1 and phosphorylates it through coupling density-enhanced phosphatase 1 (DEP-1), down-regulating FGFR signaling and cell migration (Giampietro et al., 2012).

Endostatin, a derivative of collagen XVIII, has recently been implicated in reducing angiogenesis in tumors (Dixelius et al., 2002). While the exact mechanism is unknown, the data indicate that Endostatin is important in cell matrix adhesion and cytoskeletal rearrangements by interfering with normal FGF2 binding (Dixelius et al., 2002). Another CAM expressed in NCCs, protocadherin-1, is also expressed in mouse and chick vasculature during development (Bononi et al., 2008; Redies, Heyder, Kohoutek, Staes, & Van Roy, 2008). Its function in blood vessels; however, remains unknown.
NEUROVASCULAR INTERACTIONS

Interactions of Blood Vessels and Neurons

Nerves and blood vessels depend on each other. Nerves require blood vessels to bring them oxygen and nutrients while blood vessels require nerves for vasoregulation (Eichmann et al., 2005). Blood vessels are thought to have evolved more recently than neurons. Evidence for this theory is based on the observation that simple organisms such as C. elegans do not have an extensive circulatory system because oxygen can easily diffuse through tissues in the organism. Higher evolved organisms have a more extensive vascular network because they require blood vessels to supply oxygen to organs and the nervous system (Reviewed in (Quaegebeur, Lange, & Carmeliet, 2011)). Usually nerves extend and form adjacent to blood vessels in development, but in some cases nerves form first (Bates et al., 2003). Neurovascular development has been studied more extensively in the central nervous system. Neurons and blood vessels exhibit extensive cross talk—these systems communicate and release survival signals for each other. Neurons release VEGF while blood vessels release neurotrophic factors and oxygen (Eichmann et al., 2005; Mukouyama, Gerber, Ferrara, Gu, & Anderson, 2005; Suchting, Bicknell, & Eichmann, 2006). When this cross talk is interrupted the results are devastating, leading to diseases such as ALS and Alzheimers (Quaegebeur et al., 2011; Rosenstein, Krum, & Ruhrberg, 2010).
Shared Signaling Molecules: VEGF and FGF

Neurons and blood vessels share repulsive signaling molecules, CAMs, and growth factors. Growth factors VEGF and FGF are used by each system. VEGF is a known angiogenic and vasculogenic factor for ECs, but it has also recently been shown to have important functions in CNS development (Mackenzie & Ruhrberg, 2012; Storkebaum & Carmeliet, 2004). VEGF acts as a chemotactic agent for cranial NCCs in vitro and in vivo ((Kulesa, Bailey, Kasemeier-Kulesa, & McLennan, 2010; McLennan, Teddy, Kasemeier-Kulesa, Romine, & Kulesa, 2010). Whether VEGF is chemotactic or mitogenic for trunk NCC was a focus of this study. FGF has also been shown to have major functions in each system. Basic FGF (b-FGF), alternatively known as FGF2 has promotes angiogenesis in the mouse cornea and in the chick chorioallantoic membrane (Kenyon et al., 1996) (Ribatti, 2008). FGF signaling has many roles in the nervous system (Guillemot & Zimmer, 2011). Its role in trunk NCCs was the main focus of this study. VEGF and FGF will be further elaborated on throughout this work.
SPATIAL AND TEMPORAL ANALYSIS OF
NEURAL CREST CELLS AND ENDOTHELIAL CELLS

Introduction

Neurons and blood vessels form hierarchal networks simultaneously during development. We sought to determine the juxtaposition of the dynamic leading cells of each system: NCCs and ECs. Temporal behavior of NCCs and ECs has been studied, but not in relation to one another (Wilting, Eichmann, & Christ, 1997) (Fraser & Bronner-Fraser, 1991). Previous studies have shown neurovascular interactions for the development of the SG. For example, the dorsal aorta releases SDF-1, a chemotactic agent for CXCR4 expressing NCCs destined for SG (Kasemeier-Kulesa et al., 2010; Rehimi et al., 2007; Saito et al., 2012). Similarly there have been neurovascular interactions found in the development of the digestive tract. In the chick intestine, enteric NCCs (ENCCs) were found to migrate along concentric circles formed by already positioned ECs. Removing ECs resulted in migration impairment of the ENCCs and an overall disruption in the formation of the enteric nervous system. In vitro they showed ENCCs migrating extensively on ECs via integrin signaling (Nagy et al., 2009). Additionally neurovascular interactions have been identified later in development. In vitro studies using rat DRG cultures found that cultures that contained ECs were more mitotically active compared to cultures that lacked ECs (Mompéo, Engele, & Spanel-Borowski, 2003). Furthermore, in the subventricular zone of the central nervous system, close proximity of blood vessels to neural stem cells promotes proliferation (Shen, 2008).
Using stage-by-stage static images and live dynamic imaging we sought to determine the spatial and temporal relationship of ECs relative to NCCs.

**Methods**

**Immunofluorescent Chemistry**

Quail embryos were ordered from Ozark Egg Company and were incubated at 37°C with 78% humidity. Eggs were turned on their side for 1 hour prior to removal of 1 ml of albumin. A transgenic quail model was utilized in which the ECs fluoresce with a nuclear localized yellow fluorescent protein (YFP) driven off of the Tie1 promoter, which is EC specific. Tie1 fluorescence was checked by cutting a piece of choriallantoic membrane and placing on a slide with 1X PBS. They were then observed for fluorescent ECs using a Nikon Microphot-FXA microscope (20X objective with GFP filter). Quail embryos were dissected out in 1X PBS, fixed in 4% paraformaldehyde, and brought up through a sucrose/PBS gradient (5, 15, and 30%) until they were finally embedded in OCT. Embedded embryos were sectioned using a cryostat at 16 um thickness. We used an antibody specific for quail ECs called QH1 (Developmental Hybridoma)(1:20 dilution) (Pardanaud, Altmann, Kitos, Dieterlen-Lievre, & Buck, 1987). NCCs were visualized with a monoclonal antibody, HNK or neurogenin2 (NGN2) (kind gift of Dr. Ben Novitch, UCLA). Differentiated neurons were visualized with TUJ1 that recognizes beta-tubulin on neurons and islet antibodies (developmental hybridoma). Laminin antibody was also purchased
from DH. Sections were visualized using a scanning confocal microscope Olympus Fluoview 350 with fluoview software 5.0v and 488, 543, and 633-nm wavelengths.

**Time Lapse Imaging**

pLenti PGK mCherry plasmid (Rusty Lansford, Cal Tech) was injected into the lumen of the neural tubes of Tie 1 quail embryos, followed by electroporation, at HH stage 12. Embryos were then harvested at various stages (HH st 15-21). For live imaging embryos were dissected in sterile ice cold 1XPBS and embedded into low melting point agarose for vibratoming. Embryos were then sliced at 250um thickness using a vibratome, sorted for fluorescence, and placed on a filter membrane that was presoaked with neural basal media supplemented with antibiotic, B27, and L-glutamine (Gibco). Alternatively, whole embryos were placed directly onto the membrane and imaged. The tissue was incubated at 37°C for the duration of the movie and images were taken every 4-6 minutes using the Olympus 350.

**Results**

**Endothelial Cells and Neural Crest Develop in Close Proximity**

By static analysis of successive stages of development, we found the NCCs and ECs develop in close proximity and directly contact each other during migration and development. At HH stage 14, approximately 52 hrs of development, NCCs have started migrating out of the neural tube and into the anterior somite;
simultaneously early ECs have also started migrating only they migrate dorsally emerging from the dorsal aorta (Figure 1). ECs forming the intersomitic vessel (ISV) bud off the existing dorsal aorta and migrate along the somite boundaries. Simultaneously mesoderm cells are becoming ECs through vasculogenesis in the somite (Pardanaud & Dieterlen-Lièvre, 1999). The first ECs observed in the anterior somite reside close to the ventral portion of the NT and come in direct contact with ventral migrating NCCs (Figure 2 A, B). These early ECs are establishing the persomitic vessel (Schwarz et al., 2009). As in previously published studies, we observed early migrating NCCs mixing with ECs of the ISV while later migrating NCCs were not able to cross into the ISV zone (Figure 2 A, B) (Figure 3) (Schwarz et al., 2009). Another key finding was the speed of migration of NCCs contacting ECs compared to those not. NCCs adhered to ISVs traveled very slowly while NCCs not in contact with ECs “darted” freely throughout the anterior somite (data not shown).

Later in development, HH 17 and 19, formation of the perineural vascular plexus (PNVP) has resulted from ECs migrating and surrounding the NT, while the DRG only contacts ECs medially (Figure 4 A,B). By stage 25, 4.5-5 days into development ECs have formed a web/network around NCCs in the DRG analogous to the “blood brain barrier” that will surround the CNS (Figure 4 C, D).
Figure 1. Schematic of simultaneous NCC and EC migration. Early NCCs migrate ventrally from the dorsal neural tube in streams to the SG and DRG. ECs migrate dorsally from the dorsal aorta (angiogenesis) and begin to form the perineral vascular plexus (PNVP) and perisomatic vessel. ECs are also derived through vasculogenesis from the mesoderm.
Figure 2. Spatial and temporal analysis of NCCs and ECs reveals their close proximity early in development. A,B Images show the ECs forming the Intersomitic vessel (ISV) at stage 14 and 15/16 respectively. A',B' Images show the first ECs reside ventral and lateral in close juxtaposition to the Neural Tube and will eventually extend around the neural tube to form the PNVP and form the perisomitic vessel.
Figure 3. NCCs migrate along the Intersomitic vessels (ISV). The perisomitic vessel (PSV) develops in close proximity to the ventral boundary of the DRG. Early in development NCCs intermix with the early forming ISV, but as more ECs infiltrate the ISV becomes more of a physical boundary. * EC from the developing PSV that specifically circled around “corraling” NC migrating ventrally (movie not shown and was contributed by Lynn George).
Figure 4. ECs form boundaries and encapsulate the NT and later the DRG. At stage 17 and 19, the first ECs are seen on the medial boundary of the DRG. The PNVP is formed and through angiogenesis ECs ingress to vascularize the NT. Later stages (HH 25) show the DRG encapsulated by ECs analogous to the blood brain barrier formed in the CNS.

Endothelial Cells in the Anterior Somite Directly Contact Neural Crest Cells Destined for the DRG

Analysis of static images of neurovascular development revealed novel potential interactions between ECs and NCCs that had not been described, raising new hypothesis about how ECs may influence NCC patterning. For Example, are ECs positioned at the ventral border of the forming DRG, a stopping point for migrating NCCs thereby inducing the site of DRG formation? Trunk NCCs migrate ventrally in a stream with the farthest migrating cells forming the SG. However, many migrating NCCs cease moving more dorsally and condense to form the DRG. The earliest cohort of NCCS that are destined to form the DRG express the transcription factor, Neruogenin2, which can be visualized using an antibody to Ngn2. Our analysis indicates that NCCs that are NGN2+ appear to contact the first ECs that are beginning to form the PNVP and the perisomitic vessel (Figure 5 A,B). We cannot
say definitively that filopodia make contact because our Tie1+ ECs are nuclear localized. The formation of the perisomatic vessel occurs at the same time the DRG is forming and develops at the ventral boundary of the DRG (Figure 3).

Figure 5. NCCs destined for the DRG contact early ECs. A. NGN2 positive NCCs that are destined for the DRG contact Tie1+ ECs. B. 60x magnification of NCCs directly contacting ECs through filopodial extensions.

Early Endothelial Cells in the Anterior Somite Reside at the Site of Motor Axon Emergence

Another question that arises from the juxtaposition of ECs close to the neural tube is whether they are the site for motor axons to emerge from the NT. Early ECs reside close to the site of motor axon emergence (Figure 6 A,B). Over the next in development, they start to surround the motor axons (Figure 6C). In order for motor axons to emerge from the developing spinal cord, the ECM must be degraded (Sanes, 1989). ECs are a potential source for degrading this matrix. Extracellular matrix molecule laminin surrounds the NT at HH stage 15 and has been visualized at earlier stages (S. L. Rogers, Edson, & Letourneau, 1986)(Figure 7A). Motor axons
emerge at stage 13, the same stage when these early ECs are observed (Tosney, 1991). Early ECs seemingly “embed” themselves in the laminin surrounding the NT (Figure 7B). Whether they are degrading this laminin matrix needs to be further addressed.

Figure 6. Early Migrating ECs forming the PNVP and PSV collect next to the neural tube where motor axons emerge. A, B. Stage 15/16 quail shows the juxtaposition of early ECs and axons extending from the neural tube. C. Later stage 22 quail showing ECs surrounding the ventral root but not intermixed.
Figure 7. Laminin surrounds the neural tube and the dermamyotome at HH stage 15. A, B, 60x image of ECs “embedding” themselves next to the neural tube in the lamin ECM.

**Endothelial Cells Form a Barrier for Neural Crest Cells**

Our static spatial temporal analysis and time lapse imaging suggest that ECs form a barrier for NC that are traveling ventrally via the perisomitic vessel and rostrally by ISV. NCCs appear corralled or contained within networks of ECs. This was observed in multiple embryos. NCCs do not penetrate the ISV when ECs are extensively condensed in the region (Figure 2). We also observed ECs on the lateral side of the DRG contacting migrating NCCs that were outside the boundary of the DRG. In one time-lapse analysis we observed an immediate redirection of filopodial extensions after they extended through the EC boundary (Figure 8). This phenomenon was also observed in whole embryos at earlier stages for the ventral migrating NCCs. ECs forming the perisomitic vessel surrounded NCCs that traveled
outside the DRG ventrally and changed the course of their migration, “instructing” them back towards the DRG (Figure 2*).

Figure 8. Time-lapse analysis of lateral DRG margin. NCC extending filopodia outside the lateral DRG boundary between ECs (*) are immediately retracted (**) and followed by orientation in the opposite direction (***)\. Movie contributed with the help of Lynn George.
Endothelial Cells Form a Sheath Around the DRG

Through angiogenic sprouting ECs start to surround the DRG later in development. This encasing around the DRG is analogous to the BBB of the brain (Figure 9). Similar to findings in CNS stem cell niche, we found that progenitor cells in close proximity to ECs were more mitotically active (Lynn George, data not shown). A time-lapse movie from HH stage 22 showed ECs starting to form a boundary around the DRG while a remaining few NCC are still migrating. During this time lapse a NCC contacts ECs and divides, with a daughter cell diving into the DRG (Figure 10).

Figure 9. ECs encase the DRG at HH stage 24. A. ECs were stained with QH1 are are visualized in blue while neural crest are transfected with GFP and are green or were stained with HNK and are red in the image. This formation is reminiscent of the Blood Brain Barrier. B, 60x magnification.
Figure 10. Time-lapse analysis of late migrating NCC dividing after contacting ECs. The NCC “stair-steps” down the ECs, extends filopodia through the ECs and soon after divides (*). Movie contributed by the help of Lynn George.

Discussion

This is the first analysis demonstrating the live dynamics of ECs and NCC interactions during the development of the PNS and reveals interactions not previously discerned in static analysis of fixed tissue (Miller et al., 2010). Through our analysis it is apparent that these cells extensively interact through filopodial extensions. Whether they are communicating through signaling mechanisms or independently migrating and by chance “bumping” into one another remains unknown.

The dorsal aorta is the first functional blood vessel that is formed in the developing embryo and is a reservoir for hematopoetic stem cells (reviewed in (Y.
Sato, 2012). Not only does the dorsal aorta attract NCCs that will form the SG, but it is also the birthplace of ECs that will form the ISVs (Eichmann et al., 2005). Sources of ECs have been studied previously using quail/chick chimeras. With the development of monoclonal antibody QH1 that only recognizes quail endothelial cells, these studies have been extremely productive (Ambler, Nowicki, Burke, & Bautch, 2001). The attraction between NCCs and ECs seems to be mutual. The neural tube is an attractive source for ECs because it expresses VEGF. Studies transplanting extra neural tubes resulted in EC recruitment towards these ectopic neural tubes—surrounding them forming PNVPs (Hogan, Ambler, Chapman, & Bautch, 2004).

Endothelial Cells Form Boundaries for Neural Crest

Our data indicates that NCCs and ECs only intermix during early migration stages when neither system has a wealth of cells. Very early migrating NCCs can migrate along and intermix with ECs, but as more ECs fill the ISV region, it becomes a barrier for new NCCs migrating NCCs. These results were similar to those already observed (Schwarz et al., 2009). Thus, when ECs develop into a more functional vessel they “contain” neural crest within the somite regions. The ISV could act in conjunction with inhibitory molecules in the posterior somite and help pattern the developing DRG. Although, it is possible that the ISV acts as a physical barrier to the cells that try to cross it, but promotes migration of NCCs along it. Evidence of chick ENCCs migrating on ECs is further evidence of this hypothesis (Nagy et al., 2009).
We observed NCCs migrating freely in the permissive anterior somite while NCCs that were in direct contact with ISV migrated slowly. ECs of the ISV form a boundary between the somites. It is plausible that ECs provide a substrate in a region juxtaposed next to repulsive molecules.

ECs forming the PNVP and perisomitic vessel were also shown to interact with NCCs traveling ventrally. Time-lapse imaging showed contact of an EC forming the PSV with a migrating NCC resulted in “corralling” of the NCC by ECs and redirection of migration back towards the DRG. Additionally ECs forming the lateral boundary of the DRG contact and redirect NCC filopodia back into the DRG. Whether these interactions communicate migration direction signals to the NC or are independent of contact in unknown.

While many of the factors promoting migration have been studied, the mechanisms that cause cessation are not known. The first ECs that are developing the perisomitic vessel reside in the anterior somite and contact NCCs that are specifically destined for the DRG. Whether these ECs act to prohibit ventral migration of NCCs or a signal for NCCs to differentiate has not been elucidated.

These first ECs in the anterior somite are also the site of motor axon emergence. Migratory ECs contain a variety of matrix metalloproteases (MMPs) (reviewed in (S. D. Shapiro, 1998; Stetler-Stevenson, 1999)). Perhaps these migratory ECs are responsible for degrading the laminin surrounding the neural tube at the point where motor axons emerge. Previous research has identified the phenomenon of axons following paths that have already been established by other
systems/organs (Sanes, 1989). The fact that these early ECs reside next to motor axon emergence implies their function in pre-establishing a path for axons. This degradation of the matrix could also release bound growth factors that would be imperative for neural development, survival, and perhaps outgrowth.

Endothelial Cells Promote Proliferation of Neural Crest and Progenitor Cells of the DRG

ECs surround the DRG during development. This encapsulation of the DRG is reminiscent of the blood brain barrier formation in the CNS. The cross talk and proximity of neurons and vessels in this manner are referred to as a neurovascular niche (Quaegebeur et al., 2011). Other cell types within these niches include pericytes and glia that were not analyzed in this study, but would be interesting to visualize. Similar to the CNS, progenitor cells on the boundaries of the DRG that were in close contact of ECs were more mitotically active (Lynn George, data not published). This suggests that ECs communicate or provide required molecules for proliferation. Again, since ECs have the ability to degrade ECM they are possibly able to release bound growth factors such as FGF, PDGF, EGF, to stimulate progenitor proliferation. They could also be secreting growth factors. Endothelial cells have been shown to release neurotropins such as BDNF (reviewed in (Melani & Weinstein, 2010)). The specific factors that promote proliferation for these progenitor cells in the DRG are unknown. Although our data and CNS research shows promising evidence that ECs could be a direct or indirect source.
Previous research has shown that the DRG develops normally in absence of vasculature (Miller et al., 2010). Similarly, in neurovascular development of the quail limb researchers found that blood vessels were not required for neural development and vice versa (Bates et al., 2003). In the limb neither system was required for the other’s development, but they were both regulated by a common signaling molecule, Sem3A (Bates et al., 2003). Although they quantified total blood vessels and qualified vessel growth these researchers did not look for differences in neural differentiation or survival. In a study done by Miller et al, in 2010 they used a mutant zebrafish that had compromised EC development leading to a decrease in blood vessel formation. In these zebrafish the DRG formed in the correct position without signaling or patterning of blood vessels (Miller et al., 2010). This would suggest that the formation of the DRG and the vasculature around it are not mutually dependent. However, they were not able to study neural differentiation nor proliferation because these loche mutant zebra fish die before they can analyze these events. The reverse was not studied: the development of the vascular system in the absence of NCCs (Miller et al., 2010). These researchers did not quantify the size of the DRG relative to the SG nor the total number of neurons, it was only qualitatively observed. It is possible that ECs are a stopping point for a subset of NC or involved in differentiation—these questions were not addressed.

Whether neurovascular development in the PNS arises from two independent pathways of NCCs and ECs navigating the same environment or directly communicating with mutual dependence requires further investigation. In
order to elucidate this relationship we have tried several approaches to focally eliminate ECs.
ELIMINATION OF ENDOTHELIAL CELLS BY BLOCKING FGFR SIGNALING

Introduction

In order to elucidate the function of ECs in NCC development we sought to eliminate them locally in the region of migrating neural crest. We approached this by spatially restricting the availability of FGF or VEGF in order to avoid global disruptions to the developing vasculature (Gerhardt, 2003; Kenyon et al., 1996; Nimmagadda et al., 2004; Zhang, 2003).

Methods

Elimination of Endothelial Cells In vitro

DRG were harvested from HH stage 27 tie1 quail embryos into Hanks media. They were cultured and plated on a 96 well plates that were coated with 1 mg/ml poly-D-lysine and 20 ug/ml laminin. The media used was Neural Basal Media with added glutamax, B27, antibiotic, and NGF. The media was supplemented with increasing concentrations from 0.1 uM up to 10 uM of Su5402 (FGFR blocker) or Su5416 (VEGFR Blocker), (Tocris). Previous researches have shown the viability of Su5402 as a FGF signaling blocker (Bénazéraf et al., 2010; Boscher & Mège, 2008; Eblaghie et al., 2003; J.-M. Lee et al., 2008; Lunn, Fishwick, Halley, & Storey, 2007; Martinez-Morales et al., 2011; A. Sato et al., 2011; Szarama et al., 2012; Yang, Dormann, Münsterberg, & Weijer, 2002). DRG cultures were fixed for 5-10 minutes on ice with 4% PFA and stained with primary antibodies including phosphorylated
Histone, H3 (1:250) for mitotic activity, or TUJ-1 for neural differentiation.

Cultured 250um slices from a HH stage 21 Tie1 quail embryo were also subjected to different concentrations of Su5402 or Su5416.

**Focal Elimination of Endothelial Cells *In vivo***

AG 1X2 (Bio-rad) resin beads were washed with 1XPBS and soaked in 1 or 10 mM of Su5402 or Su5416 for 1 hour at room temperature. Beads soaked in DMSO were used as a control and the different blockers were dissolved in DMSO. They were then rinsed and placed into a small petri dish under a stereoscope. Beads were inserted into Tie1 positive HH stage 20/21 quail embryos. This was done by piercing the trunk area between the forelimb neural tube with a tungsten needle under the stereoscope. Forceps were used to insert the bead into the premade incision. These embryos were fixed at HH st 27 and analyzed using IHC. Antibodies TrkA (pain and temperature receptors) (1:6000) (Rifkin et al., 2000), phosphorylated Histone H3 (1:250 Company), and islet1 and islet 2 (1:10, Developmental Hybridoma). Alternatively at younger stages 1-2 Su5402 or Su5416 soaked beads (1-10mM) were pipetted onto the trunk region of stage 9/10 quail embryos. These AG-1X resin beads were either placed in a small incision or alternatively they would “stick” to the surface of the embryo. Embryos were incubated until stage 17/18 fixed and analyzed with IHC. They were stained with HNK, QH1, and H3 and analyzed for EC survival and NCC migration.
Su5402 Filter Paper Assay

1-10mM of Su5402 (Tocris) was soaked in approximately 1mm x 1mm pieces of Whatman filter paper at room temperature for 1 hour before application. Tie1 Quail embryos were incubated at 37 degrees until HH stage 12 and were then taped and windowed for filter paper application. Embryos were visualized using neutral red dye and the filter paper was placed carefully with tweezers on the trunk region where the vitelline artery attaches to the embryo. Usually the filter paper remained in place during incubation, but not always (in control or experimental); however they were all treated the same. The quail embryos were then incubated for 24 hrs until HH 17, dissected, and fixed in 4% paraformaldehyde 30-60 minutes at 4 degrees C. Embryos were brought up in a sucrose/OCT gradient and then sectioned in 16 um sections. These sections were stained with HNK and counted using Image J. NCC numbers were counted in three regions including: dorsal 1/3, middle 1/3, and ventral 1/3. These regions were determined by measuring the length of the neural tube in each slice and dividing it into three regions. Alternatively sagittal sections were used for anterior/posterior analysis. The area NCCs occupied within the dorsal somite was measured by using a grid program on Image J. Images and analysis were taken using Leica SP8 and Olympus Fluoview 300 confocal microscopes analysis were also done with a Nikon fluorescent microscope. Statistical analysis were performed using the student-T test with averages of each embryo from at least 4 counted sections.
Results

Endothelial Cells Eliminated *In vitro*

By blocking FGFR (Su5402) signaling and VEGFR signaling (Su5416) we were able to eliminate ECs *in vitro*. For FGFR blocking this was effective at 1.0 uM concentrations in DRG explant cultures (Figure 11B) while ECs remained healthy at 0.1 uM concentrations (Figure 11B). This was also observed in cultured embryonic transverse slices (data not shown). A higher concentration of the VEGF blocker was needed to eliminate ECs (data not shown). These blockers work by intracellularly blocking the ATP binding site of the receptor tyrosine kinase (L. Sun et al., 2000; 1999). ECs that had undergone apoptosis appeared as small fluorescent punctate spots, some adhered to one another at 1.0 uM (Figure 11C). There was a slight decrease found in mitotic activity of NCC progenitors (H3), but no differences were observed in differentiation, or outgrowth of neurons (stained with tuj-1). However, these observations were not actually quantified.

![Figure 11. Su5402 eliminates ECs in vitro. Using different concentration of the FGFR blocker ECs were progressively affected and killed with 1.0 uM of the blocker. H3 an antibody for mitotically active cells in red was also decreased with decreased FGF signaling. H3= red, Tuj1=blue, and ECs= GFP.](image-url)
Focal Elimination of Endothelial Cells
*In vivo* Causes Gross Tissue Malformations

Applying Su5402 (FGFR blocker) and Su5416 (VEGFR blocker) soaked beads locally to HH st 9/10 and analyzing 24 hours later, resulted in extremely malformed tissue. One observed phenotype was the formation of a second or multiple neural tubes in areas where the bead was present (data not shown). This observation is similar to the phenotype of chimeric mouse embryos with FGFR1 nonfunctional (Ciruna, Schwartz, Harpal, Yamaguchi, & Rossant, 1997).

Decreased FGF Signaling Early in Development Eliminated Endothelial Cells and Arrested Ventral Migration of Neural Crest Cells

Since implanting beads resulted in gross tissue malformations we alternatively applied filter paper that was soaked in FGFR blocker. By decreasing FGF signaling early (filter paper applied at HH st 12 and fixed at 17/18) it was evident that NCCs forming the SG were almost completely absent in the trunk region of Su5402 treated embryos (Figure 12). Instead, NCCs “piled up” dorsally and displayed a rounded “honey comb” morphology (Figure 13B). ECs were focally eliminated dorsally, in regions where the filter paper was applied (Figure 12B, 13B). ECs in the dorsal aorta were not eliminated. To determine if NCC were dying, the total number of NCCs was quantified and while there were slightly fewer in the Su5402 treated embryos, the number was not significantly different, thus ventrally migrating NCCs weren’t simply dying. To determine the position of NCCs, the neural tube was divided into three regions and the number of NCCs in each area was
quantified using Image J. It was found that 58.3+13.6 percent of NCCs in DMSO (control) treated embryos remained in the top 1/3 of the neural tube region, compared to the 79.2+6.5 percent in control (p-value 0.043, N=5 of each condition) (Figure 12). Decreasing FGFR signaling caused NCC to pile up dorsally at the expense of the SG and ventral DRG with a decrease in ECs in the anterior somite.

Figure 12. NCCs fail to migrate ventrally to form the sympathetic ganglia, ECs are eliminated dorsally. A. Representative control embryo treated with DMSO filter paper with normal NCC migration and formation of SG, HH stage 17/18. Tie1-GFP ECs are observed forming the PNVP. B. Experimental embryo treated with 1-10mM Su5402 soaked filter paper in the trunk region characterized by migration “arrest” of NCC dorsally, at the expense of SG. Early ECs forming the PNVP are eliminated. C. Summary Chart of NCC migration counts. NCC were counted in three different regions defined by the neural tube. Dorsal 1/3, middle 1/3, and ventral 1/3 of the neural tube. N= 5 embryos Su5402, N=5 control embryos P-value: ventral: 0.043.
Figure 13. Su5402 cells differ morphologically from DMSO control. A. HH stage 17/18 control (DMSO) treated embryos display normal polarized morphology with ECs forming the PNVP. B. Su5402 treated embryos phenotypically resemble “honeycomb” with regular rounded cell bodies and an absence of dorsal ECs forming the PNVP. *Images reflect morphology and not overall total cell counts.*

Decreased FGFR Signaling Causes
Neural Crest Cells to Inappropriately Remain in the Migratory Staging Area

Not only did NCCs display an impaired ability to migrate ventrally, but NCCs inappropriately remained in the migratory staging area (MSA) of the posterior somite region (Figure 14 A, B). Furthermore, ECs forming the ISV were reduced in number in regions where filter paper was applied. NCCs in the DMSO (control) treated embryos were confined to an average of 33+4.3 percent of the dorsal somite, while the Su5402 treated NCCs spread out occupying 63+7.0 percent of the dorsal
somite (p-value 0.019, N= 3 embryos of each condition) (Figure 14 C). The decrease in total cell numbers for Su5402 treated embryos in transverse counts, although it wasn't significant, could be accounted for in the extended region of NCCs occupying the MSA of the posterior somite. Figure 14D demonstrates this phenomenon of NCCs treated with Su5402 piling up and remaining dorsally in MSA of posterior somite areas.
Figure 14. NCCs fail to obey the strict anterior/posterior somite boundary dorsally when treated with Su5402. A. DMSO embryo with normal DRG formation B. 1mM Su5402 filter paper treated embryo with NCCs spreading out inappropriately into the MSA of posterior somite with a decrease in ISVs. C. Chart depicting percent space NCCs occupy within the dorsal somite N= 3 experimental and N=3 control p-value 0.019. D. Schematic representation of NCCs response to decreased FGF signaling via Su5402 filter paper.

Focal Elimination of Endothelial Cells

_**In vivo causes a Decrease in TrkA+ Neurons**_

We were interested in the role of FGF signaling and EC elimination at later stages of NCC development. Blocking FGFR signaling at later stages did disrupt EC migration/formation in areas close to the bead, and also did not alter the overall structure of the DRG (Figure 15). This was somewhat expected since the DRG is relatively well formed when it becomes circumscribed with ECs and the beads were placed at later stages (HH stage 20/21) in development when the DRG had already started to condense. Although the DRG appeared to be undisturbed histologically; we sought to determine if there were differences in proliferation, survival, or differentiation. In order to quantify these processes we used IHC. To determine mitotic activity we again used anti-phosphorylated Histone H3, and did not find a difference in the overall mitotic activity of the cells within the DRG. We also
qualified the percentage of TrkA neurons and found that in the absence of both ECs and reduced FGF signaling there was a significant decrease in TrkA neurons. The control embryos averaged 14.0% ±2.9 of their total neurons that were TrkA positive compared to 7.8% ± 1.9 of the experimental. (P-value 0.0001 of and an N=7 experimental and 8 control embryos). There was no significant difference in the total number of neurons (determined by using islet-1 and islet-2 antibodies) suggesting that FGF signaling and/or the presence of ECs are required for neurons to differentiate into TrkA+ neurons. To determine if the cell death pathway was activated we quantified the number of caspase 3+cells and did not find any significant difference. In this study we did not compare neurons that were in proximity to ECs.

Figure 15. FGFR blocker, Su5402, kills ECs in vivo. A, image showing the placement of the AG-1x resin beads soaked with Su5402. B. Su5402 bead kills/repulses ECs that are in close proximity to the bead while in the control ECs form an intact sheath surrounding the DRG and NT.
Figure 16. Focal Elimination of Endothelial Cells with FGFR blocker (Su5402) decreased the percentage of TrkA neurons. A, A’. HH stage 25/26 chick embryos 14% of trkA neurons were counted in the control. B, B’. Decreasing FGFR signaling resulted in 7.8% trkA positive neurons. Total number of differentiated neurons were counted using islet (P-value = 0.0001 N=8 control and 7 experimental).

Discussion

Does Eliminating ECs have Direct Impacts on Neural Crest Migration?

FGFR signaling and VEGFR signaling are instrumental for EC survival. We were able to eliminate ECs in vitro and focally in vivo with filter paper and bead assays. Blocking FGFR signaling caused NCCs to accumulate dorsally in the developing embryo, and to remaining inappropriately in the dorsal portion of the posterior somite. Repulsive molecules expressed in the posterior somite normally
drive NCCs away from that region (Jia et al., 2005; Krull et al., 1997; Kuan, Tannahill, Cook, & Keynes, 2004; Wang & Anderson, 1997). However, NCCs emigrate from the NT as a continuous sheet, uniformly distributed throughout the MSA, and then are later driven into the anterior somite due to repulsion of the posterior somite (Sechrist, Serbedzija, Scherson, Fraser, & Bronner-Fraser, 1993). Possible theories for why Su5402 cells remain in the MSA of the posterior somite include: the dorsal accumulation of NCCs causes them to overflow into the posterior somite because of space restrictions, or alternatively their migration is so impaired that they cannot migrate appropriately into the anterior somite compartment either due to mechanical reasons associated with cytoskeleton and/or inability to respond to positive cues in the anterior somite (should they exist). Alternatively ECs could also be directly impacting/causing this phenomenon. It is possible ECs not only form a boundary, but the ISV form an exit route for NCCs out of the MSA, to migrate ventrally similar to ENCCs (Nagy et al., 2009). During development ECs may provide a permissive environment for a subset of migrating NCCs. Further evidence for this theory is the close juxtapositioning of ECs of the ISV relative to migrating NCC. In the central nervous system blood vessels provide a permissive substrate for migration of neuroblasts from the subventricular zone to the olfactory bulb (Saghatelyan, 2009). Rather than forming a boundary, it is possible that ISV are promoting motility directly (perhaps through ephrin/Eph signaling) or indirectly by secreting permissive molecules (Suchting et al., 2006). Through undetermined mechanisms NCCs remain very dorsal in the absence of ECs in response to
reductions in FGF signaling. In contrast, if the ISV creates a boundary for NCCs getting rid of this anterior boundary is less restrictive for NCCs and rather than follow discrete paths their trajectories are “messy” and a subset of cells are seen in the MSA of the posterior somite by default. In ENCC studies they also found an impairment of ENCC migration when ECs were eliminated using Su5402. However, they did not elucidate the function of FGF signaling in migrating ENCCs (Nagy et al., 2009). While all of these are possibilities, further research has lead us to believe that FGFR signaling is directly required in ventral migration of trunk neural crest.

However, it is still possible that ECs stimulate this indirectly and are a source of FGF ligands. Previous research has shown that in vitro ECs produce FGF (Baird & Ling, 1987) and aortic ECs produce FGF-2 (Vlodavsky, 1987).

Does Eliminating Endothelial Cells Directly Influence Neural Differentiation?

In later staged embryos the percent of differentiated TrkA neurons was decreased when we blocked FGFR signaling. This could be indirectly attributed to the elimination of ECs, or indicative of a direct role of FGF signaling in neural differentiation. Indirectly, the decrease in ECs that surround the DRG could cause a decrease in mitotic activity of the progenitor cells on the perimeter of the DRG. This is consistent with previous research: in vitro ECs were found to promote proliferation in DRG cultures (Mompeo, 2003) and in vivo ECs promote proliferation of neural stem cells in the CNS and of oligodendrocytes (Arai & Lo, 2009; Tavazoie, Van der Veken, & Silva-Vargas, 2008). Furthermore, our lab results supported this
theory because increased mitotic activity was observed in progenitor cells that were located in close proximity to ECs within the niche of the developing DRG (Lynn George, data not published). Since progenitor cells differentiate into TrkA neurons, it is possible that eliminating ECs would decrease the mitotic activity of progenitor cells, thus there would be fewer differentiated TrkA neurons. Previous research has shown ECs can secrete neurotropic factors such as NGF, BDNF, and VEGF (Sondell, Lundborg, & Kanje, 1999) (Zacchigna, Lambrechts, & Carmeliet, 2008) (Eichmann et al., 2005; Mukouyama et al., 2005). Unfortunately these embryos were not analyzed for progenitor cells. Alternatively, FGFR signaling could have direct effects on TrkA+ differentiation. This second theory will be tested in another chapter.
VEGF IN TRUNK NEURAL CREST

Introduction

Vascular Endothelial Growth Factor (VEGF) is the critical molecule linking neurons to blood vessels (Rosenstein et al., 2010; Weinstein, 2005; Zacchigna et al., 2008). Mouse models with deficient levels of VEGF display symptoms and signs of Amyotrophic Lateral Sclerosis (ALS), a severe motor degenerative disease. These phenotypes were reversed with the addition of VEGF (Zheng, Sköld, Li, Nennesmo, & Fadeel, 2007). These findings not only implicate a role for VEGFs in neurovascular diseases, but indicate that VEGF may also function in the nervous system in addition to being a key regulator of the vasculature (Storkebaum & Carmeliet, 2004). VEGF plays key roles in stroke and other ischemic traumas via neuroprotection: increasing neurogenesis and angiogenesis (Y. Sun, Jin, Xie, Childs, & Mao, 2003).

Unfortunately, mutations in VEGFR cause embryonic lethality, making VEGF difficult to study, but imperative to understand (reviewed in (Ruhrberg & Bautch, 2013)).

VEGF ligands are comprised of three isoforms due to alternate splicing. In humans VEGF isoforms are 121, 165, and 189 while in mice they are referred to as 120, 164, and 188 (one amino acid shorter). VEGF 121 is the most diffusible isoform and does not bind heparin, VEGF 165 binds one heparin sulfate, while VEGF 189 is always found bound to the extracellular matrix—binding two heparin sulfates (reviewed in (Mackenzie & Ruhrberg, 2012)). There are two receptor tyrosine kinases for VEGF: VEGFR1 (Flt-1) and VEGFR2 (Flk or KDR), both receptors are able
to bind all of the VEGF isoforms (Mackenzie & Ruhrberg, 2012; Rosenstein et al., 2010). However, the flk-1 (KDR) receptor has much stronger phosphorylation and signaling capabilities than Flt-1. Flt-1 has been hypothesized to regulate VEGF signaling by binding extra VEGF, thereby decreasing its availability for binding to flk (Ferrara, Gerber, & LeCouter, 2003). After ligands bind, these RTK dimerize and activate intracellular pathways. The intracellular routes utilized by VEGFRs include MAPK and PI3K (Rosenstein, 2004). These intracellular routes are implicated in proliferation, migration, and survival. Throughout development oxygen deprived tissue regions secrete a transcription factor known as hypoxia inducing factor (HIF-1), which binds to the hypoxia inducing region on the VEGF ligand. In this manner, areas of the developing embryos regulate vessel formation (Semenza, 2002) (Y. M. Lee et al., 2001). Another VEGF receptor called neruopilin (NRP-1), which is also expressed by NCCs, is a non-tyrosine kinase receptor and can also bind VEGF, but only the 164/165 isoform (Rosenstein et al., 2010). ECs, neurons, and cranial NC are attracted towards VEGF via NRP-1 receptors (Coultas, Chawengsaksophak, & Rossant, 2005; Kulesa et al., 2010; Mackenzie & Ruhrberg, 2012; Melani & Weinstein, 2010). Interestingly NRP-1 and VEGFR2 can bind heterotypically with the 164/165 ligand and increases EC migration (Fantin et al., 2014; Herzog, Pellet-Man, Britton, Hartzoulakis, & Zachary, 2011). This phenomenon of heterotypic binding will serve as a recurring, intriguing theme throughout this manuscript.
VEGF has been shown to have a multitude of functions in the vascular system. Its role in the nervous system has more recently been documented. In the cranial neural crest, VEGF has been shown to be chemotactic. Cranial NCCs express NRP-1 receptors and migrate toward a gradient of VEGF in vitro and in vivo. Specifically VEGF-165 is expressed in the brachial arch (ba2) and attracts cranial crest (Kulesa et al., 2010; McLennan et al., 2010). In the SVZ of rat, VEGF is chemotactic for neural progenitors through VEGFR2. Interestingly these migrating neural progenitors also require FGF-2 for migration, but FGF-2 was not shown to promote chemokinesis (Zhang, 2003).

Molecules that promote survival of neurons were discovered decades ago (reviewed in (Snider, 1994)). However, more recent research has shown that VEGF can also function as a neurotrophic factor (Sondell et al., 1999). A study in superior cervical ganglia (SCG) showed that VEGF stimulates outgrowth in vitro and increased survival. Through IFC they found flt-1 expressed on SCG and DRG in mice in vitro (Sondell et al., 1999; Sondell, Sundler, & Kanje, 2000). While many key molecules have been found for development of the PNS, the mitogens for the DRG or SG have not been determined. VEGF acts as a mitogen for ECs in rat CNS explants through MAPK and PI3K (Mani, Khaibullina, & Krum, 2003). Whether it promotes proliferation of neuronal progenitors has not been determined.

Because of these newly discovered intriguing roles of VEGF in cranial neural crest and differentiated neurons of the CNS, we preliminarily investigated VEGFs role in the development of the PNS.
Methods

Detecting Expression of VEGF with IHC

Quail embryos were fixed and sectioned at various stages and was described previously. The soluble Human VEGFR2-Fc (KDR) (R & D systems) was used to stain for VEGF ligands. Secondary antibodies to the Human Fc were used to amplify and detect the VEGF signal.

Inhibition with VEGFR-Fc Bead

VEGFR2-Fc was dissolved in sterile Hanks solution and used at a concentration of 0.5 ug/ul. Heparin acrylic beads were soaked in VEGFR2-Fc (KDR) (R &D) implanted and used as previously described.

Adding VEGF with Beads In vivo

Human recombinant VEGF-165 (Sigma) was soaked in heparin acrylic beads (sigma) or Affi-blue beads at a concentration of 0.5 ug/ul and implanted as described previously.

VEGF Chemotaxis Assay In vitro

VEGF 165 chemotaxis assay was done according to the methods described in FGF chemotaxis assay in vitro.
Results

VEGF Expression

Using VEGFR2-Fc (KDR) to determine the expression of VEGF ligands we found that VEGF is broadly expressed throughout the dermomyotome at stage 17, a stage when NCC are actively migrating and condensing to from the DRG (Figure 17). Later in development (stage 21/22) VEGF is expressed in a discreet line ventral to the dermomyotome (that is the ECM border between the dermomyotome and sclerotome) and within the DRG (Figure 18). ECs of the PNVP (medial margin of the DRG) co-localize within the zone of VEGF expression. VEGFR2-Fc binds all the isoforms of VEGF so we were not able to differentiate which isoforms these are.

Figure 17. HH stage 17 VEGF is broadly expressed in the dermomyotome and a small extent in the developing DRG. NCCs stained with HNK (green). A VEGFR2-Fc (KDR) was used to determine the expression of VEGF (red).
Figure 18. VEGF ligands are expressed in the dermomyotome close to the lateral margin of the DRG of stage 21 quail. VEGF is also co-localized with the developing vasculature of the PNVP—medial boundary of the DRG. Small quantities of punctate VEGF staining are also observed within the developing DRG.

Decreasing/Increasing VEGF Signaling *In vivo* did not Impact Neural Crest Proliferation

VEGF signaling was decreased in the developing embryo using heparin acrylic beads soaked with VEGFR2-Fc (KDR) to theoretically bind any free VEGF ligand. These beads were implanted into HH stage 21 quail embryos. These embryos were then analyzed using IHC for mitotic activity using Bromodeoxyuridine (BrdU) and neural crest (HNK). There were no obvious differences in neural crest migration compared to control 1X PBS beads. NCC were even found surrounding the bead (data not shown). Thus, there were no significant differences found in chemotactic phenotypes of NCCs because NCCs migrated normally into the SG and DRG. This was expected because beads were placed at a stage when these structures are already condensed. In order to elucidate differences in proliferation we counted BrdU positive cells on the ipsilateral portion
of the bead and compared those to the contralateral DRG. We did not find any quantitative difference in proliferation between right/left sides of embryo, or between experimental and control embryos. Similarly adding VEGF-165 soaked beads did not result in any quantitative differences for proliferation or migration. Neuronal differentiation or progenitor cells were not investigated.

**VEGF is not Chemotactic for NCCs *In vitro***

To determine whether VEGF exerted chemotactic activity on NCCs, we measured NCC migration away from dissected neural tubes *in vitro*. Prior to being placed in vitro, neural tubes were injected with pCAX (GFP expressing plasmid) and electroporated, in order to visualize NCCs. A VEGF soaked bead or control bead was placed close to the neural tubes within a matrigel matrix for 24 hours. There was not an obvious directionality of NCCs toward VEGF beads; furthermore, cultures with VEGF beads did not show an increase in overall migration distance.

**Discussion**

**VEGF is Expressed within the Developing DRG and Surrounding Areas**

During development, the somite has been shown to express VEGF, stimulated by the morphogen Sonic Hedge Hog (SHH) (Lawson, Vogel, & Weinstein, 2002). Later in development Schwann cells and differentiated neurons have been implicated in secreting VEGF (Mukouyama et al., 2005). VEGF was broadly expressed throughout the dermomyotome in early stages (HH stage 17) adjacent to
migrating NC and the developing DRG. Later in development (HH stage 21) it was expressed in a discreet line reminiscent of the boundary between the dermomyotome and the DRG and within the DRG. This VEGF expression on the lateral margin is likely an attractive source for ECs that will form the vascular around the lateral margin of the DRG because immediately following this VEGF expression, ECs start to form the sheath around the DRG. The source of this VEGF was not determined, but likely comes from the somite (Lawson et al., 2002). Within the DRG there are small amounts of punctate VEGF expression next to migrating/differentiating NCCs. Similar to ingression of blood vessels into the neural tube, it is likely that neurons are secreting VEGF in order to attract a blood supply (James & Mukouyama, 2011). This could indirectly promote their own survival (Rosenstein et al., 2010). Additionally, this could be an autocrine response: neurons secreting VEGF for their own survival/proliferation (Mackenzie & Ruhrberg, 2012; Rosenstein et al., 2010; Ruhrberg & Bautch, 2013; Storkebaum & Carmeliet, 2004). However, VEGF also stimulates survival of glia and Schwann cells, which release neurotrophins, this would indirectly promote neural survival (Mukouyama et al., 2005; Rosenstein et al., 2010). ECs that are forming the PNVP also show punctate expression of VEGF. However, it is most likely ECs are co-localizing with bound VEGF, although alternatively they could be secreting it (Cross & Claesson-Welsh, 2001). Sources of this VEGF could be the neural tube, glia, or the developing neurons within the DRG (Kutcher, Klagsbrun, & Mamluk, 2004) (Hogan et al., 2004) (Rosenstein et al., 2010).
VEGF is neither Mitogenic nor Chemotactic for Trunk Neural Crest

Trunk NCCs express the NRP-1 receptor (Lefcort and Kasemeier-Kulesa, data not shown). Migrating growth cones of neurons and filopodia of cranial NC and ECs contain NRP-1; binding semaphorins and eliciting repulsion, or binding VEGF and mediating attraction ((Bates et al., 2003; Eichmann et al., 2005; Fantin et al., 2013; Melani & Weinstein, 2010)). Unlike cranial NCCs that chemotax towards sources of VEGF via their NRP-1 receptor (Kulesa et al., 2010; McLennan et al., 2010), trunk NCCs were not found to be chemotactic toward sources of VEGF-165 in vitro. Chemotaxis assays were not performed in vivo since there was not suggestive chemotactic evidence in vitro for trunk NCC. Discrepancies could arise because different concentrations of VEGF may have pleitrophic affects within the developing embryo and in vitro. Other chemokines have been shown to have differing roles depending on concentration (Hapner, Nielsen, Chaverra, & Esper, 2006) (Garcia-Maya et al., 2006).

Additionally, VEGF was not found to promote proliferation in vivo. We compared ipsilateral (bead side DRG) to contralateral (bead free DRG). Previous researchers have used this technique to investigate angiogenesis and found differences within right/left sides of the chick embryo (Finkelstein & Poole, 2003). Furthermore, we used this same technique to investigate FGF’s role and in contrast to our findings for VEGF, found a significant influence on NCC dynamics. More studies in VEGF signaling are required to ascertain its role, if any, in proliferation.
These very preliminary studies of VEGF in the peripheral nervous system have not shown VEGF to be mitogenic *in vivo* nor attractive for NCCs *in vitro*. Ideally we would use a dominant negative NRP-1 receptor in migrating NCCs, but because NRP-1 also binds to Sema3A a known repulsive molecule of neural crest, it would make this phenotype difficult to differentiate direct effects of VEGF. Other VEGF ligands and their receptors also need to be researched. The expression of VEGF within the DRG implies that VEGF is important at least in later stages when the DRG has started to condense. Previous studies have shown VEGF has a role in neural survival, without VEGF neurons degrade (Julien & Kriz, 2006; Quaegebeur et al., 2011; Rosenstein, 2004; Rosenstein et al., 2010; Storkebaum & Carmeliet, 2004) VEGF expression within the DRG is likely stimulating ingestion of blood vessels, further indirectly and perhaps even directly promoting survival of glia and/or neurons. VEGF more than likely has multiple roles in these developing systems.
FGF EARLY STAGE: FUNCTIONS IN NEURAL CREST MIGRATION

Introduction

Fibroblast growth factors (FGF) and receptors (FGFR) exert powerful functions in cancer. FGFRs are up-regulated in a wide range of cancers from bladder cancer to myelomas (Reviewed in (Turner & Grose, 2010)). Not only does over-activation of FGFR lead to increased proliferation and survival, but an increase in FGF ligands promotes angiogenesis for a developing tumor (Cross & Claesson-Welsh, 2001; DeVore, Horvitz, & Stern, 1995; Javerzat, Auguste, & Bikfalvi, 2002; Turner & Grose, 2010). Furthermore, FGF signaling has important functions in development including: cell cycle arrest, apoptosis, survival, proliferation, differentiation, and chemotaxis. FGF signaling is so encompassing it can have polar opposite affects. An example of this includes FGFs function in proliferation and survival in a broad range of cell types such as ECs; with a distinct function in apoptosis in chondrocytes (Reviewed in (Dailey, Ambrosetti, Mansukhani, & Basilico, 2005)). Developmental systems in which FGF plays pivotal roles include lung, neural, and limb development (Eswarakumar & Schlessinger, 2007; Sheeba, Andrade, Duprez, & Palmeirim, 2010).

There are approximately 120 different FGF-FGFR interactions that have been identified in mammals (Bae, Trisnadi, Kadam, & Stathopoulos, 2012). There are four different receptors that can be alternatively splice and bind 23 ligands (Itoh & Ornitz, 2011) (Grose & Dickson, 2005; Katoh & Nakagama, 2013; Turner & Grose,
Of these 23 ligands, 8 are embryonically lethal when mutated (Itoh & Ornitz, 2011). Structurally the FGFR extracellular domain consists of three immunoglobulin like (Ig) domains, an acid box, and a cell adhesion molecule homology region. Intracellularly, FGFRs are receptor tyrosine kinases, transferring phosphate groups intracellularly. Once activated due to their conformational change they now bind intracellular proteins—thereby activating or inhibiting their responses (Christensen, Lauridsen, Berezin, Bock, & Kiselyov, 2006; Eswarakumar & Schlessinger, 2007; Nishita, Ohta, Bleyl, & Schoenwolf, 2011). FGFRs require heparin sulfate proteoglycans for stability; however, the exact binding mechanism is controversial (Mohammadi, Olsen, & Ibrahimi, 2014). In response to ligand binding FGFRs dimerize and autophosphorylate (Dailey et al., 2005; Murakami, Elfenbein, & Simons, 2008). Different isoforms of FGFR1-3 exist. These isoforms are formed by alternate splicing in the third Ig like domain which causes differences in specificity (Murakami et al., 2008). Intracellularly there are three main signaling pathways that FGFR stimulation can activate: mitogen-activated protein kinase (MAPK), phospholipase C gamma (PLC gamma), and phosphoinositide 3-kinase (PI3K) (Lunn et al., 2007) (Hu & Bouloux, 2010). These intracellular paths lead to different cellular responses. The MAPK pathway is important for proliferation and migration while the PI3 Kinase pathway is implicated in cell survival. Negative regulators of FGF signaling include Sprouty, Sef, and Map Kinase phosphatase 3 (MKP3) (Murakami et al., 2008).
To make FGF signaling even more complicated FGFR also bind CAMs, integrins, and ECM molecules: laminin, fibronectin, and proteoglycans (Christensen et al., 2006; Murakami et al., 2008). CAMs bind the acid box of the FGFR and are able to cause cellular signaling independent of FGF ligand binding. N-cadherin, L1, and NCAM have been shown to bind FGFR1 and cause neurite outgrowth in vitro (Doherty & Walsh, 1996; E. J. Williams, Furness, Walsh, & Doherty, 1994b). Furthermore, NCAM shares a 10 amino acid stretch of homology with FGFR1 (E. J. Williams, Furness, Walsh, & Doherty, 1994a). Using specific blocking antibodies researchers have shown that N-cadherin binds FGFR1 via the EC4 domain. Specifically blocking this region decreased neurite outgrowth (E.-J. Williams et al., 2001).

Previous research demonstrating FGF8 functions in chemokinesis of cardiac NCCs via FGFR1 and FGFR3 lead us to study these interactions in trunk NCCs. FGFR1c can bind 11 ligands, while FGFR3c primarily binds FGF8 (Reviewed in (Hu & Bouloux, 2010)). FGFR1 has been shown to have the strongest intracellular signaling compared to other FGFRs (Vainikka et al., 1994). FGFR1 has been extensively studied in multiple model organisms and has been found to be instrumental to development. FGFR1 and FGF8 null mice are embryonic lethal (Eswarakumar & Schlessinger, 2007). FGFR1 null mice die at E9.5 because of migration impairment in the primitive streak. FGF8 null mice die at E8.5 with defects in development of brain, gastrulation, heart, and craniofacial features (Eswarakumar & Schlessinger, 2007) (reviewed in (Stuhlmiller & García-Castro,
Mutations that cause over-activation of FGFR1 include craniosynostosis, a defect in the cranial NCCs that causes skeletal overgrowth (Petiot, Ferretti, Copp, & Chan, 2002). Other disorders that result from altered FGF signaling include Kallman and Pfeiffer syndrome, cleft lip, cleft palate, dental agenesis, and cancer (Hu & Bouloux, 2010; Itoh & Ornitz, 2011; Turner & Grose, 2010). Kallman syndrome is characterized by anosmia— inability to smell, and hypogonadotropic hypogonadism. This hypogonadism is hypothesized to occur because of the failed migration of GnRH synthesizing neurons. Mutations in the ligand FGF8 or the extracellular matrix molecule anosmosin also result in Kallman syndrome. FGF8 directly binds FGFR1 to regulate olfactory and GnRH development (Chung, Moyle, & Tsai, 2008; Dodé & Hardelin, 2004; Dodé et al., 2003; Endo, Ishiwata-Endo, & Yamada, 2012). Furthermore, anosmosin has been shown to promote FGF8 expression and is important for cranial neural crest formation (Endo et al., 2012).

FGF1 and FGF2 activation of FGFR1 is essential for EC migration and survival during vasculogenesis (S. H. Lee, Schloss, & Swain, 2000; Nourse, Rolle, Pabon, & Murry, 2007). FGF signaling has many critical functions during development of the nervous system as well. FGFs have been shown to have an early role in neural development by specifying ectoderm as neural (Stuhlmiiller & García-Castro, 2012b). FGF-FGFR signaling has also proved important for mesencephalic NCC migration (Kubota & Ito, 2000). The expression of FGF8 in the mesenchyme promoted the expression of FGF2 which lead to chemotaxis of NCCs via FGFR1 and FGFR3 in mouse (Kubota & Ito, 2000). FGFR1 functions in the induction of neural crest cells
during gastrulation. Previously it was thought to act indirectly by promoting Wnt8 expression in the mesoderm (Hong, Park, & Saint-Jeannet, 2008). Contrary to this idea researchers hypothesized FGFR1 acts directly through MAPK and is imperative for NCC induction (Stuhlmiller & García-Castro, 2012b). Cardiac NCCs have been shown to express FGFR1 and FGFR3 and chemotax towards a local source of FGF4 and FGF8 in chick embryos in vivo utilizing the MAPK pathway (A. Sato et al., 2011). Alternatively, in a similar study of cardiac development in mice, FGF8 hypomorphic mutant embryos showed normal migration of cardiac NCCs, but a decrease in their survival (Abu-Issa, Smyth, Smoak, Yamamura, & Meyers, 2002).

FGF signaling has been implicated in dorsal/ventral and anterior/posterior patterning, neural induction, and limb induction (B. Thisse & Thisse, 2005). In rostral/caudal development FGF8 opposes noggin and maintains a caudal identity within the NT. As FGF8 signaling decreases the somites develop and NCCs emigrate from the NT (Martinez-Morales et al., 2011). When FGF signaling was reduced in vivo using a dominant negative (DN) FGFR1 NCC emigrated prematurely from the neural tube (Martinez-Morales et al., 2011). Other researchers using DNFGFR1 show a decrease in mesodermal cell ability to move away from the primitive streak in mouse, with perhaps an increase in E-cadherin (Ciruna et al., 1997; Ciruna & Rossant, 2001). Furthermore, in chick, researchers has shown that the FGF8 expression in the caudal region increases randomized motion of cells and is implicated in elongation of the amniote body axis (Bénazéraf et al., 2010). Thus FGF
signaling can have multiple roles, in specific tissue compartments and at very
defined time points during development.

Our first studies with blocking FGFR signaling by using Su5402 to eliminate
ECs had an impact on ventral NCC migration. We sought to determine if this effect
was direct on NCCs or the indirect result of a reduction in ECs.

**Methods**

**DNFGFR1 Injection/Electroporation**

Dominant negative FGFR1 construct (DN-FGFR1; kind gift of Dr. Kees Weijer,
Dundee, UK) which is GFP-tagged (Martinez-Morales et al., 2011) or a dominant
negative FGFR3 construct with a myc tag were used to directly block FGF signaling
in NCCs (kind gift of Dr. M. Mina, U.Conn; Havens et al., 2008). Both have been
shown to specifically block FGFR1 or FGFR3 signaling in avian embryos
respectively. White Leghorn chicken embryos were injected at HH stage 11-12 with
1-4 ug/ul of DNFGFR1 or pEYFP-N1 control plasmid. Eggs were sealed and
incubated for 1.5-2 days longer until they reached stage 19-21. They were fixed and
embedded as described previously. Chicken embryos were sectioned at 16uM and
stained with anti GFP antibody (1:2000) (Abcam), HNK, activated caspase (1:250)
(Abcam), NCAM (DH), NCadherin (DH), chick protocadherin-1 (kind gift of Roger
Bradley), tuj, islet 1&2, MeIM (DH). RCAS-DNFGFR3 plasmid was also injected at a
concentration of 1 ug/ul as a control.
FGF8 Filter Paper Application

Whatman's filter paper cut into a 1mm x 1mm piece was soaked in 1 ug/ul of FGF8b (R &D) or 1XPBS as a control. The protocol is similar to above except that chicken embryos were used and they were fixed at stage 17/18 for counts and observation. Embryos were stained with HNK and cell migration distribution were done utilizing the same method employed with Su5402 embryos, with the exception of dividing the neural tube into 3 equal parts and also having a separate category of SG region.

Neural Tube Cultures

35 mm culture dishes (mattek) where coated with 0.02 mg/ml of poly-D Lysine (company) for 30 minutes at 37 degrees Celsius. Plates were then coated with 0.02 mg/ml of fibronectin (Invitrogen) for 2 hrs at 37 degrees Celsius. After incubation the fibronectin solution was poured off and plates were allowed to dry. Media was made prior to dissection with DMEM (Gibco), 5 % serum. Eggs were windowed and staged to HH 13/14, dissected into sterile chilled 1XPBS. Once the embryos were dissected they were placed in a solution of 2 mg/ml dispase (Abnova) in 20mM HEPES/DMEM for 10-20 minutes. Neural tubes were then rinsed with complete media and further dissected in complete media. Once all somites and extra tissue was removed from the embryo the neural tube was places in the fibronectin coated plates in a minimal amount of complete media until the neural tube adhered to the dish. Neural tubes were then submerged in media with or without 30 ng/ml of FGF8. They were then placed in a 5% CO2, 99.5% humidified
incubator for 24 hours. To stain neural tube cultures they were fixed with 4% paraformaldehyde on ice for 20 minutes before continuing with IFC. To quantify the distance of NCC migration, neural tubes were stained with HNK and TUJ-1 (neuronal marker). Three representative distance measurements were made using the distance tool (line tool) in image J. The distance from the neural tube to the farthest cell in three different regions were measured for each neural tube. These three measurements were then averaged for each neural tube.

For chemotaxis assay embryos were injected/electroporated with 0.25 ug/ul of pCAX prior to dissection at stage 11/12. Media for chemotaxis assay was changed to 50% growth factor reduced matrigel diluted in 5% serum complete media. The chemotaxis experiment was done essentially the same as the chemokinetic assays with exception of the media used. Matrigel was used because of its gel-like properties—keeping beads in place. Heparin acrylic beads (company) were soaked in FGF8 or 1XPBS for 1 hour at room temperature, and washed multiple times with 1XPBS, prior to placement in close proximity to neural tubes. To quantify the number of cells within the circumference of a bead, Image J was used. To allow for different size beads concentric circles were drawn around the beads 1, 2, and 3 concentric circles. GFP expressing cells that were within these concentric circles were counted using the cell counter tool in Image J.

**Time-Lapse Microscopy**

Time-lapse imaging was performed as previously described.
N-cadherin Plasmids with DNFGFR1

Double injections were done by mixing DNFGFR1 with Full Length N-cadherin-myc, DN N-cadherin-myc (missing the extracellular domain), or full length p120-GFP-FLAG (All N-cadherin/p120 plasmids were a kind gift of Dr. Roger Bradley). 2.0 ug/ul was used for the DNFGFR1 while 1.0 ug/ul was used for the other plasmids. In order to determine if FGF8 could rescue DN-Ncadherin—transfected NCCs, filter paper squared soaked in FGF8 were applied to the trunk region 6 hours prior to dissection. These embryos were fixed at stage 18/19 and analyzed for migration using IHC. The FL, CBR, and DN N-cadherin plasmids were visualized with an antibody to the myc tag (9E10-Developmental Hybridoma). P120 plasmid was visualized using an anti-FLAG antibody (sigma). In order to determine migration effects of the different plasmids, 5-6 sections with ventral migrating cells were imaged and compared. To quantify migration the neural tube was divided and the number of transfected cells in each region was determined.

Results

FGFR1 is Expressed on Early and Late Migrating Neural Crest Cells.

Previous research has shown the expression of FGFR1 and FGFR3 on early migrating cardiac neural crest cells (A. Sato et al., 2011). Using qPCR of neural crest cells in the trunk it was found that early wave migrating trunk neural crest cells (HH stage 13) as well as the later migrating NCCs (HH stage 16) showed equal expression levels of FGFR1 (Kasemeier-Kulesa, data not published). In contrast,
FGFR3 expression was higher in the second wave of NCC migration. This was further supported using immunohistochemistry with antibodies specific to FGFR1 (Figure 19) (gift from Margaret Kirby). Previous *in situ* hybridization data has shown the expression of FGFR1 mRNA throughout the dorsal half of chick embryos and the neural tube (Nishita et al., 2011).

**Figure 19.** FGFR1 is expressed broadly in dorsal regions of the developing chick embryo. A. Immunofluorescent Chemistry (IFC) Fof HH stage 14 chick showing FGFR1 (red) colocalizes with migrating NCCs (green). B. Later stages of NCC migration show FGFR1 expression on migrating NCCs.

**DNFGFR1 Transfected Neural Crest Cells**
**Stay Dorsal and Migrate into the Epidermis**

To directly block FGFR signaling in NCCS, compared to the Su5402 blocker, we obtained DNFGFR1-pEYFP-N1 and control pEYFP-N1 plasmids ([Martinez-Morales et al., 2011](#)). We injected and electroporated them into stage 11/12 chick embryos, before NCC migration in the trunk, and evaluated transfected NCCs at
stages 18-21. In the control embryos 24% of GFP expressing NCCs migrated in the dorsal pole and dorsally (Figure 20). The majority of control NCCs migrated ventrally and occupied regions of the central DRG, ventral DRG, and SG (Figure 20A,C). Interestingly, NCCs that expressed the DNFGFR1-GFP were hindered in ventral migration similar to treatment with Su5402. A majority of DNFGFR1 cells counted, 85%, did not migrate as far as the central DRG (Figure 20B,C). Many of the DNFGFR1 expressing cells remained in an “undecided” zone, a region characterized between the neural tube and DRG, while others migrated laterally into the epidermis (Figure 20B,C). NCCs normally migrate into the epidermis at this stage, but not to the extent that DNFGFR1 expressing cells did. We used a melanocyte antibody to determine if the DNFGFR1 cells migrating into the epidermis were differentiating into pigment cells—however since this differentiation occurs later, even in control embryos, we could not determine whether they were differentiating into melanocytes (data not shown). In order to account for the difference in expression levels of the DNFGFR1 compared to the control we increased the concentration of DNFGFR1 plasmid to 4 ug/ul. More cells expressed the DNFGFR1 and were migration impaired, but this lead to unhealthy neural tube and other tissue (data not shown). When embryos were immunostained and imaged as whole mounts, it was apparent that a small subset of cells remained between the chain of DRG in the posterior somite (data not shown). Further supporting the finding that cells expressing DNFGFR1 can remain very dorsal in the MSA, similar to our findings blocking FGFR signaling with Su5402.
Another method we employed to determine effects of blocking FGFR1 signaling in NCCs migration was co-injecting/electroporating a pLenti mCherry plasmid with each construct (control and DNFGFR1). In control embryos mCherry+ and pEYFP-N1-GFP + migrated equal distances. In contrast NCCs coexpressing mCherry and DNFGFR1 did not have equal ventral migration (Figure 21 B,B’). PGK-pLenti mCherry expressing cells migrated throughout the DRG while DNFGFR1 stayed dorsal. We also controlled by injecting/electroporating a DNFGFR3 plasmid. NCCs expressing DNFGFR3 were able to migrate ventrally throughout the DRG to the same extent as control electroporated NCCs (data not shown).
Figure 20. Expression of DNFGFR1 in NCCs impedes migration ventrally with increased movement into the epidermis. A. pEYFP-N1 control cells migrate ventrally into the SG. B. GFP positive DNFGFR1 cells stay very dorsal. C. Summary graph of NCC migration/cell location when expressing DNFGFR1 vs pEYFP-N1 for total cells counted: 292 DNFGFR1 (5 embryos), 329 pEYFP-N1 (3 embryos). D. Table representing the averages for each embryo. p-value=0.032 for Epidermis, and 0.0099 for the DRG. N= 5 DNFGFR1 embryos, N=3 control.
FGF8 is Chemokinetic *In vitro*

Since FGFR1 was found to be significant for NCC migration we decided to investigate the ligands that would also be important for this signaling interaction.

FGF8 was investigated because of previous data showing it chemotactic and chemokinetic for cardiac NCCs (A. Sato et al., 2011) and its role in GnRH migration (Chung et al., 2008; Dodé & Hardelin, 2004). FGF8 and FGF4 are normally expressed

Figure 21. DNFGFR1 expressing cells stay dorsal. A,B. Co-injecting/electroporating pLenti mCherry plasmid (red) with either pEYFPN1 control (A) or DNFGFR1 (B). A, A’. pLenti mCherry and control show equal ventral migration. B, B’. DNFGFR1 cells remain dorsal compared to pLenti mCherry plasmid.
laterally and ventrally in the myotome (Geisha Reference). To test our theory of FGF8 being chemokinetic for trunk neural crest cells, we cultured neural tubes in *vitro*, in the presence or absence of FGF8b. In order to elucidate differences we stained these cultures for NCCs (HNK) and differentiated neurons (TUJ-1) to extrapolate the distance NCCs migrated. NCCs in the presence of FGF8 migrated an average of 569 um, 1.6 fold farther compared to the average 339 um NCC migrated in its absence. (p-value 0.013, N= 8 experimental and 7 control).

Figure 22. FGF8 is chemokinetic *in vitro*. A. Control NCCs emmigrate from the neural tube and migrate along the surface of the glass dish. B. NCCs migrate farther in the presence of 30 ng/ul FGF8. C. Summary chart of average distance NCC migrated from neural tube: with FGF8 mean= 569nm, N=8 and control mean= 339nm, N=7 p-value= 0.013.
FGF8 is Chemotactic \textit{In vitro}

Next, we pursued if FGF8 was not only chemokinetic, but an attractive force for NCCs (chemotactic). Again we used neural tube cultures to elucidate this. Embryos were injected with pCAX (GFP expressing plasmid) and then harvested at HH stage 13-14 for neural crest migration assays. Beads were soaked in 100ug/ml of FGF8 or 1XPBS and were placed near neural tubes (in the same neural tube culture) and covered with growth factor reduced matrigel and media. More NCCs were found in close proximity to the FGF8 bead relative to the control (Figure 23). However, this data was not significant (p-value=0.055). NCCs were counted by tracing concentric circles around the bead using Image J to account for the different sizes of the beads. Within two concentric circles of FGF8 beads there was a total of 58 NCCs compared to 15 NCCs in the 1XPBS bead (N=4 FGF8 beads and N=3 1XPBS beads).
Figure 23. *In vitro* NCCs chemotax towards a source of FGF8. A. 60x image of electroporated NCCs close to 1XPBS bead B. 60x image of FGF8 bead. On average there were 19 GFP+ NCCs counted in close proximity to the bead, with a total of 58 GFP+ NCCs (within 2 concentric circles) compared to an average of 4 GFP+ NCCs in the control with a total of 15 GFP+ NCCs. N=3 experimental, N=5 control. P-value=0.055

Average GFP+ NCCs within 2 concentric circles: FGF8 bead = 19 ± 7.7 cells, 1XPBS bead 4 ± 2.2.

FGF8 is Chemokinetic for Neural Crest Cells *In vivo*

Since FGF8 showed considerable evidence of chemotaxis/chemokinesis we wanted to determine if it promoted migration *in vivo*. We treated embryos with FGF8 by adding filter paper that had been soaked in 1 ug/ul FGF8b to the trunk region. Embryos treated with FGF8 showed a slight increase from 19+3.3% (control) to 29+3.9% in the percent of NCCs occupying the SG region (p-value of 0.023 with N=4 control and 5 experimental) (Figure 24). Total cell number and mitotic marker H3 were also analyzed to determine if there were simply more cells, as FGF8 could function as a mitogen. There were not any significant differences in cell number or in mitotically active cells—ruling out FGF8 as a mitogen for NCCs.

Since the filter paper was in close proximity to the dorsal surface, in ovo, we
interpreted these results as FGF8 acts chemokinetically—i.e. increasing random movement in general of NCCs, not necessarily in a specific direction that would imply chemotaxis.

Figure 24. FGF8 is chemokinetic in vivo. A. Transverse section of a control embryo with 1XPBS filter paper added to the trunk, with normal SG migration. B. 1 ug/ul of FGF8 soaked filter paper added to the trunk region lead to an increase in the percentage of NCCs that migrate into SG region. C. Summary of NCC location based on 3 regions equally divided by length of the NT and the SG. Percent cells in SG region, ventral 1/3 of neural tube, middle 1/3 of neural tube, dorsal 1/3 of neural tube. There was an increased ventral migration in the SG region from 19% in the control to 29% of total NCCs in the experimental. N=4/5 control and N= 5 experimental, p-value 0.023.
Cell Morphology of DNFGFR1

The morphology of NCCs expressing the DNFGFR1 was distinct from that of control transfected NCCs while a subset of DNFGFR1 cells displayed a very round cell body with multiple filopodial extensions protruding into the environment (figure 25). The rounded cells display a similar phenotype to the NCCs in the Su5402 treated embryos “honey comb” morphology. There were three different phenotypes observed in DNFGFR1 expressing NCCs: round, multipolar and polar. Round or globular DNFGFR1 cells were found when in close contact with one another and appear “stuck” together (figure 25B). Other DNFGFR1 cells in close proximity to one another or alone display a multipolar morphology, extending multiple filopodia, this could be indicative of a “confused” or stalled cell or a cell with increased adhesion (data not shown). Isolated DNFGFR1 expressing cells were more likely to exhibit a polar phenotype although a subset did display multiple filopodia (figure 25C). To quantify these changes, measured the length, width, and number of filopodia were measured. Control transfected NCCs displayed a very polar morphology (length/width 2.4) with an average of two filopodial extensions, while DNFGFR1 transfected NCCs had a very rounded morphology (length/width ratio of 1.0) with multiple extensions (Figure 27). These dimensions were counted using live imaging. Even though DNFGFR1-transfected NCCs displayed an impairment in ventral migration, they were still very dynamic and actively extend filopodia, as observed with live time-lapse imaging (Figure 28).
Figure 25. DNFGFR1 cells display different morphologies. A. Control cells were very polar even when in contact with other cells. B. DNFGFR1-transfected NCCs in close contact display a globular morphology. C. A subset of DNFGFR1 cells in close proximity or alone display a multipolar morphology. D. Three morphologies were observed in DNFGFR1 cells: Globular, multipolar, and polar. These Globular cells were almost solely found in contact with one another while polar DNFGFR1 cells were usually alone (not contacting other DNFGFR1 expressing cells.) A total of 103 cells were counted from 3 different embryos.
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Figure 26. DNFGFR1 cells are multipolar and rounded. Length/width ratio for DNFGFR1 is closer to 1 (rounded) compared to that of control with more filopodial extensions. P-value 0.0026 and 0.0016 respectively N= 14 experimental and N=12 control, only live cells measured.

Figure 27. DNFGFR1 cells extend multiple dynamic filopodia. One cell followed through time-lapse analysis that resided between the DRG and neural tube in this “undecided” region. Images were taken every 5 minutes.
The Fate of DNFGFR1 Transfected Neural Crest Cells

Since we found that NCC migration was impaired when FGFR1 signaling was reduced, we wanted to determine whether these NCCs were able to differentiate into neurons and/or if their morphology could be explained by imminent apoptosis. To determine if these cells had impaired migration and a difference in morphology due to cell death we stained for activated Caspase 3. DNFGFR1 cells at stage 20/21 were not positive for Caspase 3 (Figure 29A,B). To further explore their fate, we stained with antibodies Tuj-1 and islet 1&2 to determine if the DN-FGFR1 transfected cells differentiated into neurons. From these experiments it can be concluded that DNFGFR1+ NCCs that migrate into the DRG are capable of differentiating into neurons, i.e. they co-express the DNFGFR1 and neural markers (Figure 29D). DNFGFR1+ cells that stay very dorsally and don’t migrate into the DRG do not differentiate into neurons (Figure 29E). To determine the fate of the very dorsal DNFGFR1 cells and even the cells that migrate into the epidermis we stained embryos that were incubated an additional day, (HH stage 24) to determine if those cells had delayed migration and would eventually migrate into the DRG. What we found was that at stage 24 the cells that were occupying the undetermined area dorsal to the DRG were now absent, even DNFGFR1+ cells in the epidermis were no longer present (data not shown). There were fewer DNFGFR1+ NCCs present and those that remained were in the DRG. Perhaps, cells that were in this “undecided” region must have died. Therefore, making it into the DRG not only leads to neural differentiation, but to the continued survival of DNFGFR1 expressing...
NCCs. Since these dorsal cells eventually die, this could also lead to or explain their altered morphology.

Figure 28. DNFGFR1 cells differentiate into neurons if they migrate into the DRG and those that stay dorsal eventually undergo apoptosis. A. Control pEYFP-N1+ NCCs cells are not undergoing apoptosis, (Activated Caspases 3-red). B. DNFGFR1+ NCCs are not caspase positive at stage 20/21. C. A subset of control neural crest that migrate into the DRG differentiate (islet-1 and islet-2). D,E. DNFGFR1 expressing cells differentiate when they migrate into the DRG (D) but do not develop into neurons in the “undecided” zone (B). A total of 158 DNFGFR1 cells and 186 pEYFP-N1 cells were counted.
Overexpression of N-cadherin Partially Rescues Loss of FGF Signaling in Neural Crest

Previous research has shown that FGFR1 can regulate Epithelial-Mesenchymal-Transition (EMT) by directly binding cell adhesion molecules (CAMs) NCAM, N-Cadherin, and L1 in the absence of FGF ligand (Doherty & Walsh, 1996; Saffell, Williams, Mason, Walsh, & Doherty, 1997; E. J. Williams, Furness, Walsh, & Doherty, 1994a). Since NCCs expressing the DNFGFR1 show impaired migration we sought to determine the role of adhesion molecules, especially given the DNFGFR1 NCC morphology. Previous studies on cranial NCC have shown that aberrant E-cadherin can impair migration and includes a decrease in N-cadherin (C. D. Rogers, Saxena, & Bronner, 2013). This phenotype was rescued by overexpressing N-cadherin. Because of this similarity we co-injected a plasmid encoding the FL-N-cadherin (kind gift from Roger Bradley) with DNFGFR1 plasmids into the neural tube. We found that NCCs co-expressing DNFGFR1 and FL-N-cadherin migrated farther ventrally (average of 58.4 + 11.4% remained in the dorsal 1/3 of NT boundary) than those embryos with DNFGFR1 alone (average of 80.0 +7.8%) (p-value = 0.037 N= 5 of each condition) hence N-cadherin partially rescued the DNFGFR1 NC ventral migration defect (Figure 30 A,B, E). A subset of double transfected cells still maintained a multipolar phenotype (data not shown). We also tried to rescue DNFGFR1 by using a plasmid encoding the Catenin Binding Region plasmid (CBR-CS2-myc, kind gift of Roger Bradley) that competes with β-catenin and would disrupt multiple cell adhesion molecules. A subset of cells that co-expressed DNFGFR1 and CBR remained in the lumen of the neural tube, thus they
were not able to undergo a complete EMT (data not shown). We also tried to rescue the DNFGFR1 cells with a p120 plasmid. P120 should increase the activity of Rac1 and Cdc42 indirectly through Vav2 and directly binds inhibiting RhoB (Anastasiadis, 2007). The p120 did not rescue the DNFGFR1 cells (only 2 embryos were observed); however, NCCs transfected with p120 alone remained dorsal, did not migrate ventrally to the same extent as control and they showed multiple filopodial extensions.

Preliminary Studies:
**DN-N-cadherin Impairs Ventral Migration**

Since FL-N-cadherin partially rescues DNFGFR1 cells we wanted to determine N-cadherin’s role in NC migration. Preliminary studies using DN-N-cadherin, lacking the extracellular domain (kind gift of Roger Bradley), showed impaired migration when compared to FL-N-cadherin co-injected with DNFGFR1. Previous research using FL-N-cadherin alone shows transfected NC cells localized to the DRG, while DN-N-cadherin expressing cells remained in the dorsal pole of the DRG with a subset of NCC in the ventral root (Bradley, data not published).

Furthermore, double electroporation of DN-N-cadherin with DNFGFR1 resulted in intensified migration impairment, compared to either construct alone in terms of the number of transfected cells remaining in contact with the neural tube as opposed to just staying dorsal (Figure 30 D, F)(anova p-value 0.0077, N=3/4 of double transfected and 3 embryos of the other conditions).
E

% NCCs within the dorsal 1/3 region

- DNFGFR1
- Ncadherin + DNFGFR1
- DN-N-cadherin

80 58.4 84
Discussion

**FGFR1 Functions in Neural Crest Migration**

In this study we have shown that a functional FGFR1 is imperative for ventral migration of NCCs in the trunk of chicken embryos, specifically for NCCs to migrate into the SG and ventral DRG regions. The generalized FGFR blocker Su5402 (which
blocks FGF signaling through all FGFR) leads to an accumulation of NCCs dorsally, with a morphology resembling “honeycomb”. Specifically blocking FGFR1 signaling with a dominant negative FGFR1 also resulted in cells staying very dorsal and with some even migrating prematurely into the epidermis. FGFR1 is expressed on migrating NCCs of the trunk as determined through RT-qPCR (Kasemier-Kulesa, data not published) and by Immunohistochemistry on cardiac NCC (A. Sato et al., 2011). FGFR1 is implicated in random cell migration in the early anterior/posterior formation in the chick embryo via FGF8 (Bénazéraf et al., 2010). Furthermore, FGFR1 has been shown to promote migration of cancer cells. Directly inhibiting FGFR1 signaling caused cancerous cells to switch from a mesenchymal, motile, fate back to a more stationary, epithelial, state. FGFR3 was not shown to have a function in ventral NCC migration, as NCCs transfected with DNFGFR3 migrated to and throughout the DRG.

**FGFR1 is Important for Cell Morphology**

FGFR1 effects NCC morphology. A subset of DNFGFR1 transfected NCCs displayed normal polar morphology, while others extend multiple thin filopodial extensions from a rounded soma. While these cells have an impaired ventral migration, they are still very dynamic—extending filopodia and sensing their environment. In normal development NCCs with multiple filopodial extension usually migrate at the leading edge of collective NCC migration, traversing large distance from the neural tube (Bianco et al., 2007; Wynn et al., 2013). These cells search their environment more, while the trailing cells are more polarized and less
exploratory. In our live imaging analysis, we saw that DNFGFR1 transfected NCCs constantly extend filopodia perhaps searching for environmental signals that they are not able to perceive, but their overall displacement is small. A rationale for the variation in morphologies observed could be the pleiotrophic affects of varying concentrations of the DNFGFR1 protein expression (Garcia-Mayar et al., 2006).

FGF8 is Chemokinetic for Peripheral Trunk NCCs In vitro and In vivo

Mutations in the genes encoding FGF8, FGFR1, and the ECM molecule ansomosin can all cause Kallman syndrome (Hu & Bouloux, 2010). FGF8 has been found to be chemotactic in multiple stages and areas of development. It is chemotactic and chemokinetic for cardiac NCCs (A. Sato et al., 2011). We have shown that FGF8 is chemokinetic for trunk NCCs in vitro. When FGF8 was directly added to the media it resulted in farther migration of NCCs from the neural tube compared to the control. This is similar to results in cardiac NCC (A. Sato et al., 2011). To determine if FGF8 was also chemotactic we implanted FGF8-soaked beads in matrigel near neural tubes. More NCCs were found in the region around FGF8 beads compared to control; however, this is difficult to prove that it is not solely chemokinesis that has caused more cells within the region of the beads to move, not in a specific direction. In vivo FGF8 was shown to be chemokinetic. By adding FGF8 soaked filter paper to the dorsal surface of the trunk more NCCs migrated farther ventrally, into the SG region, compared to the control. Although FGF8 binds FGFR1 there are other receptors that are expressed on NCCs that it
could be acting through (FGFR4 or FREK, FGFR3, FGFR2). FGF8 binds other FGFRs with greater affinity than FGFR1, such as FGFR3 (Ornitz et al., 1995BC). However our studies showed that blocking DNFGFR3 did not perturb the ventral migration of NCCs, implying that FGFR3 is not functioning (not as important at least) in NCC migration.

Consistent with the chemokinetic role rather than chemotactic role, in situ hybridization data show the source of FGF8 is lateral and ventral in the developing embryo, not in regions where NCCs migrate (geisha reference). In cranial NCCs in chick and mice the FGF source is very distinct and concentrated in the regions in which NCCs migrate and stop (Kubota & Ito, 2000; A. Sato et al., 2011; N. Trokovic, Trokovic, & Partanen, 2005). This is indicative of most chemotactic molecules with high expression in the target area or destination. In the case of FGF8, trunk NCCs migrate next to these regions, but the expression is too lateral to be a target. However its expression relative to the migratory route of NCCs is consistent with a chemokinetic function. Very low concentrations of growth factors can be difficult to detect via in situ probes or IFC. Our findings are similar to those of mouse mesencephalic NCCs in which it was shown that FGF8 was chemoattractive in vitro, but in vivo it was not apparent that it had an attractive role, although blocking it changed NCC migration compared to the control (Kubota & Ito, 2000). In this example FGF8 was thought to stimulate the production of FGF2 binding FGFR1 and FGFR3 stimulating chemotactic migration, thus FGF8 acted indirectly (Kubota & Ito, 2000).
Neural Crest Cell Targeting in the DRG is Imperative for Survival and Differentiation

A large number of NCCs expressing DNFGFR1 remain very dorsally in an undecided zone between the DRG and the neural tube. At later stages (HH 25) these transfected NCCs were absent, with a small fraction of DNFGFR1 expressing cells in the DRG. An explanation for this could be that they have undergone apoptosis without the survival factors that are located within the DRG. Hence, the DRG promotes survival of DRG-destined NCCs. Even though these cells were not undergoing apoptosis at stage 21, it is very likely that they perish before stage 24/25 because of the small number of cells expressing DNFGFR1 that were still present at that stage. Alternatively, plasmids that are electroporated become very dilute since they are not incorporated into the cellular DNA, the expression of these plasmids could diminish after a few days (Y. Sato et al., 2007). However, expression of electroporated plasmids is still observed in DNFGFR3 cells at stage 27/28, a later stage of development than what was observed for DNFGFR1, so this is not likely.

These very dorsal NCCs that appear lost are not differentiated, while the DNFGFR1 NCCs that are able to make it into the DRG do differentiate into neurons. In another study using DNFGFR1, cells were able to differentiate into neurons in vitro (Deng, 1997). Thus, in order to develop into a functional sensory neuron NCCs need to integrate into the DRG. These results are similar to studies in Xenopus. Cadherin-11 was found to influence lamellapoidia and filopodia formation and regulated migration of cranial NCCs. Similar to our results, migration was discovered to be a prerequisite for differentiation (Borchers, 2001)(Koehler et al., 2013). A subset of
cells that express DNFGFR1 migrate prematurely dorsolaterally, but do not
differentiate into melanocytes. This is evident because they were not positive for a
melanocyte marker. These cells also are absent at later stages.

N-cadherin is Required for Trunk
Neural Crest DRG Localization

In chick cranial NCCs, blocking the transcription factor Sip1 resulted in NCCs
remaining dorsal. In these experiments they found an aberrant expression of E-
cadherin with a decrease in N-cadherin and cadherin-7. Since our results were
similar to the phenotype of knocking down Sip1 in cranial NC we wanted to
determine if overexpressing N-cadherin could rescue our DNFGFR1 cells, if they too
had aberrant expression of E-cadherin. Adhesion molecules are hypothesized to
regulate one another. N-cadherin has been shown to regulate E-cadherin levels
(Gottardi, 2001). Co-expressing full length N-cadherin and DNFGFR1 resulted in
increased ventral migration of DNFGFR1 transfected NCCs, partially rescuing their
migration. Here we use the word “partial” because normal numbers of NCCs in the
SG were not quantified or compared to pEYFP-N1 control embryos. DN-N-cadherin
expressing NCCs also remained very dorsal, further implicating N-cadherin in
migrating neural crest. DN-N-cadherin lacks the extracellular domain that would be
instrumental in homotypic binding to neighboring cells. Before NC emerge from the
NT, N-cadherin is down-regulated. Previous research has shown through IHC that
NCCs don’t re-express N-cadherin until they condense to form the DRG (Bronner-
Fraser et al., 1992). Conversely, in vitro, N-cadherin is expressed on migrating NCCs
(Monier-Gavelle & Duband, 1995). It is possible that N-cadherin is expressed in small amounts on migrating NCCs and IHC is not sensitive enough to detect the signal. N-cadherin functions in collective cell movements by binding p120 and beta-catenin (Anastasiadis, 2007; Xiao, Oas, Chiasson, & Kowalczyk, 2007). P120 directly binds and inhibits RhoA and indirectly activates, through Vav2, Rac1 and Cdc4 (Anastasiadis, 2007; Wheelock & Johnson, 2003; Xiao et al., 2007). These Rho-GTPases are important for lamelapodial and filopodial formation.

Theories About FGFR1 Signaling in Neural Crest

FGFR1 Directly Impacts Neural Crest Cell Migration

Our data indicate that FGF signaling directly impacts trunk migration. Cardiac NC and cells of the primitive streak expressing FGFR1 were both shown to increase migration in the presence of FGF8. Our results also show an increased motility in the presence of FGF8 in vitro and in vivo. Both previous studies found that the MAPK intracellular pathway was stimulated (although they did not rule out other FGFR) (Bénazéraf et al., 2010; A. Sato et al., 2011). Furthermore, FGF-2 addition to cultured human epithelial cells has been shown to activate Rho/Rac GTPases (Maddala, Reddy, Epstein, & Rao, 2003). Interactions through these molecules would directly link FGFR1 signaling to actin cytoskeleton, motility, and morphology. The protein IQGAP1 has been shown to directly bind the cytoplasmic tail of FGFR1. This interaction indirectly activates Arp2/3 and functions in actin nucleation and lamelipodia formation (Benseñor et al., 2007). In our findings
DNFGFR1+ NCCs extended multiple thin filopodia, but do not form lamelipodia. These findings could implicate IQGAP1’s importance in FGFR1 signaling for ventral migration of NCCs.

**FGFR1 Signaling is Required for a Neural Identity**

FGFR1 signaling could also be a cellular signal that induces “neural” identity of trunk NCCs and a loss of functional FGFR1 results in a default “loss of identity” phenotype. Earlier in development FGF is directly implicated in neural induction of neural crest (Alvarez, Araujo, & Nieto, 1998; Storey et al., 1998). FGF signaling has been shown to be important for neural crest induction in the ectoderm in Xenopus (Stuhlmiller & García-Castro, 2012b). Expression of a dominant negative FGFR1 in chick and Xenopus lead to major malformations in primitive streak formation (Yang et al., 2002). DNFGFR1 expression in Xenopus resulted in the absence of NC transcription factor XSlug expression (Mayor, Guerrero, & Martínez, 1997). Conditional deletions of FGFR1 in mice lead to limb abnormalities, spina bifida, and neural tube defects (Deng et al., 1997). Perhaps FGFR1 continues to have a “neural” role in migrating NCCs. However, in another study using DNFGFR1, mouse embryonic stem cells were able to differentiate into neurons *in vitro* (Deng et al., 1997). Neural differentiation was based entirely on the NCC position: those DNFGFR1+ NCCs that migrated into the DRG were able to differentiate into neurons, similar to control. This argues against the “loss of identity” theory.

Alternatively, FGFR1 and Wnt are hypothesized to regulate one another. It has been
shown that Wnt/b-catenin offsets FGFR1/FGF signaling in collective migrating cells (Aman & Piotrowski, 2008). Thus decreasing FGF signaling could increase Wnt signaling, which could lead to a melanocyte morphology (Dorsky et al., 1998). The observation that DNFGFR1 cells migrate into the epidermis supports this theory. Although, they were not positive for melanocyte antibodies, but neither were control NCCs in the epidermis at the stage analyzed. It is plausible that rescuing with N-cadherin made the DNFGFR1 cells more “neural”. Thus, N-cadherin restored their identity or at least promoted ventral migration thereby getting those double transfected NCCs into the DRG and able to respond to neural cues within the DRG. In order to ascertain this “loss” of identity theory DNFGFR1 cells would need to be analyzed for transcription factor expression.

**FGFR1 Regulates Cell Adhesion**

FGF signaling has also been implicated in cell adhesion. It has been shown to regulate EMT, levels of certain cadherins, and to further complicate the story FGFR1 can directly bind CAMs. The migration impairment characterized by DNFGFR1-transfected NCCs could result from an increase in cell adhesion. In a previously published study, NCCs overexpressing N-cadherin displayed a similar morphology (Kasemeier-Kulesa et al., 2006), suggesting that overexpression or over-activation of CAMs could impair ventral migration and that blockade of FGFR1 signaling somehow increases expression or activation of a CAM. Recently, a transcription factor expressed by cranial NCCs, Sip1, was found to indirectly regulate migration in cranial NCC because of its direct influence on CAMs. When blocked it resulted in
NCCs staying dorsally, similar to our results. These researchers found that blocking Sip1 lead to an aberrant increase in E-cadherin expression and a decrease in N-cadherin and cadherin-7. They were able to partially rescue the phenotype by adding a DN-E-cadherin or full length N-cadherin (C. D. Rogers et al., 2013).

Interestingly FGF signaling has been related to Sip1 expression in early chick development. FGF signaling is important for the zinc finger protein Churchill to be expressed which directly regulates Sip1 expression and switches FGFs role in development from a mesodermal into neural (Sheng, Reis, & Stern, 2003).

FGFR1 has been shown to function in EMT in cancer (Nguyen et al., 2013) and in mesoderm development in mice (Ciruna et al., 1997; Ciruna & Rossant, 2001). Disrupting FGFR1 in chimeric mice resulted in mesodermal cells staying in the primitive streak, which was caused by an inappropriate up-regulation of E-cadherin (Ciruna & Rossant, 2001). Research in chick primitive streak formation argued against a role for FGFR1 in regulating adhesion and links cell movements through the MAP/Kinase or PI3K/AKT pathways (Hardy, Yatskievych, Konieczka, Bobbs, & Antin, 2011). It is possible that FGFR1 functions in EMT during development in trunk NCCs and even though DNFGFR1 cells emigrate from the neural tube, they do not fully undergo this transition. However, previous research with DNFGFR1 in NC showed that they emigrate early from the neural tube (Martinez-Morales et al., 2011). It doesn’t seem likely that they would migrate early if they didn’t fully undergo EMT. Another reason our studies on FGFR1 imply a role in CAM expression is because of the similarity in results to those of chick pdcdh-1:
increased cpdcdh-1 localized NCC to the DRG at the expense of the SG (Bononi et al., 2008). These alterations in CAM lead to different proportions of NCCs migrating ventrally and argue that variation in CAM levels has profound effects on the distance traveled by recently emigrated trunk NCCs.

FGFR1 has been shown to act non-autonomously by binding CAMs. FGFR1 binds N-cadherin and causes neurite outgrowth in vitro (Utton, Eickholt, Howell, & Wallis, 2001). The mechanisms of growth cone movements are similar to those of NCCs (Lefcort et al., 2007). Therefore it is plausible that NC filopodial dynamics may act through similar mechanisms. FGFR1 is capable of binding NCAM independent of FGF ligands while its binding to N-cadherin has conflicting results whether ligand binding is involved (Giampietro et al., 2012). The binding of N-cadherin to FGFR1 has been shown to promote neurite outgrowth in vitro (Doherty & Walsh, 1994; E. J. Williams, Furness, Walsh, & Doherty, 1994a) and stimulate migration of cancer cells (Suyama, Shapiro, Guttman, & Hazan, 2002) and endothelial cells (Giampietro et al., 2012). FGFR1 has been shown to bind N-cadherin in multiple immunoprecipitation assays (Boscher & Mège, 2008) (Sanchez-Heras, Howell, Williams, & Doherty, 2006; Suyama et al., 2002; Utton et al., 2001). Interestingly, ectopic expression of N-cadherin in cancer cells in vitro increased their migration, which was further increased in the presence of FGF2. They found that MMPs were up-regulated in these highly motile cultures (Nieman, Kim, Johnson, & Wheelock, 1999; Suyama et al., 2002).
N-cadherin is composed of 5 extracellular domain repeats that bind and are stabilized by calcium ions and intracellularly connects to the cytoskeleton by binding alpha and beta catenin, or p120. The farthest EC domains 1 and 2 (N-terminal) are important for the cis homotypic binding, although all EC domains can participate in trans homotypic binding (Cavallaro & Dejana, 2011) (Wheelock & Johnson, 2003). EC4 domain of N-cadherin has been shown to be the domain most important for metastasis in cancer cells (J. B. Kim et al., 2000). Intriguingly, it has been shown through blocking proteins that the region between the 1st and 2nd Ig domain of FGFR1 binds the EC4 region of N-cadherin (Sanchez-Heras et al., 2006; E.-J. Williams et al., 2001). Whether it is the CAM homology domain (HAV domain) between the two Ig domains that is binding N-cadherin is controversial (Doherty & Walsh, 1996; Sanchez-Heras et al., 2006). The specific mechanism that regulates the separation in these opposing functions of migration verses adhesion for N-cadherins are not yet determined. Interestingly in studies of N-cadherin/FGFR1 binding, the two proteins do not co-localize in regions where tight cell-cell contacts are formed by N-cadherin, rather they co-localize in other regions of the cell (Sanchez-Heras et al., 2006). It is plausible that binding to EC4 from FGFR1 or other proteins disrupts N-cadherin’s ability to bind homotypically and form tight junctions, thus the cells are more motile. Although, this does not explain why increasing N-cadherin would increase migration. N-cadherin is thought to stabilize the expression of FGFR1 extracellularly, resulting in stimulated FGFR1 signaling (Suyama et al., 2002). N-cadherin is expressed is increased when NCCs start to condense to from the DRG
(Bronner-Fraser et al., 1992). Another possible reason the N-cadherin partially rescues the DNFGFR1 phenotype cells could be that the DNFGFR1 + transfected cells are becoming more localized to the DRG and not an increase in migration; perhaps DNFGFR1 are able to transiently adhere to one another and form the DRG.

NCAM has also been shown to bind FGFR1 promoting migration and sustaining FGF signaling (Doherty & Walsh, 1996; Kiselyov, 2009; Kochoyan, Poulsen, Berezin, Bock, & Kiselyov, 2008). NCAM is expressed on NC condensing to form the DRG (Bronner-Fraser et al., 1992). In an interesting study, researchers found that binding of FGF2 lead to proliferation while NCAM binding FGFR1 promoted migration in vitro in HeLa cells. These researchers implied that NCAM was a carrier protein to promote FGFR signaling in areas of the cell where it is normally degraded (Francavilla et al., 2009). NCAM's role in NCC migration has not been researched. FGFR1 has also been shown to bind non-autonomously to integrins, ligand independent, and stimulate the migration of liver ECs (Zou, Cao, Kang, Huebert, & Shah, 2012).

FGF signaling has diverse functions in development. The promiscuity of receptors, plethora of ligands, and non-autonomous signaling complicate the story of FGFs in development (view model1 and model2). From our research we have shown that FGFR1 is required for ventral migration of trunk NCCs. The morphology of the DNFGFR1 cells indicates a perhaps a difference in cell adhesion or polarization. Moreover, FGFR1 has been directly linked to CAM in neural induction, emigration from the neural tube, neurite outgrowth, and cancer. A role for FGFR1
and CAM in NCC migration would not be surprising given the various other instances where they interact.

Figure 30. Model 1: Intracellular pathways possibly mediating NC migration. 1. FGFR1 signaling could act independently by activating the MAP kinase, PLC, PI3K, or directly binding IQGAP1. 2. Alternatively it could be acting by directly binding to the EC4 domain of N-cadherin. This interaction would promote FGFR1 signaling and has been found in neurite outgrowth and cancer metastasis. The rescue of N-cadherin could be mediated by the direct interaction of FGFR1 and N-cadherin, or the independent intracellular pathways linked to N-cadherin. 3. N-cadherin intracellularly binds B-catenin and p120. B-catenin activates alpha catenin that directly impacts the actin cytoskeleton. Alternatively cytosolic p120 (p120 not bound to cadherins) can directly bind and inhibit RhoA while indirectly activating Cdc42 and Rac1, both mediate lamellapodial formation and migration.
Figure 31. Model 2. The intracellular pathways that are inhibited using DNFGFR1. Double knockdowns of N-cadherin and FGFR1 (DN-N-cadherin and DNFGFR1) lead to even further migration impairment in migration. This could imply that two separate pathways are at work, are both altered or the same pathway has been even further impaired.
Introduction

The mechanisms that drive differentiation of neural crest into a heterozygous population of neurons and glia contained in the DRG remain largely undetermined. Neurogenesis—birth of neurons, occurs in two distinct waves within the developing DRG. The first wave of neurogenesis is characterized by the expression of the basic helix-loop-helix (bHLH) transcription factor Neurogenin 2 (Ngn2). These cells differentiate into the large diameter TrkC and TrkB neurons that become proprioceptors and mechanoreceptors respectively (George et al., 2010; Lefcort et al., 1996; Rifkin et al., 2000; Snider, 1994). The majority of early differentiating neurons express TrkC (Lefcort et al., 1996). The second wave of neurogenesis is characterized by the expression of a different bHLH transcription factor Ngn1, these cells differentiate into pain (nociceptors) and temperature (thermoreceptores) receptors and express TrkA. Interestingly a subset of these cells derive from the contralateral side of the neural tube and the neural progenitors on the perimeter of the forming DRG (George et al., 2007) (George et al., 2010).

The DRG is sculpted by an intriguing phenomenon of programmed cell death (Reviewed in (Nikoletopoulou et al., 2010)). Neurons survive by binding specific neurotrophins that are expressed within the DRG and by target tissue that axons are seeking to innervate (Rifkin et al., 2000). It is hypothesized that the amount of target tissue determines the number of neurons that survive (Snider, 1994).
Our preliminary studies with FGFR blocker, Su5402, stimulated our interest in the hypothesis that FGFR signaling may function directly in the differentiation of TrkA neurons. FGF signaling functions in multiple steps that regulate development from neural induction to neurite outgrowth (Storey et al., 1998; E. J. Williams, Furness, Walsh, & Doherty, 1994a). We have shown its importance in NCC migration, but its role in differentiation of the DRG has only recently been studied (Hadjab et al., 2013). From using Su5402 to block FGFR signaling we found a decrease in the percentage of trkA neurons. We sought to determine if ECs were involved in trkA differentiation, or if FGF signaling directly mediates neural differentiation.

**Methods**

*In Situ Hybridization*

In situ hybridizations were performed by Martha Chaverra as described (Nelson, Claes, Todd, & Chaverra, 2004; Rifkin et al., 2000).

*DNFGFR3 Injections/Electroporations*

DNFGFR3-CS2-myc plasmids and control CS2-gfp plasmids were injected into HH stage 11/12 chick embryos and fixed at stages 20/21 or 27/28. Embryos were analyzed using IFC with antibodies to TrkA, TrkB, TrkC (Lefcort et al., 1996) Tuj-1, and 9E10 (myc). The fate of transfected cells was determined in five sections from the trunk region of each embryo condition.
Results

FGFR3 is Expressed within the Developing DRG

To explore the function of FGFR in the developing DRG we determined the spatial and temporal expression of the different isoforms. From our in situ hybridization data we found that FGFR3c mRNA is expressed within the developing DRG at stage 19 and 21, specifically concentrated in the dorsal pole and along the DRG perimeter (Figure 31). However, by stage 24 FGFR3 mRNA expression is gone.

FGFR3 is also expressed in the NT, with a distinct localization in the floor plate and in the ventricular zone (VZ). Both the VZ and the DRG perimeter are established progenitor zones. FGFR2 was expressed dorsally in the ectoderm, but not within the developing DRG (data not shown). FGFR1 and FGFR4 (FREK) were not studied, although previous studies in situ indicate FGFR4 expression in the developing DRG (geisha). Furthermore, FGFR1 has also been shown to be expressed in the developing DRG (Hadjab et al., 2013). The expression of FGFR3 was further confirmed through q-PCR which showed that FGFR3 was expressed by migrating NCC with an increase in expression during the second wave of migration (HH stage 16) compared to the first (HH stage 13) (p-value=0.0018) (data not shown)(Kasemier-Kulesa, unpublished).
Figure 32. *In situ* hybridization images showing expression of FGFR3 in the DRG at stage 19 and 21. FGFR3 is localized to the DRG at stage 19 and strongly expressed in the dorsal pole at stage 21. By stage 24 FGFR3 expression is gone from the DRG. *In situ* data/images were performed by Martha Chaverra, Lefcort Lab.

DNFGFR3 transfected Neural Crest Cells Differentiate at Early Stages

DNFGFR3-transfected NCCs migrated appropriately to the region of the DRG and throughout the DRG, and differentiated into neurons similarly to control. This was supported by quantifying GFP or myc + cells that were also differentiated neurons (control 88.0 ± 6.5% GFP+/Tuj-1 neurons compared to 79.4 ± 5.0 % myc+DNFGFR3/ Neurofilament+) but was not statistically significant (p-value = 0.21 N= 3 DNFGFR3, N= 4 control) (Figure 32). DNFGFR3 expressing NCCs were able to differentiate at this early stage into neurons. These neurons correlate temporally with the early wave of neurogenesis of the large diameter trkC and trkB neurons (George et al., 2010; Lefcort et al., 1996).
Figure 33. DNFGFR3 expressing Neural Crest Cells are able to differentiate into neurons at stage 21/22 similar to control. A. 88.0 % of control CS2-gfp expressing cells differentiate into neurons, visualized with Tuj-1 antibody. B. 79.4 % of DNFGFR3 cells differentiate into neurons, visualized with neurofilament (BGNF). BGNF and Tuj-1 co-localized completely in separate tests.

DNFGFR3-transfected Neural Crest Cells
Undergo Reduced Differentiation into TrkA+ Neurons: Preliminary Studies

Preliminary studies injecting/electroporating DNFGFR3 into chick embryos and analyzing at stage 27/28 showed a decrease in trkA positive neurons. Very early data shows a decrease in the percentage of DNFGFR3+, TrkA+ neurons (control 12.0±1.1 % trkA+ transfected/ total transfected compared to 5.6 ± 1.2 DNFGFR3+ TrkA+). However, only two embryos of each condition have been analyzed (2004 transfected control cells counted and 889 transfected experimental) (Figure 33). These experiments are very preliminary and more embryos need to be counted in order to definitively determine that FGFR3 functions in TrkA differentiation.
Figure 34. DNFGFR3 transfected NCCs do not differentiate into TrkA neurons to the extent that control (CS2-GFP) NCCs do. A. Control DRG with CS2-GFP NCCs 12% differentiate into trkA neurons (red), i.e. they colocalize compared to DNFGFR3 6%

**Discussion**

**FGFR3 is Expressed in the Dorsal Pole of the DRG where TrkA Neural Progenitors Reside**

FGFR3 is expressed throughout the DRG, but most concentrated at the dorsal pole. The mRNA expression FGFR3 spatially correlates with the localization of progenitor cells, and is also similar to the chick pcdh-1 expression (Bononi et al., 2008; George et al., 2007; 2010). FGFR2 was not found in the DRG, and FGFR1 and FREK (FGFR4) were not studied but could also be implicated in neurogenesis and/or differentiation. Recent research has shown that FGFR1 has important functions for differentiation of TrkA neurons in mice through the MAP Kinase and PI3K pathways (Hadjab et al., 2013). The ligands FGF1 and FGF2 also increased the
expression of RunX1+, late born TrkA neurons; however the mechanisms are unknown (Hadjab et al., 2013). Our latest findings proving FGFR1 functions in ventral NCC migration could also explain a decrease in neural differentiation, observed in Hadjab et al, 2013, especially if a majority of NCC are not migrating into the DRG.

**FGFR3 may Function in TrkA Differentiation**

Our previous studies blocking FGFR signaling using Su5402 resulted in a decrease in trkA+ neurons. Preliminary studies decreasing FGFR3 lead to a decrease in the percentage of trkA neurons. Further studies are required to determine if reducing signaling through FGFR3 alters total neuronal number. Also progenitor cells were not quantified to determine if the trkA difference starts with a difference in progenitor cells. Because we did not observe a statistical difference in neural differentiation at stage 22 when TrkB and TrkC neurons are present, we hypothesize that FGFR3 does not function in trkB or TrkC differentiation.

Our lab has shown previously that the TrkA+ progenitors in the dorsal pole express the transcription factor Pax3—in future studies, we can determine if the Pax3+ progenitors die and/or drop out of the cell cycle. FGFR3 expression at stage 21 in the dorsal pole is earlier than observed Pax3 expression (trkA progenitors) at stage 23 (George et al., 2010). This is evidence that FGFR3 is ideally positioned to impact function of progenitor cells. Possible roles include regulating progenitor survival, self-renewal, or differentiation. FGFR3 acts redundantly with FGFR1 and FGFR2 in the CNS supporting neural progenitor survival, specifically in the
rhombomere 1 of the midbrain (Saarimäki-Vire et al., 2007). FGFR3 has also been found to function in differentiation of oligodendrocytes in the CNS through the MAP Kinase intracellular pathway. Mice deficient in FGFR3 had less oligodendrocytes because progenitor cells were not differentiating into oligodendrocytes (Oh et al., 2003). Thus, FGFR3 regulates the amount of oligodendrocytes by promoting progenitor differentiation (Fortin, Rom, Sun, Yayon, & Bansal, 2005; Oh et al., 2003). Whether it has a similar role in neural progenitors needs to be further addressed. These functions of FGFR signaling of glia progenitors and neural progenitors will need to be encompassed into further investigations.

The decrease in trkA neurons we found by broadly blocking FGFR signaling most likely indicates that FGFR signaling directly functions in differentiation. Our preliminary studies with FGFR3 and previous researcher with FGFR1 further support FGFR functions in TrkA differentiation (Hadjab et al., 2013). However, since ECs were also decreased with blocking FGFR signaling and were shown to impact progenitor cell proliferation, the direct participation of ECs in neural progenitors proliferation cannot be discounted. FGFR signaling and EC proximity may both play a role in progenitor proliferation and differentiation. One possibility is that ECs are the source of FGF ligands (Vlodavsky et al., 1987). The exact mechanism underlying ECs and FGF signaling in TrkA+ neural differentiation needs to be further addressed.
CONCLUSION

There are multitudes of signaling molecules that function in neural crest migration and differentiation in the peripheral nervous system (PNS). Simultaneously, endothelial cells migrate and form blood vessels in the same tissue regions. While neurovascular interactions have been found important in the adult brain because of implications in disease such as trauma and stroke, their interactions in development have not been thoroughly investigated. Whether ECs influence neural crest patterning was a focus of this study. Using time-lapse confocal microscopy to observe live cell dynamics, we found that ECs and NCCs interact extensively. Specifically, NCCs contact and migrate along the ECs forming the intersomitic vessels. This interaction appears permissive for a subset of NCCs, but could also serve as a boundary constraining NCCs to the anterior somite in conjunction with repulsive molecules in the posterior somite. Other vessels, such as the perisomitic vessel, develop at the ventral boundary of the DRG and seem to restrict NCCs from migrating further ventrally. These are the first reported studies showing live extensive filopodial interactions of NCC and ECs during PNS development. Whether ECs are directing or shaping neural crest migration requires further investigation.

Later in development ECs circumscribe the DRG and were found to stimulate proliferation of progenitor cells on the perimeter of the DRG. It is possible that ECs are secreting mitogens that NCCs are responding to, especially given their other supportive functions in neurovascular interactions. Since mitotic activity was
increased in cells that were in direct contact with ECs it is conceivable that contact of ECs stimulates proliferation by cell/cell communication.

To investigate the molecular basis for neurovascular interactions we used the FGF receptor (FGFR) blocker, Su5402, which lead to a reduction in ECs in vitro and in vivo. Blocking FGFR signaling during NCC migration impaired NCC migration ventrally and focally reduced ECs forming intersomitic vessels. Neural crest migration was so impaired that NCCs remained inappropriately in the migratory staging area of the posterior somite. We later discovered that FGFR1 signaling functions in ventral migration of NC. If ISVs function as a permissive substrate, reducing them would also impair migration. However, because FGFR signaling in NCCs directly impacts their migration we concluded that the impairment in NCC migration in response to Su5402 is likely due to blocking FGFR signaling directly in NCCs and not a reduction in ECs. It is possible that NCCs treated with Su5402 were further impaired than our DNFGFR1, but since we are unable to focally eliminate ECs we cannot determine their role. FGF could be a common signaling molecule used for communication between the ECs and NCCs, ECs may secrete FGF stimulating chemokinesis of NCCs. The role of ECs is difficult to ascertain because of the shared signaling molecules between neural crest and ECs.

Blocking FGFR signaling with Su5402 during neural differentiation decreased the number of ECs and reduced TrkA+ pain and temperature receptors. Since ECs were found to promote proliferation of progenitors in the DRG it is possible that this reduction in TrkA+ neurons is due to a decrease in ECs contacting progenitors, thus
a reduction in progenitors. However, preliminary studies with a dominant negative FGFR3 also reduced TrkA+ neurons. While the role of ECs has not been determined the role of FGFR3 is promising, indicating that FGFR3 directly functions in trkA+ neural differentiation.

The signaling molecules VEGF and FGF were a primary focus of our research because of their impact on both the vascular and nervous system. VEGF was not found to be chemotactic nor mitogenic for trunk NCCs, however FGF signaling was found to have multiple functions in neural crest development. FGFR1 functions in neural crest migration ventrally while FGFR3 may function in TrkA+ neural differentiation. NCCs transfected with a dominant negative FGFR1 (DNFGFR1) showed similar migration impairment to NCCs treated with FGFR blocker, Su5402. However, we did not observe the numbers of NCCs remaining in the MSA of the posterior somite. We cannot rule out that ECs may also play a role in ventral NCC migration. We found that stimulating FGFR signaling \textit{in vitro} and \textit{in vivo} was chemokinetic for NCCs. It is possible that increasing FGF signaling leads to a proliferation or survival of ECs, which could indirectly increase ventral migration of a subset of NCCs, if indeed ECs of the ISV are a permissive substrate. However, given our findings with DNFGFR1 it is most likely that stimulating FGF signaling directly impacts NCC migration ventrally.

The exact mechanisms of migration via FGFR1 were undetermined. Given the morphology of the DNFGFR1+ NCCs, we hypothesize that lamellopodia formation, cell polarization, cell adhesion molecules, and/or cytoskeletal
reorganization are disrupted and decrease migration. It is possible that FGFR1 normally functions in migration through the MAPK and/or PLCγ pathways. FGFR signaling has been shown to function in cancer metastasis and migration of cardiac NCCs, but its role in ventral migration of trunk neural crest was previously unknown.

FGFR1 can directly bind CAMs such as N-cadherin and NCAM, complicating investigations. Our finding that N-cadherin was able to rescue DNFGFR1+ transfected NCCs implies that N-cadherin and FGFR1 may cooperate in ventral migration or DRG localization of NCCs. Further studies of N-cadherin and FGFR1 need to be performed in order to determine if they function independently or in concert.

From our findings and previous research FGF signaling functions in multiple stages of neural crest development from neural induction, migration, differentiation, and later neurite outgrowth. FGF signaling is complex because of the wealth of binding partners and intracellular pathways. The role of FGF signaling in neural crest migration and differentiation is likely integrated by multiple pathways and may indirectly involve endothelial cells.
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