Evaluating the role of Nck adaptor proteins in the endothelial to mesenchymal transition

by

Cameron Harris

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The formation of a functional cardiovascular system depends on a collection of cells undergoing a coordinated endothelial to mesenchymal transition (EnMT) during development. This EnMT is responsible for providing the septations and valves of the mature heart, with defects causing congenital heart malformations. The Nck family of adaptor proteins has previously been linked to heart development, with indications it is through this EnMT process. By measuring well characterized markers of EnMT in a developmental mouse model, and establishing an endothelial cell line, the mechanism behind EnMT signaling in heart development can begin to be elucidated. Understanding the role of Nck in EnMT will provide new insights into the pathways involved in the mesenchymal transition in heart development and extrapolated further to pathological processes utilizing EnMT such as organ and tissue fibrosis.
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<tr>
<td>AVC</td>
<td>Atrioventricular canal</td>
</tr>
<tr>
<td>BAEC</td>
<td>Bovine aortic endothelial cells</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Cdc42</td>
<td>Cell division control protein 42</td>
</tr>
<tr>
<td>CD31</td>
<td>Cluster of differentiation 31</td>
</tr>
<tr>
<td>CNKO</td>
<td>Conditional Nck knockout</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>E10.5</td>
<td>Embryonic day 10.5</td>
</tr>
<tr>
<td>EC</td>
<td>Endocardial cushions</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ECGS</td>
<td>Endothelial cell growth supplement</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>En</td>
<td>Endocardium</td>
</tr>
<tr>
<td>EnMT</td>
<td>Endothelial to mesenchymal transition</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>Flx</td>
<td>Flanked by LoxP</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
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<tr>
<td>IFB</td>
<td>Immunofluorescence buffer</td>
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IFNγ  Interferon gamma
Im   Immortalizing gene
MAPK Mitogen activated protein kinase
MEF  Mouse embryonic fibroblasts
MHC  Major histocompatibility complex
MRTF-A Myocardial related transcription factor-A
My   Myocardium
N-WASP Neuronal Wiskott Aldrich Syndrome protein
Nck  Non-catalytic kinase
OT   Outflow Tract
PAK  p21 activated kinase
PBS  Phosphate buffered saline
PCR  Polymerase chain reaction
PFA  Paraformaldehyde
RBC  Red blood cells
SH   Src homology
SMA  Smooth muscle actin
STAT Signal transducer and activator of transcription
SV40 Simian virus 40
TGFβ Transforming growth factor beta
TGFβR Transforming growth factor beta receptor
VE-cadherin Vascular endothelial cadherin
VEGF Vascular endothelial growth factor
ZO-1 Zonula occludens-1
1. Introduction

1.1. Cardiovascular development

During embryonic development, the mesoderm germ layer in the embryo is established during the morphological process of gastrulation via a series of cell conversion events known as epithelial to mesenchymal transition (EMT) (Larue and Bellacosa, 2005). The mesoderm will differentiate into several tissues including the cardiovascular system, which consists of the heart, blood, and blood vessels. Formation of a functional vascular system during embryonic development depends in large part on two other major processes: vasculogenesis, and angiogenesis. Vasculogenesis is the formation of new blood vessels de novo. The first vascular network is established through the differentiation of angioblasts into endothelial cells which undergo vasculogenesis to create a primitive plexus of vessels (Risau and Flamme, 1995). Angiogenesis enables the formation of new vessels from pre-existing blood vessels by facilitating endothelial cell motility. Over time, angiogenesis sculpts the primitive plexus into the hierarchical vascular arrangement seen in adult physiology.

The cardiovascular system is the first organ system to develop, with the heart beginning to beat at around embryonic day 8 (E8) as a primitive tube in the mouse (Lucitti et al., 2007). The embryonic heart tube is comprised of an inner layer of endocardial cells, surrounded by an outer myocardial layer with an acellular matrix termed the cardiac jelly in between the layers. The primitive heart tube will undergo a series of coordinated asymmetrical folds and bulging to create pockets which will become the chambers of the adult heart. Beginning at approximately E9.5, the primitive heart valves begin to form as swellings between the endocardial and myocardial layers in the region of the atrioventricular canal (AVC) and then subsequently the
outflow tract (OT) (Kaufman and Navaratnam, 1981) (Figure 1). As this is occurring, the cardiac jelly aggregates in these AVC and OT swellings while being displaced from the rest of the heart tube (Wessels and Sedmera, 2003). The budding swells in the AVC and OT will develop into the endocardial cushions, which contribute to the formation of the heart valves and septation of the mature heart (Nakajima et al., 2000).

![Figure 1: Illustration of the developing embryonic mouse heart.](image)

The green portion represents the truncus arteriosus that will develop into the aorta and pulmonary trunk. The yellow and beige areas represent the right and left ventricle respectively, with the purple area developing into the left and right atrium. The blue and brown areas represent the endocardial cushions that will provide the septations and cardiac valves of the adult heart.

1.2. Endothelial to mesenchymal transition (EnMT)

A process analogous to EMT, known as endothelial to mesenchymal transition (EnMT) (Potenta et al., 2008), is required to allow the migration of cells from the endocardium into the cardiac jelly so that the endocardial cushions may form properly (Boyer et al., 1999). The process of EMT is more widely studied than EnMT; however both are characterized by the loss of cadherins and acquisition of mesenchymal traits facilitated by many of the same mediators of
activation (Arciniegas et al., 2007). As such, many of the principles of EMT are used as a basis to study EnMT. During EnMT, an endothelial cell changes from a polarized, immobile, interconnected endothelial cell, to a motile mesenchymal cell devoid of specialized cell-cell contacts, and these phenotypes are correlated with changes in marker expression (Larue and Bellacosa, 2005; Xu et al., 2009) (Figure 2). EnMT is seen during tissue repair, regeneration, while being linked to angiogenesis, and required for the proper development of the embryonic heart (Potenta et al., 2008). EnMT is a tightly regulated process and loss of this regulation can lead to tissue/organ fibrosis, pathological ossification and developmental defects including congenital heart malformations, with indications it may be involved in angiogenesis and production of cancer associated fibroblasts (Kalluri and Weinberg, 2009; Medici et al., 2010).

**Figure 2: Illustration of the endothelial mesenchymal transition.** In response to induction of EnMT, interendothelial contacts dissolve, gene expression is altered, and the actin cytoskeleton is rearranged. The cellular changes allow markers to be assessed to characterize a cell as endothelial or mesenchymal. Blue cells represent endothelial cells, green represent mesenchymal.
The cells that have invaded into the endocardial cushions are highly proliferative mesenchymal cells with the highest rate of proliferation at E9.5 days (de Boer et al., 2012). Lineage tracing in mice using the endothelial specific promoter Tie2 has shown that during endocardial cushion formation, the mesenchymal cells of the AVC and OT are derived from the overlying endothelial cells which migrate towards the underlying myocardium during development (Snarr et al., 2008). This is further reinforced through two separate Tie2-Cre lineage tracing analyses showing that the mature AVC valves are mostly, if not entirely compromised of cells of an endothelial origin (de Lange et al., 2004; Lincoln et al., 2004). Several signaling molecules are released from the underlying myocardium to induce this EnMT, including but not limited to transforming growth factor (TGF) β2, bone morphogenetic protein (BMP) 2, and canonical Wnt signaling (Camenisch et al., 2002; Hinton and Yutzey, 2011).

1.2.1. EnMT signaling

Growth factors associated with EnMT include fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and BMP4. However, the TGFβ superfamily are the most widely used growth factors to induce and study EnMT and as such, have a more thoroughly characterized response and mechanism of action (Camenisch et al., 2002; Docherty et al., 2006). In mice, the primary isoform of TGFβ released from the underlying myocardium to induce the transition is TGFβ2 (Camenisch et al., 2002). Initiation of EnMT through the TGFβ family is mediated by many signaling molecules, highlighted by Smad dependent and Smad independent pathways in response to TGFβ receptor (TGFβR) activation.
TGFβR consists of a heteromeric complex of type I and type II serine/threonine kinase receptors (Dijke and Hill, 2004). The TGFβ ligands have a higher affinity for the type II TGFβR, and upon binding, recruit a second TGFβR type I which gets phosphorylated by the type II receptors (Wrana et al., 1994; Feng and Derynck, 2005). This induces a conformational change in the TGFβR type I allowing the recruitment and phosphorylation of Smads (Wieser et al., 1995).

**Figure 3: Smad dependent and Smad independent TGFβ responses.** In response to TGFβ, Smad-dependent and Smad-independent pathways become activated. In the Smad-dependent pathway, phosphorylation of Smad2/3 activates them allowing them to complex with Smad4 and translocate to the nucleus. In the nucleus, the Smad complex is able to regulate expression of various genes involved in the mesenchymal transition process. The Smad-independent pathway activates a variety of GTPases, including but not limited to Ras, Rho, Cdc42, and Rac. Rho, Rac and Cdc42 regulate JNK which is involved in Smad3 phosphorylation and enhances its nuclear translocation. Cdc42 and Rac are involved in activation of p38MAPK which induces actin polymerization and stress fiber formation. TGFβ and Ras cooperate to induce Snail activation, and modulate the Erk MAPK pathway. All the GTPases listed affect cytoskeletal rearrangement either through lamellipodia formation, stress fiber formation, or both.
Smads are a group of transcription factors that act as nuclear effectors for TGFβR by propagating extracellular signals to the nucleus. The canonical pathway of TGFβ involves the C-terminus of TGFβR type I phosphorylating Smad2 and Smad3, which can then form a trimer with Smad4 and move into the nucleus to alter target gene expression (Heldin et al., 1997).

The Smad mediated pathway is the primary mechanism of TGFβ induced EnMT, but several non-Smad signaling mechanisms are also involved including the Rho family of GTPases, particularly RhoA; the p38 class of mitogen activated protein kinases (p38MAPK); and Ras-related protein (R-Ras) (Figure 3). RhoA has been shown to be a major GTPase in the EnMT process. In vivo RhoA expression is increased in the AVC at the developmental time points corresponding with EnMT, and RhoA expression is induced in vitro by TGFβ stimulation without affecting Smad activation (Bhowmick et al., 2001a; Tavares et al., 2006). Additionally, inhibiting Rho can significantly suppress TGFβ2 mediated EnMT (Mihira et al., 2011). RhoA activation, facilitated by guanine nucleotide exchange factors (GEFs), is required for TGFβ stimulated actin reorganization into stress fibers and the formation of smooth muscle actin (SMA) (Bhowmick et al., 2001a; Tavares et al., 2006; Tsapara et al., 2010), both of which are critical for EnMT. Interestingly, TGFβ stimulation has a temporal effect on RhoA expression as it is initially inactivated by GTPase activating proteins (GAPs) to facilitate the degradation of cell junctions (Tsapara et al., 2010). A series of experiments showed that p38MAPK is phosphorylated by TGFβ and is required for TGFβ induced transformation in mice without affecting Smad phosphorylation, but that it is not sufficient to induce the transition by itself (Bhowmick et al., 2001b; Bakin et al., 2002; Yu et al., 2002). The Ras pathway works in conjunction with intracellular Smad2 and Smad3 proteins in response to TGFβ signaling,
inducing several families of transcription factors that cause major changes in gene expression and facilitate the cellular transition into a mesenchymal state (Zeisberg et al., 2007a; Xu et al., 2009). While the Ras-related proteins (R-Ras) are key proteins in integrin signaling, and are shown to be involved in the autocrine release of TGFβ to induce and maintain a cell’s transition into a mesenchymal phenotype (Osada et al., 1999; Janda et al., 2002; Erdogan et al., 2008).

1.2.2. EnMT cell dynamics

Endothelial cells have strong cell-cell adhesion due to both adherens junctions (comprised of vascular endothelial (VE) cadherin) and tight junctions (containing claudin 5 and zona occludens (ZO)-1) (Dejana, 2004). In order for endothelial cells to become motile, their cell-cell contacts need to be removed. This is largely accomplished through the up regulation of the transcriptional repressor Snail1, which is shown to cause the dissolution of VE-cadherin junctions (Timmerman et al., 2004), reduced transcription of claudin 5 (Kokudo et al., 2008), and the cytoplasmic relocalization of ZO-1 from tight junctions (Ikenouchi et al., 2003). The actin cytoskeleton aligning with these adherens and tight junctions becomes drastically altered upon their loss and forms stress fibers anchoring to focal adhesions that facilitate cell movement (Xu et al., 2009).

1.2.3. Markers of EnMT

EnMT causes gross morphological changes to the cell’s actin cytoskeleton and alters gene expression, giving the cell migratory and invasive capabilities (Song, 2007). These changes create several distinct and well characterized markers which can be analyzed to classify cells as
either endothelial or mesenchymal (Figure 2 and Table 1). Some of the more prominent endothelial markers include VE-cadherin, cluster of differentiation 31 (CD31), and claudin5. The transmembrane adhesion protein VE-cadherin is a commonly used endothelial marker since it is constitutively and exclusively expressed at the adherens junctions between endothelial cells as early as E7.5 in mice (Lampugnani et al., 1992). VE-cadherin is a major component of endothelial cell dynamics as its forced expression is sufficient to induce fibroblasts to adopt an endothelial phenotype (Lampugnani et al., 2002). The removal of VE-cadherin is a necessity for cells to undergo EnMT and is mediated by the expression of Snail, whereby the Snail protein binds the promoter region of VE-cadherin preventing transcription (Timmerman et al., 2004). The addition of ectopic Snail1 is sufficient to induce a mesenchymal phenotype, and Snail1 is expressed during the natural progression of EnMT in endocardial cushion development, inversely correlating with cadherin expression (Cano et al., 2000; Carver et al., 2001). The expression of VE-cadherin is known to be involved in cross talk mechanisms with tight junctions, including another endothelial marker claudin5 (Taddei et al., 2008). The homotypic binding of VE-cadherin sequesters β-catenin at the cell membrane, and through intracellular pathway involving PI3K, Akt, and FoxO1, claudin5 expression is increased (Taddei et al., 2008). Claudin5 is an endothelial specific component of tight junctions which is primarily involved in the regulation of para-cellular permeability. The downregulation of claudins is a critical event for the mesenchymal transition (Kokudo et al., 2008). Claudin5 is known to be downregulated in response to TGFβ2 expression and is lost as cells acquire a mesenchymal phenotype (Ikenouchi et al., 2003; Kokudo et al., 2008; Shen et al., 2011). Another known endothelial marker is CD31. It has been proven to be an effective marker of endothelial cells since it is not expressed in epithelial cells, and is lost during the EnMT process (Zeisberg et al., 2007b). CD31 is one of
the primary proteins involved in endothelial cell junctions promoting homotypic binding, and is expressed from the primitive angioblast stage of development (Schmidt et al., 2007).

As endothelial cells undergo the EnMT, they also acquire several key mesenchymal markers, including αSMA and S100A4. αSMA is a commonly used marker to identify the switch to a contractile phenotype characteristic of mesenchymal cells. The acquired αSMA allows for and is sufficient to promote stress fiber and focal adhesion assembly, aiding the locomotion of the cell (Hinz et al., 2001). In response to TGFβ stimulation, GEFs stimulate RhoA which in turn enhance the activity of the αSMA promoter inducing its expression (Tsapara et al., 2010). S100A4 is another mesenchymal marker acquired after cells undergo the mesenchymal transition (Okada et al., 1997). Exclusively found in mesenchymal cells, S100A4 is a calcium binding protein that is being used as a prognostic marker for the potential of cancer metastasis. It does not have enzymatic activity, but has been proposed to be involved in cell motility, invasion, and metastasis (Kriajevska et al., 1994). The colocalization of S100A4 and myosin at the leading edge of cells lends support to the theory that S100A4 is involved in a functional interaction with myosin enabling active migration (Kim and Helfman, 2003). S100A4 is also associated with cytoskeletal rearrangements and cell motility through its interactions with actin filaments (Watanabe et al., 1993) and non-muscle tropomyosin (Takenaga et al., 1994). The specific pathways S100A4 is involved in are poorly characterized since it acts in calcium dependent and independent pathways through intracellular and extracellular mechanisms (Helfman et al., 2005).
1.3. Biology of cell movement

Cell migration is a naturally occurring process in response to growth factors that, among other effects, is required for angiogenesis, and is the final step in the EnMT process. There are five general steps enabling cell migration: filopodia formation, lamellipodia formation, focal adhesion assembly, stress fibre formation, and focal adhesion disassembly from the trailing edge (Figure 4). Cell movement requires the coordinated activity of cell signaling pathways. The first step for cell migration is the extension of filopodia, which are specialized structures of parallel actin filaments that extend from the cell surface to sense chemical gradients guiding migration (Gerhardt et al., 2003). Lamellipodia extend the leading edge of the cell in the direction of the filopodia via a neuronal Wiskott Aldrich Syndrome protein (N-WASp) mediated activation of Arp2/3, causing actin polymerization (Urban et al., 2010). In filopodia and lamellipodia formation, the cell division control protein 42 (Cdc42) and Rac1 play a role in the signaling pathway leading to actin polymerization (Ridley, 2006), while other Rho family GTPases

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<tr>
<th>Marker</th>
<th>Predominant expression</th>
<th>Function</th>
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<tr>
<td>VE-cadherin</td>
<td>Endothelium</td>
<td>Exclusively and constitutively expressed between endothelial cells to promote homotypic adhesion.</td>
</tr>
<tr>
<td>CD31</td>
<td>Endothelium</td>
<td>Promotes homotypic binding exclusively in the vascular system</td>
</tr>
<tr>
<td>Claudin5</td>
<td>Endothelium</td>
<td>Component of tight junctions in endothelial cells. Regulates paracellular movement through pores</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Endothelium</td>
<td>Anchor to actin filaments that contributes strength and integrity to tight junctions</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Mesenchyme</td>
<td>Component of heart myocardium and tunica media of blood vessels that aids in contractile forces.</td>
</tr>
<tr>
<td>S100A4</td>
<td>Mesenchyme</td>
<td>De novo production seen after EnMT. Enhances migratory and invasive properties of a cell</td>
</tr>
<tr>
<td>Snail1</td>
<td>Mesenchyme</td>
<td>Transcription factor leading to regionalized EnMT by down regulating cell-cell adhesion</td>
</tr>
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mediate the stability of the actin filament extensions (Etienne-Mannville and Hall, 2002). The new lamellipodium adheres to its environment via focal adhesions mediated, in part, by the activity of Rho family GTPases, including RhoA (Ridley and Hall, 1992; Playford et al., 2008), and focal adhesion kinase (FAK) (Playford et al., 2008). The formation of stress fibers, mediated by RhoA and p38 MAPK, provides a means for motile cells to contract from the newly formed focal adhesion, dragging the rest of the cell along (Hotulainen and Lappalainen, 2006) as the trailing end of the cell loses its adhesion to the extracellular matrix (ECM) mediated by p21-activated kinase (PAK) (Brown et al., 2002). A protein known to be crucial in this migration process is non-catalytic kinase (Nck).

Figure 4: Schematic illustrating the process of cell movement with some of the associated molecules involved at each step. Blue puncta represent existing focal adhesions, blue extensions represent filopodia, purple extensions represent lamellipodia, green puncta represent new focal adhesions, and red lines represent stress fibers.
1.4. Nck adaptor protein

The Nck family of intracellular adaptor proteins functions to connect extracellular signals to intracellular signaling responses. In endothelial cell morphogenesis, they have been shown to be key adaptor molecules by orchestrating polarized cytoskeletal changes (Chaki et al., 2012). This is accomplished through one carboxy terminal Src homology (SH) 2 domain, and three SH3 domains that play a role in regulating the actin cytoskeleton (Buday et al., 2002; Jones et al., 2006). The SH2 domain preferentially binds to phospho-tyrosine residues on receptor tyrosine kinases (RTK) causing recruitment of the Nck protein, typically to the plasma membrane (Rivera et al., 2006; Blasutig et al., 2008). The SH3 domain of Nck binds to proline rich regions linking the signal to downstream effectors (Antoku et al., 2008; Abella et al., 2010). Recent evidence is also suggesting that Nck may function not only in transmitting signals from RTKs, but may be involved in stabilizing downstream target effectors, even in the absence of an active RTK (Guan et al., 2009). The Nck protein family has many downstream effector molecules including, but not limited to, Rho family GTPases (Guan et al., 2009), p38 MAPK (Lamalice et al., 2006), R-Ras (Erdogan et al., 2008), particularly interesting cysteine and histidine-rich (PINCH1) (Tu et al., 1998), and N-WASp (Abella et al., 2010). There are two members of the Nck protein family, Nck1 (Nckα) and Nck2 (Nckβ/Grb4), which are collectively referred to as Nck. Nck 1 and 2 share 68% of their amino acids in common, with most of the variation between the region linking the SH2 and SH3 domains, and the murine Nck collectively is 96% similar to the human counterpart (Buday et al., 2002). Loss of both Nck proteins in mice results in embryonic lethality at E9.5 due to gross morphological changes, proving Nck expression is critical during development (Bladt et al., 2003). Selective removal of either Nck1 or Nck2 does not result in phenotypic or functional changes, suggesting redundant functions between the two proteins.
(Bladt et al., 2003). However, Nck1 has been found to be expressed in higher levels in the heart, kidney and brain, while Nck2 has higher expression in the thymus, spleen and lungs, which indicates a possibility of some distinct purposes (Bladt et al., 2003). Accordingly, some recent evidence has also emerged that Nck1 and Nck2 may play slightly different roles in response to the same growth factor, though this has not been shown in the developing heart (Guan et al., 2009). In this study, Nck1 and Nck2 had non-compensating roles in response to platelet derived growth factor, with Nck1 signaling through Cdc42, and Nck2 through RhoA pathways to mediate cell migration (Guan et al., 2009). It has also been shown that Nck2, but not Nck1, binds to the protein PINCH1 through its SH3 domains (Tu et al., 1998). PINCH proteins play critical roles in integrin dependent pathways relaying signals from the ECM to pathways involved in anchoring the actin cytoskeleton during migration (Hehlgens et al., 2007). Preventing Nck2 and PINCH1 from interacting caused defects in cell spreading and migration, thus implicating a role for Nck as a mediator between integrin signaling pathways and RTK pathways (Xu et al., 2005).

Nck has been shown to be required and sufficient to induce actin polymerization, a requirement for cell migration (Rivera et al., 2006; Hu et al., 2009). Nck is involved in each of the major pathways involved in the migration process (Figure 4). Migration begins with actin polymerization and extension of actin filaments to extend the membrane at the leading edge producing filopodia and lamellipodia, a process mediated by Nck via Cdc42 (Bladt et al., 2003; Lamalice et al., 2007). Adhesion of the leading edge is mediated, in part, by FAK activation to create focal adhesions that allows RhoA to facilitate the formation of stress fibers pulling the cell along from these new adhesions. Both RhoA and FAK activation have been linked to Nck (Choudhury et al., 1996; Rivera et al., 2006; Guan et al., 2009), and this RhoA dependent actin remodelling has been shown specifically in endothelial cells (Chaki et al., 2012). PAK binds the
SH3 domains on Nck to mediate the release of trailing edge focal adhesions and the release of stress fibers (Brown et al., 2002). Finally, Nck binds and removes the auto-inhibition on N-WASp, creating regions of localized actin polymerization clustered around Nck (Abella et al., 2010). Since Nck is recruited to active RTKs, this allows directed cell movement in response to chemotactic gradients of growth factors. Consistent with these findings, deletion of Nck in mouse embryonic fibroblasts (MEF) significantly impaired cell motility and restoration of Nck expression returned the migratory capabilities of the MEF cells (Bladt et al., 2003). The combined effects of Nck deficiency on actin cytoskeleton ultrastructure were shown by electron microscopy, whereby Nck null MEFs had less branched and less dense actin network at the leading edge of the cell, with shorter actin filaments, likely resulting in the motility defects (Bladt et al., 2003). Additional studies in MEFs null for Nck showed severely decreased activation of the RhoA, Rac, and Cdc42 GTPases in response to PDGF (Ruusala et al., 2008). Specifically in endothelial cells, time-lapse differential interference contrast (DIC) imaging has shown Nck null cells fail to establish cellular polarity with fewer, and unstable membrane protrusions, indicating Nck is required for migration as it coordinates the spatiotemporal activation of various cytoskeletal regulators, including GTPases (Chaki et al., 2012).

1.4.1. Role of Nck in widespread physiological systems

Nck is a prominent adaptor protein that has wide ranging effects through various physiological systems, but all focus around actin cytoskeleton dynamics. Within the immune system, Nck has been shown to be required for proper functioning of various immune cells. In monocytes, Nck is required to complex with Rac1 to mediate modification of the actin cytoskeleton to form lamellipodia (Konakahara et al., 2012). As well, Nck is a prerequisite in
the recruitment of the WAVE complex which mediates phagocytosis of bacteria via actin polymerization (Pils et al., 2012). Tissue-specific removal of Nck from T-cells shows over a 50% decrease in circulating peripheral T-cells, which are also hypo-responsive to T-cell receptor activation (Roy et al., 2010). Nck deletion from T-cells also reduced the capability of ERK phosphorylation and movement of calcium, all of which combined to severely reduce T-cell response to antigens (Roy et al., 2010).

Many studies have also implicated a role for Nck in the nervous system. A conditional deletion of Nck from the developing nervous system using Cre-loxP technology shows a non-lethal phenotype, but with severe locomotor, corticospinal tract, central pattern generator, and axon guidance defects (Fawcett et al., 2007). Since external cues are required to provide direction to the polymerization of actin for axon guidance, it makes sense that Nck would play a role. A family of membrane receptors responsible for guiding axon growth, Robo, requires Nck to guide the outgrowth of cortical axons and dendrites (Round and Sun, 2011). In this model, Nck acts atypically in that it is constitutively bound to the Robo receptor in the absence of a ligand, then upon ligand binding Robo becomes activated and Nck is released (Round and Sun, 2011). This abnormal binding pattern of Nck is also seen on another neural receptor DCC (deleted in colorectal cancer) that is responsible for axon growth cone migration (Li et al., 2002).

Within the urinary system, Nck has been shown to be a key protein in the maintenance of kidney function. Within podocytes of the blood filtration barrier, Nck is required for both the formation and maintenance of their unique actin-based foot processes, loss of which leads to proteinuria (Jones et al., 2006, 2009). Additionally, Nck is involved in the intracellular phosphorylation events that maintain podocyte function (Jones et al., 2009; New et al., 2013).
1.4.2. Nck in cardiovascular development

In addition to the immune, neural and urinary systems, our lab has recently determined that Nck is required during development of the cardiovascular system. Selective removal of Nck 

\textit{in vivo} from endothelial cells of mice results in embryonic lethality at E11.5 with severe vascular defects (Clouthier et al., unpublished). These defects include, but are not limited to, inhibited sprouting angiogenesis resulting in vascular disarray and a major reduction in caliber of the vitelline vessel. Primary mouse lung endothelial cells isolated from these mice were cultured and examined via wound healing assays analogous to Bladt et al. 2003, and showed migration defects in response to a variety of growth factors including VEGF, similar to MEFs lacking Nck. Furthermore, histological analysis showed that Nck null embryos have thinner and disrupted cell layers within the developing heart, with associated defects in the cardiac cushions in the AVC and OT. A complementary \textit{ex vivo} endocardial cushion outgrowth assay from E9.5 Nck mutant embryos showed a reduced number and outgrowth of mesenchymal cells from the atrioventricular explants (Clouthier et al., unpublished). Given that the AVC and OT regions are required to undergo EnMT to form the endocardial cushions; these results implicate Nck in aspects of EnMT.

1.5. Hypothetical role of Nck in EnMT

There have been no studies directly identifying a role for Nck in EMT or EnMT. Furthermore, our lab’s unpublished work implicating a potential role for Nck in EnMT may actually be a result of the migratory defect, and not necessarily the cells being unable to
transition into a mesenchymal state. However, several lines of evidence support a role for Nck in the mesenchymal transition. Firstly, signaling molecules involved in EnMT including RhoA, p38 MAPK, and R-Ras can all associate with Nck. In addition, Nck is required to facilitate RhoA signaling, and without expression of Nck, RhoA is unable to form stress fibers or stabilize lamellipodia and filopodia extensions (Ruusala et al., 2008; Guan et al., 2009). There is also evidence that Nck is a vital component in signaling cascades linking phosphorylated VEGF receptors to the downstream effector protein p38MAPK via Cdc42, which reorganizes the cytoskeleton and forms stress fibers (Lamalice et al., 2006). In this process, it is believed that Nck is recruited to a phosphorylated tyrosine on an RTK, enabling the phosphorylation and activation of Fyn and Cdc42, sequentially leading to the activation of p38MAPK (Lamalice et al., 2004, 2006). Granted that this study was performed on the VEGF receptor, and TGFβ is the known primary inducer of EnMT, it nevertheless proves Nck binds and regulates a known effector of the EnMT pathway. A final connection between Nck and EnMT is through R-Ras. Nck binds to the proline rich region of R-Ras. Inhibition of this binding prevents R-Ras from stimulating the autocrine release of TGFβ and prevents the activity of integrins, thereby affecting cell adhesion properties (Wang et al., 2000).

1.6. Research proposal

Nck is a widely expressed adaptor protein that participates in a variety of signaling pathways to coordinate actin cytoskeletal dynamics during cell migration. Within the developing heart, loss of Nck expression in embryonic endothelial cells results in defects in angiogenesis and cardiac cushion formation, the latter of which suggests a role for Nck in the EnMT process. To examine the hypothesis that Nck is involved in EnMT, markers of EnMT will be analyzed in
the developing cardiac cushions of mice with or without endothelial expression of Nck. In parallel, an endothelial cell line deficient for Nck expression will be established. Using complementary *in vivo* and *in vitro* approaches, these experiments will provide insight into the potential role for Nck signaling in this critical developmental and pathological process.
2. Methods:

2.1. Mouse colony

All work with transgenic laboratory mice was pre-defined under Animal Utilization Protocol #11R011, which was reviewed and sanctioned by the Animal Care Committee of the Senate Research Board at the University of Guelph.

The transgenic mouse lines were maintained by registered laboratory animal technicians at the Central Animal Facility at the University of Guelph. Mice were kept under a regulated 12 hour light 12 hour dark photoperiod. For timed breeding pairs, a male was set up with up to 2 females in the same cage in the afternoon and left overnight to mate. The following morning was considered to be embryonic day 0.5. The females were checked for vaginal plugs to indicate sexual activity, then separated from the male mouse. Pregnancy was confirmed by continual weight gain (minimum gain of 10 percent of the original weight) over the first 9 days. Confirmed pregnancies were either left to go to term, or euthanized at embryonic day 9.5 or 10.5 for embryo isolations.

Two separate mouse lines were used and maintained over this time frame: endothelial specific Nck knockout line, and an “immortalized” Nck knockout line. The endothelial specific knockout line is achieved through the Cre-LoxP mechanism. In this case the Nck1 allele is genetically knocked out of the mice, while the Nck2 allele is flanked by 2 LoxP sites. Meanwhile the expression of the Cre-recombinase protein is under the control of the endothelial specific Tie2 promoter to remove the LoxP flanked Nck2 allele. This genotype (Nck1\(^{+/−}\), Nck2\(^{lof}\), Tie2-Cre\(^{+/−}\)) results in an endothelial specific, conditional Nck knockout (CNKO). Since the resulting phenotype is not viable, CNKO embryos are obtained by breeding Nck double
mutant females (Nck1<sup>−/−</sup> Nck2<sup>+/−</sup>), with Tie-2-Cre expressing males (Nck1<sup>+/−</sup>, Nck2<sup>+/−</sup>, Tie2-Cre<sup>+/−</sup>). The resulting CNKO embryos occur roughly at the expected frequency of ¼.

The immortalized Nck line was derived by selective crossing of Nck double mutant mice to an Immortomouse® from Charles River. The Immortomouse® has a temperature sensitive simian virus (SV) 40 large T-antigen under the control of the ubiquitously expressed major histocompatibility complex (MHC) class-I promoter H-2KB which in the presence of interferon-γ (IFNγ) pushes the cell into the cell cycle. Mice expressing the immortalizing gene (Im) were crossed to the existing Nck double mutant, with subsequent breeding of certain genotypes to yield the desired Nck1<sup>−/−</sup>,Nck2<sup>+/−</sup>, Im<sup>+/−</sup> genotype. Once the mice were Nck1<sup>−/−</sup> Nck2<sup>+/−</sup> and had been exposed to the immortalizing gene, they were kept separate from the remaining stock double mutant mouse colony. Mice expressing the immortalizing gene tended to have a short life expectancy (4-6 months from birth). As such, when breeding pairs were set up, the males selected would express the immortalizing gene while the females would not, to therefore prevent the chance of their passing while nursing pups.

For embryo isolations, females were euthanized via cervical dislocation, sprayed with 70% ethanol, then the uterine horns containing the embryos were dissected out through the abdomen and placed in phosphate buffered saline (PBS). Using fine forceps and scissors, the uterine tissues were removed to expose the embryo contained within the visceral yolk sac. The yolk sac was removed and used for genotyping while the embryo was stored in 4% paraformaldehyde (PFA) for 48 hours at 4°C.
2.2. Paraffin embedding, sectioning, and immunofluorescence

Fixed embryos were put through sequential washes of 50, 70, 80, 95, and 100% ethanol washes for 30 minutes each, with a final 100% ethanol wash for 15 minutes. Embryos were then moved into a 1:1 ethanol and xylene solution for 30 minutes, followed by 2 washes in xylene for 20 minutes each. Embryos were then transferred to a molten paraffin wax treatment at 60°C and left there overnight. The following morning, the embryos were put through a second wax treatment for 2 hours before being paraffin embedded in a cassette. Cassettes were stored at 4°C until ready for use. Paraffin blocks were sectioned using a microtome at a thickness of 5μm while being kept cool on an ice block between sectioning. Ribbons were smoothed using a 45°C warm water bath before being transferred onto glass slides. Slides were placed on a slide warmer at 40°C overnight to fully adhere. If the slides were not used immediately they were stored at 4°C.

Antigen retrieval for staining began by washing the slides twice in xylene for 10 minutes each in coplin jars followed by 2 washes in 100% ethanol for 2 minutes each. Slides were then put through a 20 minute methanol/3% H₂O₂ solution to quench internal peroxidases. Slides were next rehydrated though a sequential series of ethanol washes (100%, 95%, 70%, 50%, 30%) for 2 minutes each followed by 2 washes in distilled water for 5 minutes each. A 10mM tris (hydroxymethyl) aminomethane (Tris) - 1mM Ethylenediamine tetra-acetic acid (EDTA) buffer in PBS was heated to 95°C and used for the antigen retrieval for 20 minutes and then allowed to cool for 15 minutes in solution. The sample area was outlined by a histological PAP pen and washed twice in PBT (PBS + 0.2% bovine serum albumin (BSA) + 0.1% Tween-20) for 5 minutes each. Samples were blocked in immunofluorescence buffer (PBS + 2% BSA and 2% foetal bovine serum) (IFB) for 1 hour in a humidified chamber at room temperature followed by
2 washes in PBS for 5 minutes each. SMA required IFB with 3% BSA and 3% FBS in PBS for specific staining. The primary antibodies were applied [VE-cadherin (Abcam, ab33168), SMA (Sigma, A.2547), S100A4 (Abcam, ab27957), claudin-5 (Santa Cruz, SC-28670)] in IFB at a dilution of 1:200 and left overnight at 4°C in a humidified chamber. Samples were washed 3 times in PBS for 5 minutes each followed by applying the appropriate secondary (Invitrogen, Alexa-Fluor) for 1 hour at room temperature in a humidified chamber at a 1:400 dilution in IFB. Three subsequent washes in PBS for 5 minutes each followed. Coverslips were mounted on the slides using Pro-Long Gold Antifade reagent with DAPI (Life Technologies), allowed to adhere overnight at 4°C then sealed and imaged the following morning. Slides were imaged on a Leica DMIRE2 using a 20x objective lens and Volocity software was used for image acquisition. The sections were all imaged at the same exposure and magnitude of fluorescence for each antibody during replicates. All staining procedure were accompanied by secondary only controls.

2.3. Cryo-embedding, sectioning and immunofluorescence

Stored embryos were rinsed 3 x 5 minutes in PBS and placed in a 25% sucrose solution followed by a 35% sucrose solution dissolved in PBS and allowed to equilibrate in both solutions before proceeding. This took roughly one hour to equilibrate at 25%, and an additional 2 hours to equilibrate at 35% sucrose. Embryos were transferred to embedding molds and covered with Shandon Cryomatrix then snap frozen on dry ice. Blocks were stored at -80°C until used. Blocks were sectioned at 8μm using a cryotome set at -19°C. Sections were allowed to adhere to the slide at room temperature for 2 hours and were stored at -20°C for up to a month.
Sections were brought to room temperature before proceeding with immunostaining. The areas of interest on the slides were circled using a histological PAP pen and rehydrated using PBTD (PBS with 0.1% Tween-20, and 1% DMSO) in a humid chamber for 3 minutes. Sections were blocked using 10% goat serum in PBTD at room temperature for 1 hour. The blocking agent was replaced with primary antibody diluted in block at a 1:400 dilution and incubated overnight at 4°C in a humid chamber. The primary was carefully removed and sections were rinsed 3 times in PBS for 10 minutes each. The appropriate secondary was added in a 1:500 dilution in block and incubated at room temperature for one hour in a humid chamber. Sections were rinsed 3 more times in PBTD for 10 minutes each, and then mounted using ProLong Gold antifade reagent with DAPI.

2.4. Immunoanalysis quantification

Image J software was used to quantify the intensity and abundance of marker stain and DAPI from within the endocardial cushion. The endocardial cushion space was highlighted roughly 5 pixels inside the endocardial and myocardial layer, so only the cells occupying the cardiac jelly from the images are quantified. Image J software returned several analytical measures, including the average intensity of staining from within the highlighted area. Using stacks of merged images allowed the exact same area to be highlighted between marker and DAPI staining. To account for discrepancies in the number of cells that migrate into the endocardial space, the amount of marker was taken as a ratio to the abundance of the nuclear stain DAPI.
2.5. Immortalized primary cell isolation

Following genotyping, the desired mice were euthanized by cervical dislocation, pinned down, and rinsed with ethanol before making the incision along the thoracic cavity. The lungs and heart were removed carefully while removing all attached tissues and placed into isolation medium (20% FBS, 1% pen/strep in DMEM). The samples were transferred to a sterile laminar flow hood and both heart and lungs were flushed with the isolation medium in an attempt to reduce erythrocyte contamination. The samples were then finely minced and placed in pre-warmed tissue dissociation medium (2.4U/mL Dispase (Sigma D4818), and 0.1% glucose in D-PBS) for 45 minutes on a shaker at 37°C. The mixture was then triturated with a 22G metal cannula until homogenous and pipetted through a 70μm cell strainer into a 50mL falcon tube and washed with 10mL isolation medium. The suspension was spun down at 3600rpm for 10 minutes at room temperature and the cell pellet was re-suspended in 1.5mL sorting buffer (0.1%BSA in D-PBS) and each lung and heart isolate was divided into three 500μL aliquots in Eppendorf tubes. Dynabeads M-450 sheep anti-rat IgG were conjugated the day before at 4°C to purified rat α mouse CD31 (BD Pharmingen 553325) according to manufacturer’s protocol. 15μL of the bead mixture was added to tubes containing each heart and lung isolates and incubated on a rotator for 15 minutes at room temperature. The tubes were mounted on a magnetic separator for 2 minutes, rinsed thoroughly with sorting buffer, and then repeated 4 times. The final wash was in 1mL of endothelial growth medium (DMEM + 15% FBS, 1% pen/strep, and 100μg/mL endothelial cell growth supplement (ECGS) (Sigma E2759)), followed by plating in collagen coated wells and storage at 33°C.

Cells were grown until confluent, at which point they underwent a secondary selection using the Dynabeads conjugated to intercellular adhesion molecule (ICAM2) since it is
constitutively expressed at high levels on vascular endothelial cells. Cells were trypsinized and allowed to bind with the Dynabeads for 15 minutes at room temperature. The same wash and rinse cycles were used as described above, and the cells were plated on a collagen coated plate stored at 33°C with ECGS.

Adenovirus transduction of Cre-recombinase was performed once the cells were 90% confluent. Cells were lifted off the plate with trypsin and EDTA and the number of cells was counted to enable a multiplicity of infection of 500 for the adenovirus. Cells were divided into adeno-Cre (Ad-Cre), adeno-GFP (Ad-GFP) and no adenovirus (no-Ad) groups and plated on collagen coated plates in serum free media for 24 hours. The media was aspirated off and replaced with the endothelial growth media, with transgene expression being apparent after ~48 hours.

2.6. Genomic DNA extraction from cell cultures

Verification of successful Cre-recombinase transduction was done via a combination of DNA and protein analysis. Genomic DNA was extracted from 95% confluent 10cm plates by collecting the cells into 1.5mL of PBS. Cells were pelleted at 4°C for 5 minutes at 1.1 x g. The supernatant was gently removed, and the cell pellet re-suspended in 50μL PBS. 1mL of DNAzol (Invitrogen – 10503027) was added and incubated overnight at 4°C. The mixture was then centrifuged twice at 14 000 rpm for 10 minutes twice, each time moving the supernatant to a new tube. 500μL of cold 95% ethanol was added to precipitate genomic DNA into a cloudy precipitate in the tube for 30 minutes. More ethanol was added if required to precipitate the DNA. The pellet was centrifuged at 13 000rpm for 30 minutes at 4°C. The pellet was washed
with 70% cold ethanol and centrifuged again 2 times repeating the wash in between. The supernatant was removed and the pellet was allowed to air dry until it became clear before being re-suspended in 100μL of PCR water.

2.7. Cell lysis

Confluent cells to be lysed were rinsed twice with PBS while on ice. Lysis buffer (PLC (10% glycerol, 50mM Hepes pH 7.5, 150mM NaCl, 1.5mM MgCl₂, 1mM EGTA, 100mM NaPPi, 100mM NaF, 1% Triton X-100) + 0.1% aprotinin, 0.1% leupeptin, 1% sodium orthovanadate, and 1% phenylmethylsulfonyl fluoride) was added to the cell culture. Cells were scraped and collected into microfuge tubes and then centrifuged at 13 000 rpm for 10 minutes at 4°C. The supernatant was transferred to a new tube. All assays were performed on the supernatant. Protein concentration of cell lysates was determined using a BSA standard curve.

2.8. Western blotting

SDS polyacrylamide gels were cast using 8%, 10%, or 12% acrylamide depending on the protein(s) of interest. Protein concentration of cell lysates was determined by BSA standard curve analysis and an equal amount of protein was loaded into each well. Separated proteins were transferred to PVDF membrane on a semi-dry transfer apparatus at 0.5A for 35 minutes. The membrane was blocked (in either 5% BSA or 5% skim milk in TBST) for 1 hour before primary antibodies were applied and left at 4°C overnight on a nutator. Membranes were washed 3x10 minutes in TBST and incubated 1 hour with the appropriate secondary antibody conjugated to horseradish peroxidase at room temperature. Membranes were washed 3x10 minutes again
before detection using enhanced chemi-luminescence (ECL). Application of film, fixation and developing was all performed under red light.

2.9. shRNA knockdown of Nck in bovine aortic endothelial cells

Bovine aortic endothelial cells (BAEC) certified as being of endothelial origin were kindly donated by Dr. B. Coomber (Guelph, ON). A stable knockdown of Nck1 and Nck2 was attempted using shRNA transduction via lentiviral particles with an accompanying puromycin resistance gene in the plasmid. Various shRNA clones were purchased from Thermo-Scientific (TRC-Mm1.0), packaged and transduced into cultured BAEC as per manufacturer’s protocol. Briefly, 5x10⁴ cells were seeded in a 6-well plate the day before transduction. 100μL of plasmid DNA was diluted in 1mL of endothelial growth medium (DMEM + 15% FBS + 1%P/S) for each well. Cells were cultured at 37°C, and 24 hours later were switched into 1μg/mL puromycin selection in the endothelial growth medium.

2.10. Genotyping

Tissue samples for genotyping were prepared for analysis using a HotShot technique. Briefly, 75μL of alkaline lysis buffer was added to the microfuge tube containing the tissue sample and incubated 30 minutes at 95°C. The samples were put on a vortex until they became clear and then put on ice. 75μL of a neutralization buffer was added and mixed well. For polymerase chain reactions (PCR) to assess the genotype, 3μL of this HotShot solution was added per PCR reaction. Refer to Table 2 for primer sequences.
Table 2: Primer sequences for PCR based genotyping

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tie2-Cre</td>
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<td>gcagtaaaactccagcaa</td>
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</tr>
<tr>
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<td>cacatacagatacacaagctgaag</td>
<td>170</td>
</tr>
<tr>
<td>Nck2-flx</td>
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<td>gtgctatttgacagaagctgac</td>
<td>510</td>
</tr>
<tr>
<td>Immorto-wt</td>
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<td>gatggcatcactgtcatga</td>
<td></td>
</tr>
<tr>
<td>Immorto SV40</td>
<td>agtctcagatctgttcag</td>
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</tr>
</tbody>
</table>
3. Results

3.1. Re-establishment of the mouse colony

Prior to the onset of this study, mice with the appropriate genotypes required for this study had been established. However, generations of genetic inbreeding had resulted in an infertile mouse colony. Between March 2012 and December 2012, 91.8% (56/61) of breeding mice pairs did not have any pups, while the remaining breeding pairs having small litters. In order to efficiently carry out the proposed studies, we decided to restore genetic variability within our colony by backcrossing our existing Nck double mutant (Nck1\textsuperscript{-/-}, Nck2\textsuperscript{f/f}) males on a

![Genotyping results from the progeny of a CD1 female with an Nck double mutant male (F0). The first filial generation (F1) resulted entirely in mice heterozygous for Nck1 and Nck2. The resulting F2 retained expression of either Nck1 or Nck2 while the other allele was knocked out (F2\textsuperscript{a} and F2\textsuperscript{b}). Although a double mutant genotype was possible in the F2, this was not obtained until the F3.](image)

Figure 5: Re-establishment of the mouse colony. Genotyping results from the progeny of a CD1 female with an Nck double mutant male (F0). The first filial generation (F1) resulted entirely in mice heterozygous for Nck1 and Nck2. The resulting F2 retained expression of either Nck1 or Nck2 while the other allele was knocked out (F2\textsuperscript{a} and F2\textsuperscript{b}). Although a double mutant genotype was possible in the F2, this was not obtained until the F3.
mixed outbred background to wild type CD-1 females. Using ear notches for identification and genotyping, selective breeding allowed for larger and more frequent litters, with all 10 breeding pairs from progeny involving the backcross producing offspring. Continued selective breeding resulted in all mice in the colony being Nck1−/−, Nck2 ff (Figure 5).

To generate the appropriate mice with Nck deleted from the endothelium, Nck double mutant mice were crossed to Tie-2 breeders (Nck1+/− Nck2 ff Cre+/−). Analysis of embryos collected at E10.5 confirmed that the CNKO genotype (Nck1−/− Nck2 ff Cre+/−) was obtained at the expected ¼ ratio. In the studies that follow, all other genotypes are considered as controls since they are expressing at least one Nck allele. The yolk sac surrounding the embryos was used to confirm the genotype through PCR.

3.2. Verification of endothelial-specific Nck knockout in vivo

Refer to Figure 6 for an overview of the embryonic heart anatomy that will be stated. Within the developing embryo, the endothelial specific Tie-2 promoter becomes active at E8.5 (Mustonen and Alitalo, 1995), which would allow roughly 48 hours for Cre to be produced and eliminate Nck from the endothelium of the CNKO embryos. In order to visualize this, E10.5 embryos were immunostained with an antibody recognizing both Nck1 and Nck2 (pan-Nck) (Figure 7). The control hearts showed ubiquitous staining for Nck throughout the embryo as expected (Figure 7A). In the CNKO embryonic heart, it appears that both the endocardial layer (arrow) and the area inside the developing endocardial cushions (asterisk) are devoid of Nck expression, without affecting Nck expression in the myocardium (Figure 7B). This is consistent with the literature which states that the mesenchymal cells in the endocardial cushions are of an
endothelial origin. The control embryos show abundant staining of Nck in the endocardial cushion (asterisk) and endocardial lining (arrow). To verify that the secondary antibodies were binding specifically to the expected primary antibodies, appropriate secondary antibody only controls were included in all immunofluorescence experiments (Figure 7C). The results show few puncta of intense staining that do not appear related to structures within the embryo. This indicates that the secondary antibody binds specifically to the primaries. Similar secondary only controls were included for each experimental replicate with similar results (data not shown).

Figure 6: Overview of E10.5 developing heart at the specified field of view (A) shown at 10x magnification (B) and 20x magnification (C). The pericardium is the outermost layer surrounding the heart. The myocardium is the muscular layer compromising the majority of the tissue. The endocardium is the innermost single layer of squamous endothelial cells. The endocardial cushions are encompassed by the red boxes, and then viewed at 20x magnification in ‘B’. The cardiac jelly is a specialized ECM highlighted in red (one side only) that occupies the space within the endocardial cushions.
Figure 7: Staining pattern of Nck in the E10.5 mouse heart. A) The control embryo shows ubiquitous staining of Nck throughout the heart including the endothelial lining (arrow) as well as the presence of Nck within the endocardial cushions (asterisk). The top panel shows the image at 10x magnification, bottom panel is at 20x magnification. B) In the CNKO embryos, there is an absence of Nck in the endothelial lining of the heart (arrows) as well as within the endocardial cushions (asterisk). The top panel shows the image at 10x magnification, bottom panel is at 20x magnification. C) Secondary only control fluorescing at a wavelength of 488 (green) shown at 10x magnification. EC indicates endocardial cushion; En, endocardium; My, myocardium; RBC, red blood cell.
3.3. Assessing endothelial and mesenchymal markers in the endocardial cushions \textit{in vivo}

In order to visualize the markers associated with EnMT, mouse embryos were isolated at E10.5 corresponding with the completion of endocardial cushion formation. Embryos were isolated prior to the onset of lethality associated with the CNKO phenotype. Sections were taken in the sagittal plane and immunostained with EnMT markers, as indicated. Images are of the atrioventricular canal at 20x magnification with the scale bar representing 35μm. CNKO embryos are classified as embryos of the genotype Nck1\textsuperscript{-/-}, Nck2\textsuperscript{ff}, Cre\textsuperscript{+/-}, whereas control embryos are any other genotypes expressing at least one allele of Nck. All image quantification was done using MacBiophotonics ImageJ by taking the ratio of the marker of interest relative to DAPI in order to account for any discrepancies of cell density within the area under visualization. It should be noted that in the CNKO embryos, there was substantial hemorrhaging throughout the embryo resulting in uncontained erythrocytes throughout the embryo.

3.3.1. VE-cadherin

VE-cadherin is a vital component of adherens junctions found exclusively in endothelial cells, and expression is lost upon mesenchymal transition. In the control embryos (Figure 8A) (n=10), strong staining of VE-cadherin is visible throughout the endocardial lining of the heart, as well as the outer layer of the endocardial cushion partitioning the atrial and ventricular space. Focusing on the endocardial cushions, the most abundant staining is along the endocardial lining, with the cells closer to the underlying myocardium showing a less intense and reduced abundance of the VE-cadherin stain.
The CNKO embryos (Figure 8B) (n=10), display strong staining for VE-cadherin throughout the endocardial lining, although not as abundant relative to the control. However, focusing on the endocardial cushions, it is clear that the morphology is distinctly different than the control. The cushions do not appear to have the same ability to distinctly separate the atrial and ventricular space. The cushions in the control embryos meet to form the adult valves at their apices, whereas the mutant embryos meet along their lateral edges. The VE-cadherin immunostaining pattern also differs between the mutant and control. The mutant embryos show VE-cadherin along the outer edge of the endocardial cushion, although not as abundant as the control. Interestingly, the cells within the endocardial space do not appear to lose expression of VE-cadherin in the CNKO embryos as they become more proximal to the underlying myocardium. The intensity of VE-cadherin staining remains similar between the cells migrating into the endocardial cushion and the overlying endocardium.

Quantitation of VE-cadherin expression within the endocardial cushions of control embryos showed an average ratio of 2.20 units of VE-cadherin stain to DAPI, with a standard deviation of 0.85, while, the CNKO embryos had an average of 5.58 staining ratio with a standard deviation of 1.01 (Figure 8C). Using a Student’s t-test, the difference in VE-cadherin expression within the endocardial cushions of the control and CNKO is significant with $p>0.005$ (actual $p = 1.40E-7$).
Figure 8: Endocardial cushions at E10.5 stained for VE cadherin with a DAPI nuclear stain. Immunofluorescence reveals strong VE-cadherin stain around the outer edges of the cardiac cushions (arrow) in both the wild type (A) and the CNKO (B) embryos. The inside of the endocardial cushions (asterisk) shows signal decreasing in the wild type embryo (A) as the cells invade towards the underlying myocardium. The CNKO embryo (B) retains abundant expression of VE-cadherin throughout the endocardial cushions (asterisk). C) Quantifying the intensity and abundance of VE-cadherin stain within the endocardial cushions relative to DAPI shows a significant increase in VE-cadherin staining in the CNKO embryo compared to the wild type. EC indicates endocardial cushion; En, endocardium; My, myocardium.
3.3.2. Claudin 5

Claudin5 is an endothelial specific component of tight junctions that regulates paracellular permeability of endothelial cells. Reduced expression of claudin5 is a critical event in the EnMT process. The control embryos (n=5) show robust Claudin 5 expression throughout the heart and into the lining of the endocardial cushion (Figure 9A). Once the cells move into the cardiac jelly, claudin 5 expression appears to decrease in a gradient fashion, becoming less intense and abundant as it becomes more proximal to the underlying myocardium.

The CNKO embryos (n=4) stained for claudin 5 show similar gross morphological defects to those stained for VE-cadherin in that there is not a definitive point where the cushions meet to create the separation between atrium and ventricle (Figure 9B). The abundance and intensity of claudin 5 expression is similar throughout the layers of the endocardium and myocardium. The CNKO appears to retain the expression of claudin 5 within the cardiac jelly as few differences in the intensity and abundance of claudin 5 staining are observed, regardless of the cells positions within the cardiac jelly.

Statistical analysis of the claudin 5 staining pattern between the control and CNKO embryos supports the observation of a more robust signal within the cardiac jelly of the CNKO relative to the control (Figure 9C). The average intensity of claudin 5 expression in the endocardial cushion of control embryos relative to DAPI was 3.25 +/- 0.92, while the CNKO embryos had an average of 7.35 +/- 1.70. This is a significant difference between the control and CNKO embryos with \( p<0.005 \) (actual \( p=0.0018 \)).
Figure 9: Staining pattern for claudin5 at E10.5 in the developing endocardial cushion. Immunofluorescent patterns for claudin5 show strong staining around the endocardial cushions (arrow) in both the wild type (A) and CNKO (B) embryos. A) As the cells invade into the cushions (asterisk) from the overlying endocardium the expression of claudin5 is decreasing in the wild type embryo as the cells migrate closer to the underlying myocardium. B) In the CNKO embryos, the expression of claudin5 is maintained throughout the endocardial cushion (asterisk). C) Quantitative analysis of the staining patterns in the cushions shows that claudin5 expression within the endocardial cushion is significantly greater in the CNKO embryo relative to the control. EC indicates endocardial cushion; En, endocardium; My, myocardium.
3.3.3. S100A4

S100A4 is a mesenchymal protein acquired after the mesenchymal transition that associates with the actin cytoskeleton and is believed to be involved in cell motility, invasion, and metastasis. The staining pattern of S100A4 in the control embryos is fairly uniform throughout the heart, while staining outside of the heart area (not shown) was minimal or non-existent (n=8). In the endocardial cushion, the staining pattern appears to be slightly more intense in the endocardial layer encompassing the cardiac cushions separating the atrium and ventricle compared to the underlying myocardium, particularly at the endocardium near the apex of the endocardial cushions (Figure 10A). As the cells move into the endocardial cushions, the expression of S100A4 remains abundant with a consistent intensity.

The CNKO embryos show expression of S100A4 throughout the heart (n=3), while minimal amounts of stain appear in the rest of the embryo (not shown). Through the section including the cardiac cushions, there is a stronger stain for S100A4 in the underlying myocardium than in the overlying endocardial layer surrounding the cardiac jelly (Figure 10B), which is quite different than in the control embryos. The cells that have migrated into the endocardial cushions appear to retain the same intensity and abundance as the overlying endocardium.

Quantifying the staining patterns of S100A4 in the cardiac cushions reveals significantly less S100A4 staining in the CNKO embryos relative to the control (p<0.01) (Figure 10C). The average staining intensity ratio between S100A4 and DAPI in the control was 10.60 (+/- 3.86), whereas the CNKO was 2.50 (+/-1.00).
Figure 10: Endocardial cushion staining patterns for S100A4 at E10.5 in the wild type (A) and CNKO embryos (B). A) Wild type embryos show a consistent expression of S100A4 throughout the endocardial cushion, whether it is at the overlying endocardium (arrow) or the maintenance of this expression into the cushion (asterisk). B) The CNKO embryos revealed an overall weaker staining pattern of S100A4 in the endocardial cushion, with the exception of the underlying myocardium. The overlying endocardium (arrow) had a weaker signal in the CNKO embryo and the expression of S100A4 was not increased as the cells invaded into the endocardial cushions (asterisk). C) Analysis of S100A4 in the endocardial cushion space reveals significantly greater expression of S100A4 in the wild type embryos relative to the CNKO embryos. EC indicates endocardial cushion; En, endocardium; My, myocardium.
3.3.4. SMA

SMA is required for a cell to have a contractile phenotype which aids in locomotion. It is not found in endothelial cells and is acquired after the EnMT process. In the control embryos stained for SMA (n=8), there is strong staining in the underlying myocardium, but the endocardial lining does not have any SMA stain with the exception of the cells encompassing the endocardial cushions, which show weak staining for SMA (Figure 11A). From the apex of the cardiac cushions through to the underlying myocardium, it appears that the cells within the cardiac jelly maintain their SMA expression throughout the cushion.

SMA immunostaining in the CNKO embryos (n=6) also showed strong staining in the underlying myocardium (Figure 11B), however the staining pattern in the area of the endocardial cushions is drastically different from the controls. There does not appear to be any staining in the endocardial layer of the heart, and no consistent expression of SMA in the endocardium surrounding the cushions. Within the endocardial cushions there is no acquisition of SMA or any major staining patterns.

Using the same methods of quantification as the other markers, there is significantly more staining of SMA within the endocardial cushions of the control embryos than the CNKO embryos ($p<0.001$) (Figure 11C). The average staining ratio of SMA to DAPI was 0.23 (+/- 0.10) in the CNKO while the control had a ratio of 0.79 (+/- 0.26).
**Figure 11:** Staining patterns for SMA in the developing endocardial cushions at E10.5 between wild type (A) and CNKO (B) embryos. Both wild type and CNKO embryos show string expression of SMA in the underlying myocardium of the primitive heart. In both embryos there is minimal expression of SMA in the endocardial lining overlying the cushions. Within the endocardial cushion (asterisk) there is mild acquisition of SMA in the wild type embryos (A), whereas in the CNKO embryos there is almost no acquisition of SMA (B). C) Quantifying the expression levels of SMA within the endocardial cushions between the wild type and CNKO embryos reveals significantly more SMA staining in the wild type than the CNKO cushions. EC indicates endocardial cushion; En, endocardium; My, myocardium.
3.4. *In vitro* development of tools to assess effects of Nck

Parallel to the *in vivo* studies, *in vitro* tools were being developed to aid in elucidating the involvement of Nck during TGFβ signaling in endothelial cells. Several methods were attempted for the development of such tools: 1) shRNA mediated knockdown of BAEC previously validated as endothelial in origin, and 2) primary culture of immortalized endothelial cells.

3.4.1. shRNA knockdown of Nck

To begin preliminary experiments testing the effects of Nck on EnMT *in vitro*, shRNA mediated knockdown of Nck1 and Nck2 was attempted in BAEC. 10 plasmids in total were used, 5 for each of Nck1 and Nck2. Each plasmid targeted a slightly different sequence of Nck mRNA. Plasmids were transduced into the cell cultures with no visible knockdown of total Nck levels (Figure 12). It was believed that the plasmids may have required the combined effect of different target sequences, so to reduce this, different permutations of the plasmids were transduced into the cell cultures. With different combinations of plasmids there was still no visible knockdown of pan-Nck expression levels in the BAECs (Figure 12). Although the loading control (GAPDH) shows there is less overall protein with certain combinations of shRNA plasmids (ie: lane 4), all of these lanes still show roughly the same expression levels of Nck, further confirming shRNA transduction did not achieve a knockdown of Nck.
3.4.2. Introducing Immortomouse® transgene into Nck mutant mouse colony

Immortomouse® was crossed with the existing Nck double mutant mice to initiate our temperature sensitive SV40 transgenic mouse colony. The first progeny were all heterozygous for Nck1 and Nck2, and were roughly at a 50/50 ratio between heterozygous expression of the immortalizing gene, or wildtype (not expressing the immortalizing gene). Multiple generations of selective mating eventually yielded mice of the genotype Nck1<sup>+/−</sup>, Nck2<sup>+/−</sup>, and SV40<sup>+/−</sup> (Figure 13). Unexpectedly, we did not obtain mice with this genotype at the expected Mendelian frequency. It took 43 pups before obtaining one pup with this genotype when the expected ratio was at worst a 1/16 chance depending on the genotype of the parents. The ratio was variable as the several prospective parents had varying genotypes altering the expected ratio. Mice that did not carry the SV40 transgene were also used for breeding purposes in this colony since the presence of this transgene greatly reduced the lifespan of the mice. Mice heterozygous or
homozygous for the expression of the SV40 transgene died between 4-6 months of age. Post mortem evaluation performed by CAF revealed significant thymic hyperplasia increasing the mass of the thymus to approximately 10x the normal size and occupying 85% of the thoracic cavity. The enlarged thymus took on a soft irregular shape that appeared to block the airways.

Figure 13: Breeding in the temperature sensitive SV40 gene to Nck double mutant mice. Crossing an Immortomouse ® to existing Nck double mutants resulted in the F1 generation being heterozygous for Nck expression while approximately half the progeny express the SV40 immortalizing gene. Selective breeding resulted in the F2 and all future generations being homozygous null for Nck1 expression and homozygous floxed for Nck2 expression while alternating SV40 gene expression.
3.4.3. Isolation of immortalized endothelial cells and adenovirus transduction

Cells were isolated from mice that were either Nck1+/−, Nck2ff, and SV40+/− or Nck1−/−, Nck2ff, and SV40+/− mouse, ensuring that after adenovirus transduction with Cre recombinase, all permutations of Nck expression would be available. Upon isolation of immortalized lung endothelial cells using Dynabeads, cell cultures were expanded, lysed and tested for expression of endothelial markers to confirm their lineage. Antibodies for S100A4, Claudin5, CD31, and ZO-1 were not effective at detecting protein expression via western blot, so the majority of analysis from western blots is via the endothelial marker VE-cadherin and the mesenchymal marker SMA. Successful isolations had at least one culture expressing high levels of VE-cadherin and no SMA even after long exposures (Figure 14A). Not all isolations were successful, as some isolates yielded no VE-cadherin expression while abundant SMA was observed. Primary isolates were split into 6 well collagen coated plates for adenovirus transduction of Cre. After culturing the cells for 3 more passages, collected whole cell lysates were analyzed to determine levels of Nck expression and verify that they maintained their endothelial nature after being cultured. At this point, there were some discrepancies in the results. Some experimental replicates showed there was an acquisition of SMA, and a slight decrease in VE-cadherin protein expression, yet from the same whole cell lysate, there were replicates showing maintenance of strong VE-cadherin expression and no SMA expression (Figure 14B). It is thus unclear whether the cultures are of endothelial nature, or whether they have acquired mesenchymal traits as they were passaged during the cell culturing process.
Figure 14: Characterizing the primary endothelial cell line established from the Nck double mutant Immortomouse ®. A) Isolated cells were cultured, and the lines producing sustained cell growth were lysed and characterized to identify their origin as endothelial. All isolations from both the heterozygous and null cell lines showed strong VE-cadherin expression, with little to no smooth muscle actin expression. The cell lines with the least smooth muscle actin were propagated, while the other isolates were frozen down. B) After several passages, isolates were tested again to verify their endothelial origin. Mixed results were obtained. In some scenarios (left) there would appear to be less VE-cadherin in the cells, with a simultaneous acquisition of SMA. Whereas several other test runs revealed that the isolates had retained their VE-cadherin expression and had not acquired any SMA (right) indicating they were likely still endothelial in nature.
The success of the adenovirus transduction of Cre or GFP control was determined using a combination of PCR and western blots. Confluent cells were genotyped to confirm the genomic deletion of Nck post adeno-Cre infection. The control cell line showed the same genotype for Nck as the isolation was performed on \((\text{Nck}^{1+/-}, \text{Nck}^{2\text{fr}})\) except where Cre was added to facilitate the removal of the Nck2 allele (Figure 15A). It can be seen that the Nck null cell line with the addition of Cre is null for Nck1, and has the floxed Nck2 allele removed as well, compared to the Nck null lines with the adeno-GFP transduction and the no virus control which both retain expression of the non-recombined Nck2 floxed allele (Figure 15B). Thus, it appears that the transduction of Cre-recombinase is able to remove the floxed Nck2 allele from the genome.

To complement the PCR work, western blots were used to measure the expression levels of Nck between the cell lines. Blotting for Nck1, it can be seen that the control cell lines all retain expression of Nck, while Nck1 expression is lost in the null cell lysates. Staining for Nck2 shows all cell lanes retain Nck2 expression except for the lysates that were exposed to the adeno-Cre virus to facilitate the removal of the floxed Nck2 allele (Figure 15C). Although the GAPDH loading control for the heterozygous cell lysates is not loaded equally despite conducting a BSA curve and densitometry from previous Western blots, it is still sufficient to show expression of Nck1 and lower Nck2 expression than its corresponding controls. Taken together, this indicates that the addition of Cre-recombinase is able to reduce the expression of the floxed Nck2 allele.
Figure 15: Genotyping of the primary Nck null and control cell isolates after Adeno-Cre infection. The control cell line is Nck1+/−, Nck2fl/fl, and the null cell line is Nck1−/−, Nck2fl/fl prior to adenovirus infection. A) Control cell line is heterozygous for Nck1, and it can be seen that Adeno-Cre infection (Ad-Cre) had no effect on Nck1 expression. The Nck2 allele was floxed and with the addition of Cre-recombinase, the Nck2 allele is removed from the isolate (lane 1). No effect on Nck2 expression was seen in the Adeno-GFP (Ad-GFP) or no-adenovirus controls (No-Ad) (lanes 2 and 3). B) In the null cell line, the Nck1 allele was null in all the samples genotyped and was not affected by the adenovirus. The Nck2 allele was floxed and is excised by the addition of the Adeno-Cre (Ad-Cre) (lane 1), while the Adeno-GFP (Ad-GFP) and no adenovirus (No-Ad) controls had no effect on the genotype (lane 2 and 3). C) Het lysates are from Nck1+/−, Nck2fl/fl and null lysates are from Nck1−/−, Nck2fl/fl primary immortalized isolates. Blotting for Nck1 shows the Nck null cells devoid of Nck1 while the cell lysates heterozygous for Nck1 retain expression. Stripping and re-probing the membrane for Nck2 shows loss of Nck2 expression in both cell cultures exposed to Cre as expected, while controls retain expression of Nck2.
4. Discussion

4.1. Future considerations within the mouse colony

As the majority of experiments described in this thesis were based around the breeding success of the Nck double mutant mice, the regeneration of that colony was of utmost importance. The backcross of the CD1 wild-type mouse to the existing mutants proved to be successful in enabling successive generations to breed. However, this was not without its drawbacks as it took 27 weeks to complete the backcross and subsequently intercross Nck double mutant animals. While this was not a labour intensive process, it did greatly hinder the ability to obtain embryos for immunostaining analysis and breed the Nck double mutants to Immortomouse for primary cell isolations. Given the amount of time it took to regenerate the colony, it would be a worthwhile investment to integrate a backcross to a wild type CD1 mouse annually within the breeding of the maintenance mice. If this is done prior to the onset of breeding issues, there would be a ¼ chance of obtaining an Nck1<sup>−/−</sup> and Nck2<sup>−/−</sup> in the second generation, and given the large litter sizes this would be highly probable. Regularly completing this backcross would ensure that the genetic pool for the colony would remain diverse and thus greatly reduce the risk of future setbacks. This would also eliminate the need to perform a backcross on the Tie-2 Cre breeders as the female Nck double mutants crossed with these males would already have increased genetic diversity.

4.2. In vivo effects of Nck on EnMT

Using immunoanalysis, a near complete absence of Nck in the endothelium of the developing heart of CNKOs at E10.5 has been confirmed. As such, all further discussions
relating to Nck in the endocardium are under the assumption that neither Nck allele is present in the endothelia of the developing heart.

It is interesting to note that the CNKO embryos had major hemorrhaging throughout the embryo, and especially near the primitive heart (data not shown). This is likely a result of the inability of Nck deficient endothelial cells to remodel and integrate with the other layers of blood vessels, impairing vascular integrity (Clouthier et al., unpublished). This underscores a confounding variable associated with the CNKO embryos as the impaired vasculature would decrease hemodynamic forces. Hemodynamic forces do play a role in remodelling the developing heart (Lucitti et al., 2007), but given the magnitude of Nck’s role in signaling cascades associated with the mesenchymal transition, the heart defects seen in the CNKO embryos are more thoroughly explained as alterations in Nck signaling pathways.

The greatest instigator of the mesenchymal transition as cells invade the cardiac jelly is the release of TGFβ2 from the underlying myocardium. Smad-dependent and Smad-independent pathways in response to TGFβ have been intensely investigated. There is little evidence to suggest that Nck is directly involved in the Smad-dependent activation pathway, although this possibility cannot be ruled out. It is more likely based on the observations and background literature that Nck is playing a role in the activation of GTPases and other downstream effectors of the Smad-independent pathway following TGFβ activation.

Immunoanalysis of CNKO embryos revealed a greater retention of endothelial markers (VE-cadherin and claudin5), and less acquisition of mesenchymal markers (SMA and S100A4) relative to the control embryos (Figure 16). Meta-analysis shows that the coordinated activation of GTPases is a common element involved in the loss of endothelial markers as well as the
increase in the mesenchymal markers during EnMT. Given Nck’s known requirement in activation of many GTPases, and the work presented here implicating Nck in induction of EnMT, it suggests that Nck could provide a link between TGFβ mediated EnMT and the activation of GTPases. Such a role for Nck in EnMT in the endocardial cushions is further supported by previous findings as well.

Figure 16: Illustration of the proposed role Nck has on protein expression within the endocardial cushions. At E10.5 when Nck is selectively removed from the cells originating from the endothelial tissue of the heart (blue) embryonic cushions show elevated expression of VE-cadherin and claudin5 (endothelial markers), with reduced expression of S100A4 and SMA (mesenchymal markers) relative to the control embryos.
4.2.1. VE-cadherin

VE-cadherin is an important component of adherens junctions wherein it maintains inter-endothelial integrity, but it also has much more extensive functions in endothelial cells. VE-cadherin acts as a communication hub by maintaining endothelial junctions, interacting with the actin cytoskeleton, and regulating intracellular signaling cascades (Taddei et al., 2008). As such, the internalization of VE-cadherin to degrade endothelial junctions is intimately linked to the acquisition of mesenchymal characteristics (Schmidt et al., 2007). Tyrosine phosphorylation on VE-cadherin (and claudin5) as a result of TGFβ stimulation mediates further downstream signaling including internalizing VE-cadherin to the cytoplasm and para-nuclear region (Shen et al., 2011). Immunoanalysis of embryos revealed a significantly higher abundance of VE-cadherin in the endocardial cushion space in a model null for Nck. This could in part be explained by Nck’s role in the activation of GTPases, particularly the Rho and Rac family for VE-cadherin. The coordinated spatial activation of Rho and Rac is critical for VE-cadherin function and the appropriate intracellular responses (Sander et al., 1999).

Upon phosphorylation of conserved residues in the cytoplasmic tail of VE-cadherin, the internalization of VE-cadherin is through endocytosis of the protein complex into clathrin coated vesicles (Gavard and Gutkind, 2006). Endocytosis is mediated, in part, through the coordinated activity of several members from the Rho family of GTPases. The coordinated activation of specific Rho proteins are responsible for determining the fate of endocytic vesicles via intracellular trafficking (Ellis and Mellor, 2000; Moravec et al., 2012). If Nck is responsible for coordinating the activation of these Rho family proteins involved in endocytosis it could explain the abundance of VE-cadherin in the endocardial cushions of the endothelial Nck knockout embryos.
4.2.2. Claudin5

Little work has been done investigating the mechanism behind claudin5 degradation over the course of the EnMT, but some of the preliminary work implicates a role for GTPases in this process. The immunoanalysis of the CNKO embryos shows a preservation of claudin5 expression within the endocardial cushions after the cells have invaded this space. Similar to VE-cadherin, it has been proposed that TGFβ phosphorylates claudin5 initiating its internalization and targeting it for degradation (Shen et al., 2011). Activity of two Rho family members (RhoK and RhoA) is linked to the phosphorylation events on claudin5 (Yamamoto et al., 2008; He et al., 2012). Again, given Nck’s requirement for activation of Rho proteins, preventing this phosphorylation event on claudin5 could prevent its internalization and degradation, which would explain its abundant expression within the endocardial cushions of the CNKO embryos. An alternative possibility explaining this is through direct relationship between VE-cadherin and claudin5. VE-cadherin can induce PI3K activation, and through a cascade allows the phosphorylation of FoxO1 removing its inhibition on the claudin5 promoter, leading to an up regulation of Claudin5 (Taddei et al., 2008). Since the CNKO embryos retain expression of VE-cadherin FoxO1 remains phosphorylated and is never able to inhibit claudin5 expression. In both cases, the removal of Nck as a facilitator of Rho family activation explains the retained expression of claudin5 in the embryonic endocardial cushions.
4.2.3. S100A4

Our immunofluorescence study showed significantly less S100A4 acquired within the endocardial cushions of CNKO embryos relative to the controls. S100A4 has become a widely studied marker to assess when cells have gained mesenchymal characteristics, but similar to claudin5, there is little study as to how its production is increased in the mesenchymal state. One of the mechanisms seen to increase the transcriptional activation of S100A4 is through STATs (signal transducer and activator of transcription) (Liu et al., 2012a). STATs become phosphorylated via SH2 domains and translocate to the nucleus to alter gene expression (Darnell, 1997). The inability of CNKO embryos to acquire S100A4 in the endocardial cushions can be related back to the activation of Rho family of GTPases. RhoA activity is required for the transcriptional activation of STATs, while Rac1 is crucial in the phosphorylation events on STATs that cause their translocation to the nucleus (Pelletier et al., 2003). If Nck is involved in GTPase activity, then without Nck to facilitate the activation Rac1 and RhoA less STATs would become active, and the expression levels of S100A4 would be lower, which is seen in the CNKO embryos.

4.2.4. SMA

Looking at the staining pattern of SMA within the endocardial cushions of the CNKO embryos, it is clear that Nck is affecting the acquisition of SMA, as there is almost no expression of SMA within the cushions. The acquisition of SMA within the control embryos is not as intensely stained as previous literature suggests it should be, but it is present. This discrepancy may be explained using Nck’s affiliation with RhoA activation. RhoA activity is widely detected in the AVC during the periods corresponding with endocardial cushion formation in
response to TGFβ (Larue and Bellacosa, 2005; Tavares et al., 2006). In response to TGFβ stimulation, RhoA specific promoters are increased resulting in enhanced SMA production through RhoA pathways (Krendel et al., 2002; Tsapara et al., 2010). Furthermore, upon TGFβ stimulation, myocardial related transcription factor-A (MRTF-A) expression is increased and localizes in the nucleus, greatly increasing the production of α-SMA in a Rho dependent manner (Small et al., 2010). Given the intimate relationship between RhoA activity and SMA production, and that depletion of Nck prevents RhoA activation (Ruusala et al., 2008), it is plausible that there is no SMA production in the CNKO embryos.

4.2.5. Previous unexplained work implicating Nck in GTPase activity

Nck has not been shown to be involved in TGFβ mediated induction of EnMT during heart development, but it does appear based on immunofluorescence (IF) that it plays a role, which may be in the activation of GTPases which coincides with previous findings. Activated Rac1 is required by VE-cadherin to maintain microvasculature stability and integrity (Waschke et al., 2004), and if Nck is involved in Rac1 activation, it could explain the hemorrhaging seen in the CNKO embryos. Unpublished work from our lab shows a significant inhibition of migration in endothelial cells under VEGF stimulation and angiopoietin-1 treatment when Nck is knocked out. The inhibition of migration is far greater though in response to angiopoietin-1. This is easily explained if Nck is involved in GTPase activation as VEGF utilizes the coordinated activation of Rho, Rac and Cdc42 in its downstream signaling cascade, but their activation is not always required (Ferrara et al., 2003). Whereas angiopoietin-1 requires the coordinated activation and inactivation of RhoA and Rac1 to maintain vascular function (Mammoto et al., 2007).
The severely impaired vasculature and heart formation in the CNKO embryos brought about the concern of hemodynamic forces being a confounding variable to the proper formation of the heart. As such, it is interesting to note that RhoA is a major protein involved in the mechano-transduction of the hemodynamic forces mediated the development of the embryonic heart and vasculature (Tan et al., 2012).

Previous work has shown that Nck is critical for cell movement and migration (Buday et al., 2002; Bladt et al., 2003), creating the possibility that the malformed endocardial cushions are a result of migratory defects, and not related to an EnMT defect. This does not seem likely though as the cells within the endocardial cushion of the CNKO do not appear to have undergone EnMT evident by the retention of endothelial markers and failure to acquire mesenchymal markers. Given the critical role of GTPase activation in cell migration (Ruusala et al., 2008; Chaki et al., 2012) and in the mesenchymal transition (Kalluri and Neilson, 2003; Yilmaz and Christofori, 2009), it is possible Nck is involved in both pathways through GTPase activity.

4.2.6. Future directions

To examine whether Nck signaling plays a role in the activation of GTPases in response to TGFβ, complementary in vitro experiments could be set up. Ideally this could be done in endothelial cells, but given the difficulty in establishing our own endothelial Nck knockout line, a different cell type could be used and extrapolated back. Many GTPase activation assays are commercially available and could be used to assess which GTPases are affected by an absence of Nck in response to TGFβ. Fluorescent resonance energy transfer microscopy could also be performed on cell cultures to determine the spatiotemporal activation of GTPases in response to
TGFβ in the presence and absence of Nck (Komatsu et al., 2011). Given that Nck has previously been linked to the activation of GTPases, and based on the potential connection between the \textit{in vivo} findings of the CNKO embryos and GTPase deregulation, it would be logical to explore a novel role for Nck in GTPase activity in response to TGFβ.

To further analyze and complement the findings from the immunofluorescence studies, real time/quantitative PCR could be utilized. This would unveil any changes in transcriptional activation of endothelial and mesenchymal markers in CNKO embryos. Only the heart would be examined to minimize background noise. Preliminary trials have shown that sufficient amounts of RNA can be extracted from a single embryonic heart to allow proper assessment. This approach could also allow analysis of other markers that were not successfully detected in the paraffin sections, including CD31, Slug, ZO-1, and Tie-2. Altogether, having quantified levels of RNA transcripts would be very useful when combined with immunofluorescence-based protein analysis in understanding the role of Nck in EnMT.

4.3. \textit{In vitro} Discussions

4.3.1. Lentiviral shRNA knockdown of BAEC

Initially, lentivirus-mediated knockdown of Nck in a previously validated endothelial cell line, BAEC, was going to be used as a preliminary model to investigate Nek’s role in EnMT. This approach was unsuccessful as knockdown of Nck was not obtained despite various efforts. Although this could be a result of using shRNA targeted to Nck in the mouse genome to knockdown the bovine Nck counterparts, we pre-determined that bovine and mouse Nck are quite conserved, thus this was expected to work. Considering a knockdown of Nck using these
shRNA plasmids was ineffective in mouse and human cell cultures through various other efforts in the lab (data not shown), there was likely another issue. Various groups have published results using shRNA or siRNA mediated knockdown of total Nck in cells before. The transduction process into the BAEC was successful as the cells gained puromycin resistance; it could be a result of our plasmids having internal ribosome entry sites allowing the translation of the puromycin resistance gene without translating the upstream shRNA targeted to Nck.

4.3.2. Isolation of primary endothelial cells

Isolation of primary immortalized endothelial cells proved to be more successful than the attempts at a knockdown model. In the majority of cases, isolations using Dynabeads worked well and initially yielded cultures that appeared to be endothelial, showing strong expression of VE-cadherin but not α-SMA. After isolation, but prior to the first passage, the cells would grow very slowly, then after the first passage the primary immortalized cells would grow rapidly. Over the course of growing and passaging, the isolates would appear to acquire expression of mesenchymal markers. We reasoned that this could have been from either spontaneous transition into a mesenchymal phenotype, or from trace amounts of mesenchymal cells from the initial isolation out-competing the endothelial cells. A spontaneous acquisition of mesenchymal characteristics has been documented before (Liu et al., 2012b), and may be a by-product of sub-confluent cell cultures. Endothelial cells require the homotypic binding of VE-cadherin to maintain their underlying cytoskeletal organization and adhesion dynamics. To try and minimize this potential effect, primary cell cultures were never allowed below 50% confluency so there were always cell-cell contacts and therefore continuous VE-cadherin binding. The other issue of mesenchymal cell contamination was addressed as well. Given that endothelial cells are more
adhesive than mesenchymal cells, the first cells to lift off the culture plates would be the mesenchymal cells. Therefore, cells were washed for 2 minutes in trypsin, or until the first cells started to lift off the plate before passaging the cells. In order to determine which of these issues might be the underlying cause of this mesenchymal acquisition, a clonal selection of a specific endothelial cell could be done using a process of serial dilutions. If a clonal population of endothelial cells is still acquiring mesenchymal characteristics, then the cause might be from sub-confluent cultures affecting the underlying dynamics of the endothelial cell. If the spontaneous acquisition of mesenchymal traits was from the sub-confluent cell cultures, the endothelial cells could be grown on cadherin coated plates in a similar manner to Nagaoka et al., 2006. This would have an advantage over our collagen coated plates as it would maintain homotypic binding of VE-cadherin and more closely mimic the natural environment of endothelial cells. Growing the cells on cadherin coated plates has shown to yield endothelial cells with greater proliferative capacity, transfection efficiency, and maintenance of unique endothelial characteristics (Nagaoka et al., 2006).

Analysis of the endothelial and mesenchymal markers from the primary cell cultures yielded inconsistent results. Several attempts showed an acquisition of α-SMA, while others showed no α-SMA acquisition while maintaining strong VE-cadherin expression. Despite using the same samples for repeated runs, results were varied, making it difficult to proceed without having a validated cell line. Unfortunately, many of the markers available including CD31, claudin5, S100A4, Tie2, Slug, and ZO-1 were not able to detect any protein expression in samples or controls via Western blots. Using another analytical technique such as flow cytometry could be used to verify the Western blot findings while hopefully increasing the number of markers that can be used to validate the endothelial nature of the primary isolates.
With an established endothelial cell line, and knowing that the adenovirus is efficient at recombining the floxed Nck2 allele, it would be interesting to assess the impact of exogenous TGFβ application on the cell cultures. With the kinetics of the TGFβ response mapped out in the BAEC (data not shown), it will hopefully be a seamless transition to assess the effects of Nck on TGFβ-induced EnMT. Of particular interest would be whether or not Smads can become activated via phosphorylation in response to TGFβ in the absence of Nck. This would either validate the previous hypothesis that Nck is exerting an effect on the mesenchymal transition via Smad independent pathways, or if the Smads cannot be phosphorylated, it would provide new insights into the role Nck plays in response to TGFβ. Smad activation, as well as the endothelial and mesenchymal markers, could be assessed using combinations of Western blots, flow cytometry and quantitative/real time-PCR. This could be complemented by immunofluorescence on the cell cultures to determine the localization of the various protein markers.

5. Concluding remarks

Overall it is clear that Nck plays a role in the ability of endothelial cells to acquire mesenchymal traits in the developing heart based on the in vivo immunofluorescence analysis. With the continued development of in vitro tools to complement these in vivo findings, it could be definitively shown that Nck plays a role in the mesenchymal transition. It is well established that Nck is involved in Rho family activity, thus teasing out which family members are affected by Nck deficiency in response to TGFβ would be a useful step in mapping out the Smad independent pathway of TGFβ. Determining whether Nck plays a role in the activation of Smad’s in response to TGFβ during the mesenchymal transition would also be of particular
interest. Further elucidation of the role Nck plays in EnMT would be valuable knowledge as EnMT relates to congenital heart defects, tissue and organ fibrosis, and is linked to tumour metastasis.
6. References:


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