THE ROLE OF Nck ADAPTORS IN ENDOTHELIAL CELL POLARITY

A Senior Scholars Thesis

by

DEEPIKA RAM

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

May 2012

Major: Biomedical Science

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Approved by:

Research Advisor: Gonzalo M. Rivera
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ABSTRACT

The Role of Nck Adaptors in Endothelial Cell Polarity. (May 2012)

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The establishment of a polarized endothelial layer is essential for normal vascular organization. This project examined the role of actin cytoskeleton remodeling by Nck adaptors in the polarized organization of endothelial cells. We assessed the role of Nck in the subcellular distribution of markers of polarity in cells cultured in two dimensions. Cellular levels of Nck were manipulated using tools of molecular genetics. Time-course experiments using control cells demonstrated that an incubation period of 2 h following the wounding of a confluent monolayer is optimum for the analysis of cell polarization. Our experimental results showed a significantly decreased index of cell polarization in Nck-depleted (shNck) vs. control or rescued cells (control ~59%; shNck, ~32%, and rescued ~55%). These findings strongly suggest an important role for Nck-dependent actin rearrangements in the establishment of polarity of endothelial cells. These studies open new avenues of research into the molecular mechanisms controlling the formation of polarized vascular networks.

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CHAPTER I

INTRODUCTION

The cytoskeleton of mammalian cells, consisting of microtubules, intermediate filaments, and actin filaments, is a dynamic and interconnected network that determines cell shape and provides the necessary structural components for cell motility (Fletcher and Mullins, 2010). Actin filaments, polymers of the abundant cellular protein actin, undergo continuous reshaping (i.e, assembly and disassembly) into subcellular structures involved in the organization and function of cells. Actin filaments contribute to the establishment and maintenance of cell polarity, which is the differential distribution of macromolecules and organelles across the cell. Cell polarity is an integral component of many developmental processes, and the loss of cell polarity is frequently linked to diseases including cancer (Wodarz and Nathke, 2007). The development of the vascular network of an organism through vasculogenesis and angiogenesis involves cell polarity. The establishment of a polarized endothelial layer is essential for normal vascular organization. The cytoskeletal architecture, including the organization of cell-cell junctions, underlies the establishment of apical-basal polarity in endothelial cells. The small GTPases Cdc42, Rac, and RhoA, well characterized regulators of cytoskeletal dynamics, play an essential role in the organization of cell polarity by modulating the subcellular distribution and activity of polarity proteins, including the partitioning defective (PAR) polarity complex, Crumbs, and Scribble (Iden and Collard, 2008).

This thesis follows the style of *Journal of Cell Biology.*

One of the research interests in the Rivera lab concerns the regulation of the actin cytoskeleton by non-catalytic proteins –generically termed adaptors– that bridge various molecular components in signaling cascades. The Nck family of adaptors includes two closely related proteins, Nck1 (or α) and Nck2 (or β), that link extracellular signals with cytoskeletal remodeling (Li et al, 2001). These adaptors, consisting of three Src Homology (SH) 3 and one SH2 domain, are well suited to coupling signals mediated by tyrosine phosphorylation with cytoskeletal effectors. In previous studies we showed that an increased local concentration of Nck induces N-WASp/Arp2/3-dependent actin polymerization in living cells (Rivera et al, 2009) (Rivera et al, 2004). Importantly, these adaptors have been involved in vascular endothelial cell motility stimulated by the activated VEGF receptor (VEGFR-2) (Stoletov et al, 2004) (Lamalice et al, 2006). Modulation of actin dynamics by Nck adaptors may also play a role in the establishment of endothelial cell polarity and vascular lumen organization. For instance, Nck has been involved in EphA-ephrinA-dependent enhancement of E-cadherin-based cell-cell contacts and the establishment of apical-basal polarity in MDCK cells (Miura et al, 2009). Since EphA activation plays a critical role in VEGF-stimulated angiogenesis (Cheng et al, 2002), it is likely that Nck adaptors provide a physical link between these converging signaling pathways. Indeed, our preliminary data show that Nck depletion by siRNA impairs endothelial cell morphogenesis, presumably, by altering cell polarity and VE-cadherin-dependent cell-cell junctions (Figure 1.). However, the molecular mechanisms by which Nck promotes cell polarity are unknown.

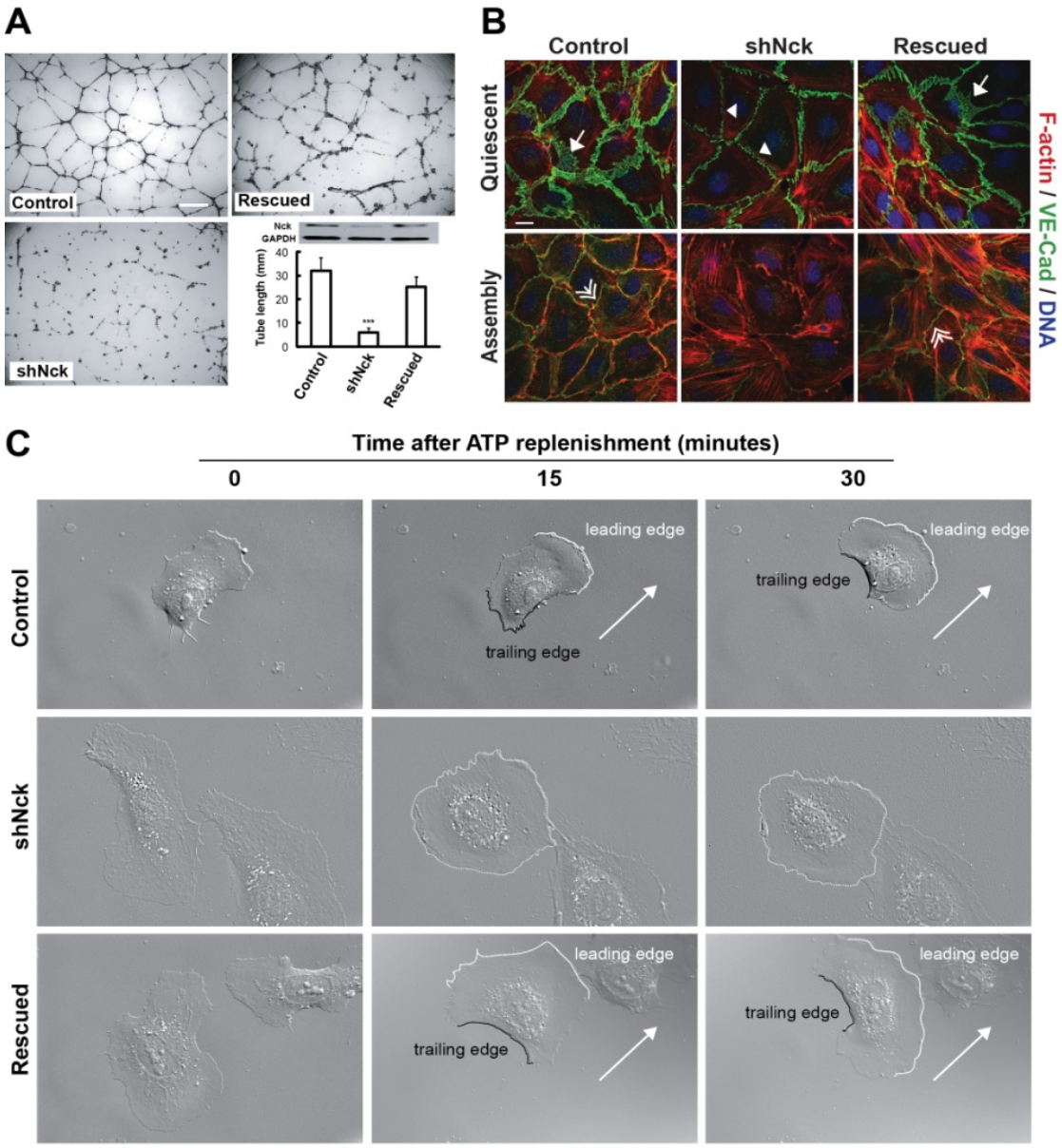

Figure 1: Nck presence is important for cell polarity and morphogenesis. Endothelial cell cytoskeletal remodeling and polarity. Nck depletion (shNCK) disrupts *in vitro* tube morphogenesis (A), the organization/assembly of cell-cell junctions (B), and the establishment of cell polarity (C).

CHAPTER II

METHODS

Materials

Human Umbilical Vascular Endothelial Cells (HUVEC) were obtained from Lonza Walkersville Inc. C2519A. Primary Antibody GM130 was acquired from BD Transduction Laboratories 610822 and BSA was obtained from Sigma-Aldrich Co. A3059-100G. For the secondary antibodies Tx-Red Phalloidin was obtained from Invitrogen Molecular Probes and DAPI was collected from Sigma-Aldrich Co. D8417. DPBS was obtained from HyClone Laboratories Inc. SH30028.02.

Virus preparation and infection

Virus preparation was performed using 293T cells. The plates prepared were p-super puro (-), p-super puro – Nck1c, PSP – Nck2a, and MSCV – mNck-2. P-super puro (-) was used for the control p-super puro – Nck1c and PSP – Nck2a were used in the double knock-down and MSCV – mNck-2 was used for the rescue. Virus collection was performed for 48 hours. A double infection was then performed on HUVEC cells.

Cell culture and scratch induced migration

For the scratch induced assays, cells were seeded on pre-coated Fibronectin coverslips and grown to a confluence of 50×10^4 in Complete Media (CM). Individual scratch wounds were then made using a small pipette tip. The cells were then incubated during various time intervals.

Immunofluorescence and image quantification

Cells were first fixed with paraformaldehyde and permeabilized using Triton X-100. Then cells were blocked with 2% BSA and incubated with GM130 antibody to visualize the Golgi Apparatus. Then they were incubated with secondary antibody goat and mouse 488 mixed with DAPI to visualize the nucleus and Tx-Red Phalloidin to visualize filamentous actin. Slides were mounted using Fluoromount G and taken for fluorescent microscopy. Images were captured using an Olympus IX70 microscope equipped with a 10x, 20x, and 40x objectives. Images were then analyzed using ImageJ.

Nucleus and Golgi Apparatus orientation and cell polarity

Golgi orientation relative to the nucleus and the wound area was quantified by dividing the cell into three 120° sectors with the nucleus in the center. One sector faces towards the wound edge, and cells with the Golgi Apparatus oriented within this sector were considered as polarized. Approximately 100-150 cells per experimental group were analyzed to calculate the total percentage of polarity.

CHAPTER III

RESULTS

The effect of incubation times on endothelial cell polarity

We observed polarity in a wound-induced migration assay of HUVEC cells through a time course experiment. Time points were set to 1, 2, 4 and 6 hours. Confluent monolayers were starved overnight and, after wounding by scratching with a pipette tip, cells were incubated for various periods of time. After incubation, the cells were fixed, permeabilized, blocked, incubated with 1° antibody (GM130), incubated with 2° antibodies (goat and mouse 488, DAPI, and Tx-Red Phalloidin). It was found that the 1 hour plate showed mixed results with approximately 50% polarization of the cells (Fig. 2 A). For the 2-hour incubation, cells show a good percentage of polarization at around 60% (Fig. 2 B). During the 4-hour (Fig.2 C) and 6-hour (Fig. 2 D) incubation time point, cells showed a high amount of polarization at around 80%. At these time points, cells were also observed to have migrated into the wound site and the monolayers became less compact. When the results were analyzed it was seen that the percentage of polarization in cells increased linearly from the 0-3 hour time points. At around 4 hours, the indexes of cell polarity plateaued and no significant increases were observed after incubation for 6 hours (Figure 3). Thus, we considered 2 hours of incubation as optimum because at this time the increase in polarity was linear and the monolayers still offered a compact/confluent appearance. In subsequent experiments cells were incubated for 2 hours following wounding of monolayers.

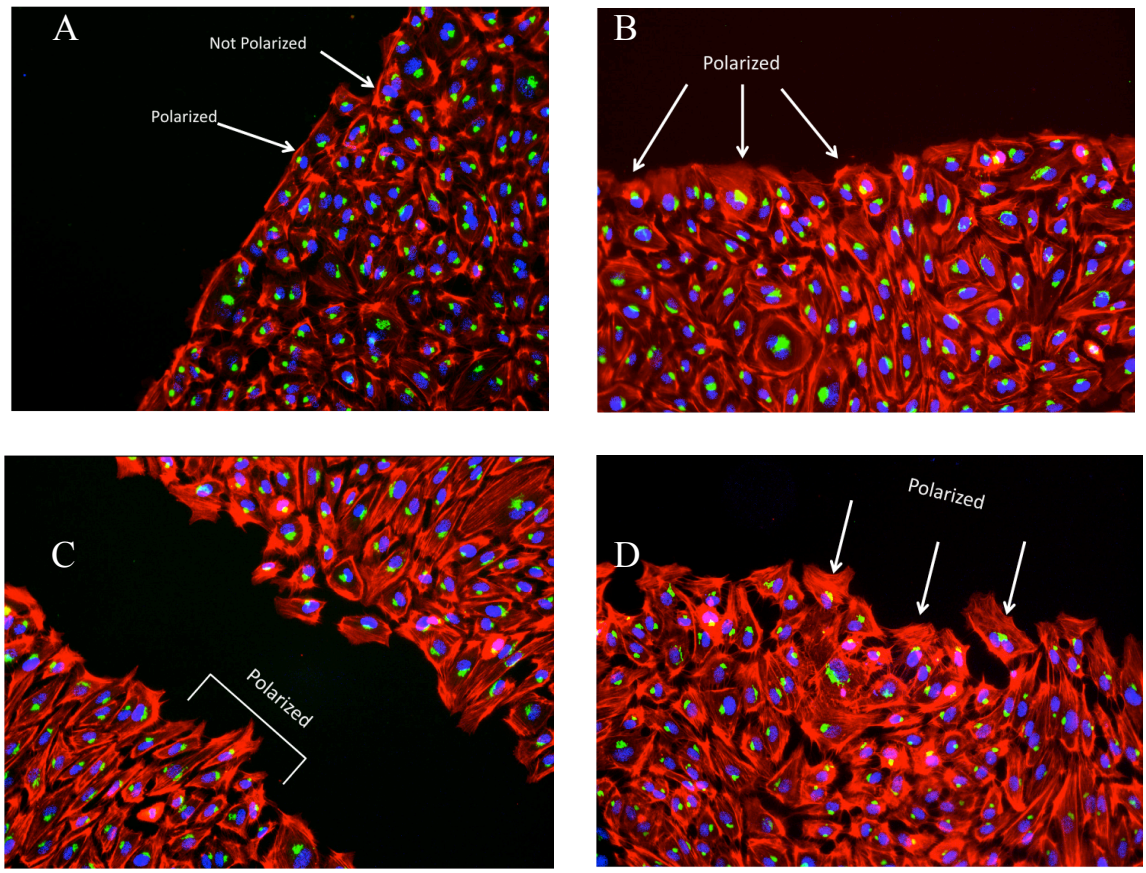

Figure 2: Time course scratch assay. The Cell polarization after a scratch shows 1 hour of incubation (**A),** 2 hours of incubation (**B**), 4 hours of incubation (**C**), and 6 hours of incubation (**D).**

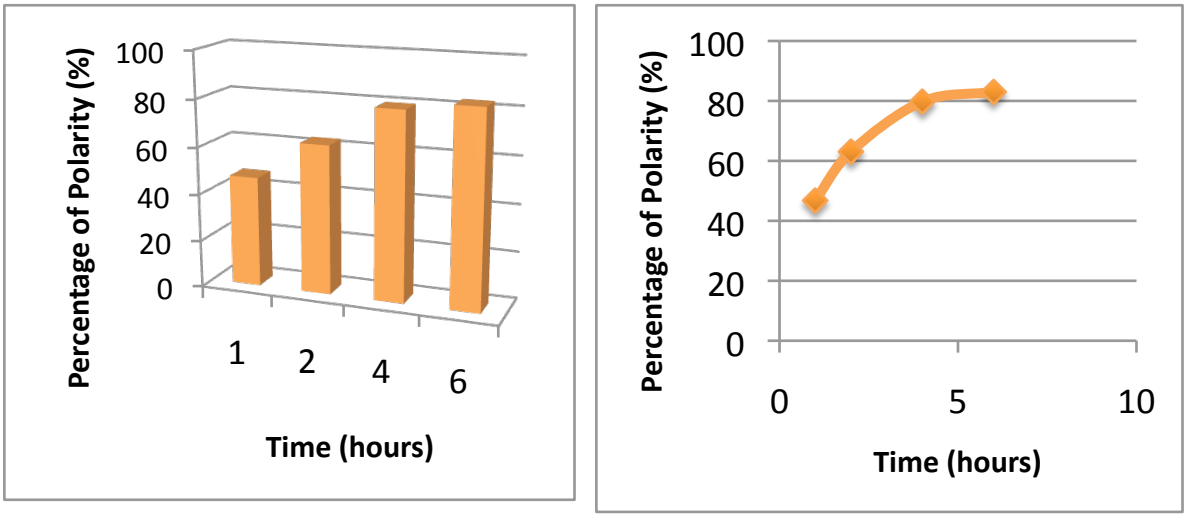

Figure 3: Percentage of polarity in time course experiment. The above figures show the percentage of polarity over the different time intervals.

Scratch assay with experimental groups – control, shNck, rescue

To examine the role of Nck adaptors in the establishment of cell polarity during cell migration, we performed a wound-induced migration assay of HUVEC cells with or without altered Nck signaling. Thus, we compared control, Nck-depleted (shNck), and rescued cells. In this experiment, 3 confluent (50×10^4) 18mm coverslips were used. Immunofluorescence staining and imaging was performed as described above. As expected (Table 1), the control cells showed significant polarization (~ 60%) following 2 h of incubation. In the Nck-depleted cells, in contrast, the index of cell polarity was only ~30%. Re-expression of Nck protein in Nck-depleted cells recovered the index of polarity to levels similar to those observed in control cells (rescued cells ~ 55%) (Figs. 4-5) .

Table 1: Percent polarity for experimental groups.

Cells	Percentage of Polarity
Control	59.29 % n = 113
shNck	32.25 % n = 124
Rescued	55.14 % n = 107

Wound induced cell polarization shows a high percentage of polarity seen in the control and rescue experimental groups after 2 hours. Compared to the control, shNck showed approximately half the percentage of polarity. Around 110-125 cells were analyzed.

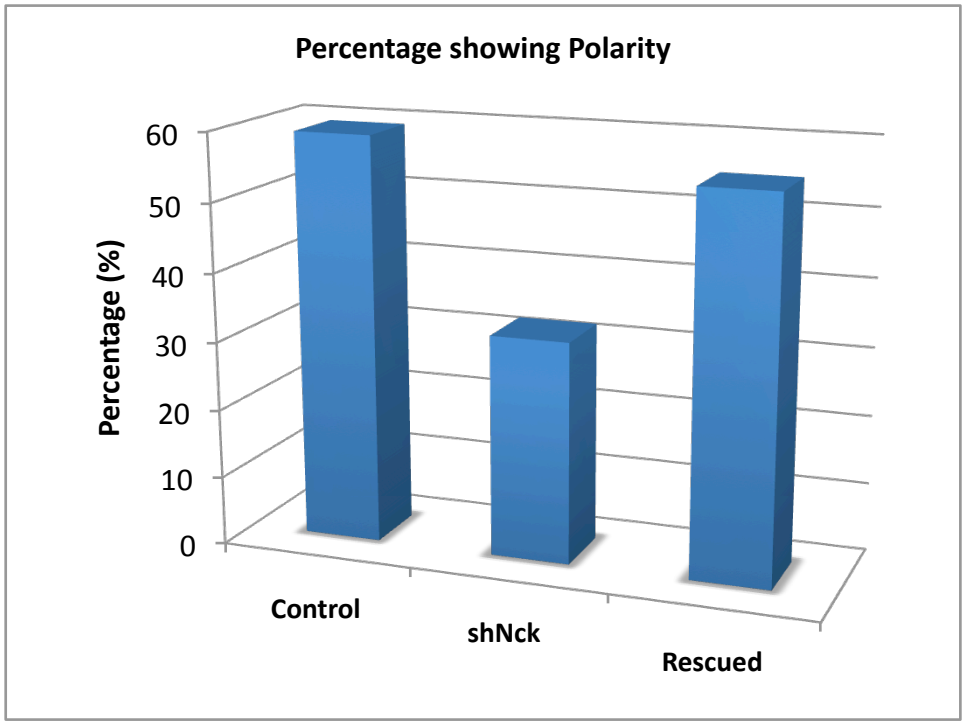

Figure 4: Percentage of polarity in experimental groups. Results show that shNck has reduced expression of polarity whereas control and rescue groups show good percentage of polarity in HUVEC cells.

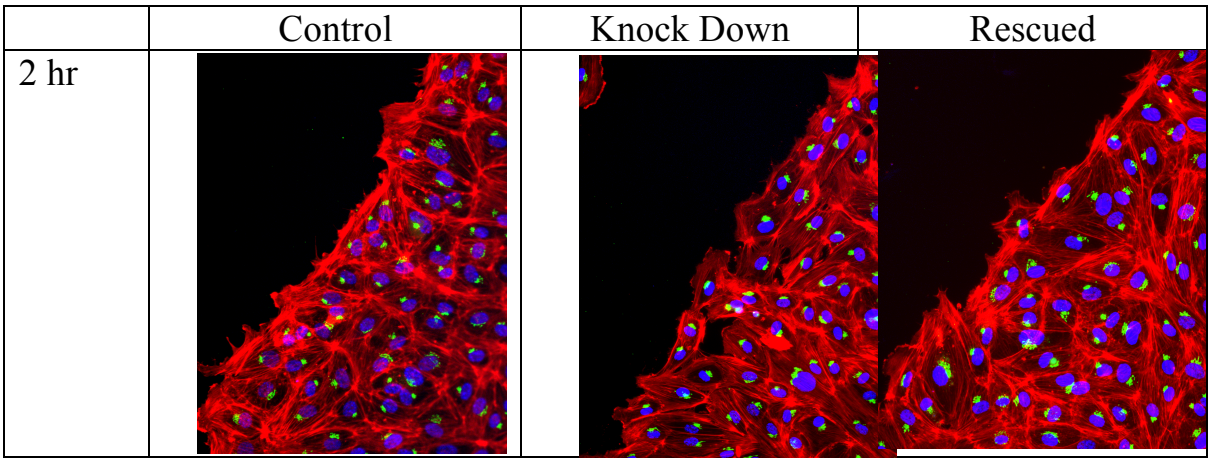

Figure 5: Polarity of control, knockdown and rescue cells. Wound induced cell polarization was observed in control, shNck and rescued cells at 2-hour incubation. After 2 hours of wounding, the cells were fixed and immunostained with GM130 for the Golgi, DAPI for the nucleus, and Tx-Red Phalloidin for microfilaments.

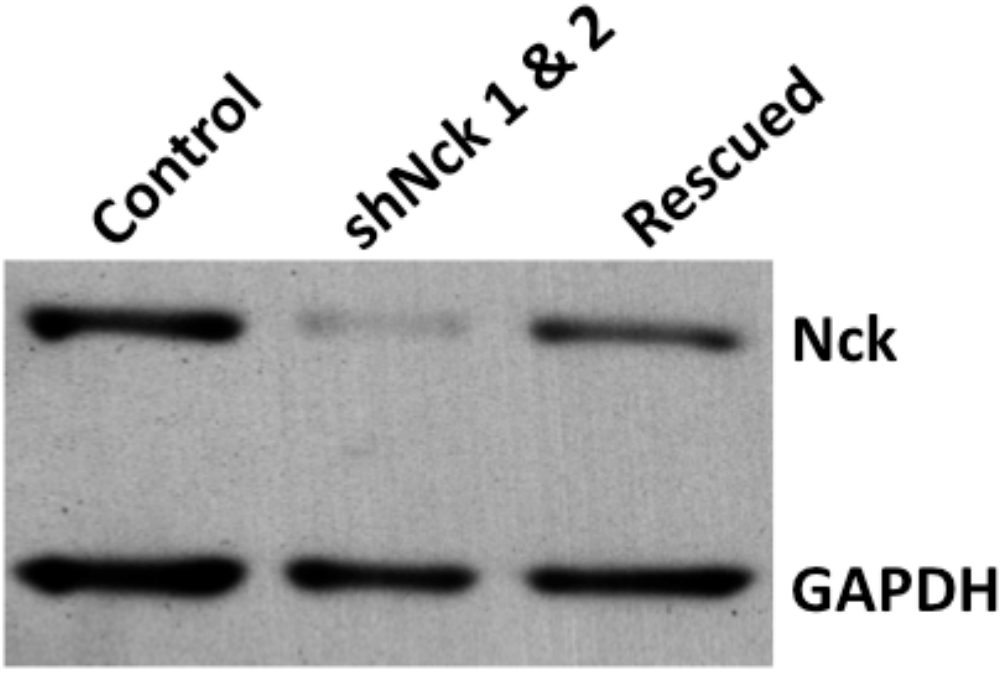

Figure 6: Western blot analysis. Cell licates obtained 96 hours after infections were subjected to Western Blot analysis using and anti-[ban] Nck antibody. shNck 1&2 resulted in ~80% reduction of human Nck. Double knock down cells were rescued by expression of siRNA-resistant Nck 2 cDNA. GAPDH served as loading control.

To determine if the Nck adaptor protein had been properly manipulated for the experimental groups, a western blot was performed. The western blot analysis shows that Nck expression in knockdown was effectively reduced and was effectively re-expressed in rescue (Fig. 6).

CHAPTER IV

SUMMARY

Effect of Nck adaptor proteins

Our results demonstrate a significant reduction in the indexes of cell polarity in Nck-depleted vs. control and rescued cells. We conclude that actin remodeling induced by the Nck adaptor family is required for the establishment of polarity in endothelial cells. These results suggest an important role of Nck in the formation of polarized vascular networks.

Future research

Having studied the role of Nck in polarity of endothelial cells in 2 dimensions, future studies will focus on the analysis of the significance of these signaling molecules using 3-dimensional models. We hypothesize that Nck plays an important role in the organization of apical and basolateral surfaces during tube formation by endothelial cells cultured in 3D.

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