MODULATION OF TRANSFORMING GROWTH FACTOR β-INDUCED ENDOTHELIAL-MESENCHYMAL TRANSITION BY NCK

An Undergraduate Research Scholars Thesis

by

THUY TIEN TRAN

Submitted to the Undergraduate Research Scholars program at
Texas A&M University
in partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by Research Advisor: Dr. Gonzalo Rivera

May 2019

Major: Biomedical Sciences

TABLE OF CONTENTS

ABSTRACT

Modulation of Transforming Growth Factor β-induced Endothelial-Mesenchymal Transition by Nck

Thuy Tien Tran
Department of Veterinary Medicine
Texas A&M University

Research Advisor: Dr. Gonzalo Rivera
Department of Veterinary Pathology
Texas A&M University

Fibrotic disorders, including atherosclerosis, are known to concur with endothelial-to-mesenchymal transition (EndMT) induced by cues from the tissue microenvironment such as transforming growth factor β (TGF-β). Nck, a family of adaptor proteins linking tyrosine phosphorylation with cytoskeletal remodeling, is involved in cardiovascular morphogenesis. Although EndMT underlies cardiovascular development, a potential role for Nck in TGF-β-induced EndMT is yet to be determined. To address this gap in the knowledge, naïve human umbilical vein endothelial cells (HUVEC) or HUVEC with short interference RNA (siRNA)-mediated silencing of Nck were left untreated or treated with TGF-β2 for 48 hours. Expression levels of Nck, endothelial and mesenchymal cell markers were determined by western blotting. With the successful replications of TGF-β induced EndMT, the results from silencing Nck with the presence of TGF-β presented the downregulation of both EC, CD31 and VE-Cad, and MC, vimentin, specific markers. The results suggest that Nck plays a critical role in TGF-β induced EndMT, while also provide a guide for future research of the signaling mechanisms modulated by Nck in TGF-β-stimulated cells.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my research professor, Dr. Gonzalo Rivera, for giving me an opportunity to do research in his lab. With his careful and patient guidance, I gain valuable wisdom in cellular biology for my future endeavors. Not only do I gain a one of a kind knowledge, but I was also able to enhance my independence, self-confidence, and endurance skills when faced with creating my own experiments and experiencing setbacks.

My greatest appreciation to the research assistant, Briana Bywaters, for guiding and supporting me through every step of my research journey. Without her help, I would not be able to be where I am right now in this research. Her willingness to spare her time generously to assist my research is very much appreciated.

I would like to give special thanks to my lab partners, Thao Nguyen and Gladys Pedraza, for inspiring me to produce the best work I can do.

Thank you to the National Education Foundation, American Heart Association Foundation and the Veterinary Pathology Department for funding of this research.

Finally, I thank my family and friends for their warm words encouragements whenever I needed their voice the most.

NOMENCLATURE

BSA	Bovine Serum Albumin
CM	Complete media
EndMT	Endothelial-to-mesenchymal transition
HUVEC	Human umbilical vein endothelial cells
Nck	Non-catalytic region of tyrosine kinase
NFDM	Non-Fat Dried Milk
pSmad2/3	phospho-Smad2/3
siRNA	short interference RNA
TGF-β2	Transforming growth factor-beta 2
VE-Cad	VE-Cadherin
αSMA	Alpha-smooth muscle actin

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

TABLES Page

CHAPTER I

INTRODUCTION

Endothelial cells form a thin layer lining the lumen of blood vessels to regulate the movement of fluid, nutrients, and gases in and out of tissues (Aman et al., 2016). The endothelium regulates blood flow and contributes to tissue homeostasis. Depending on external signals, it may favor antithrombotic or prothrombotic states (Yau et al., 2015). Under physiological conditions, the endothelium of mature blood vessels regulates permeability and tissue perfusion.

EndMT is a physiological process that entail partial or full conversion of endothelial cells into mesenchymal cells. EndMT is required during cardiovascular formation. In adults, the occurrence of EndMT may contribute to pathologies involving vascular dysfunction and fibrosis (Dejana & Lampugnani, 2018). During EndMT, endothelial cells downregulate endothelial cell-specific markers and begin to express markers typical of mesenchymal cells (Figure 1). In addition, cells undergoing EndMT remodel their cytoskeletal organization, cell-cell junctions, and acquired increased motility (Cho et al., 2018). Characteristic endothelial markers are represented by proteins such as CD31 and vascular endothelial cadherin (VE-cadherin) whereas typical mesenchymal markers include, among others, alpha-smooth muscle actin (αSMA) and vimentin. EndMT contributes to a pro-thrombotic state and is linked to increased vascular permeability control and the release of growth factors (Dejana & Lampugnani, 2018). EndMT is also a process contributing to fibrotic diseases, including atherosclerosis. The current understanding is that EndMT represents a continuum of intermediate cellular stages between fully differentiated endothelial and mesenchymal phenotypes (Schwartz et al., 2018). It is

hypothesized that EndMT may represent an attempt of endothelial cells to regain homeostasis following vascular damage.

It is well known that TGF-β is a key driver of EndMT. TGF-β is a family of cytokines capable of activating SMAD-dependent (canonical) as well as Rho-A-dependent (non-canonical) pathways downstream of TGF-β receptor stimulation (Heldin & Moustakas, 2016). TGF-β has been shown to induce EndMT through activation of SMAD proteins (Figure 2). Although induction of EndMT by TGF-β is well documented, how Nck modulates TGF-stimulated EndMT remains unknown. The Nck family are adaptors proteins that regulate cell adhesion and cytoskeletal remodeling during cardiovascular morphogenesis (Clouthier et al., 2015). There are two paralogues in the Nck family; Nck1/Nckα and Nck2/Nckβ. Nck adaptors consists of three SH3 domains followed by one C-terminal SH2 domain, thus they facilitate the formation of signaling complexes in response to activation of tyrosine phosphorylation (Lettau et al., 2009).

This investigation addresses the role of Nck in TGF-β-induced EndMT. To this end, we combined culture of human umbilical vein endothelial cells (HUVEC), molecular genetics, and assessment of endothelial/mesenchymal marker expression by western blotting.

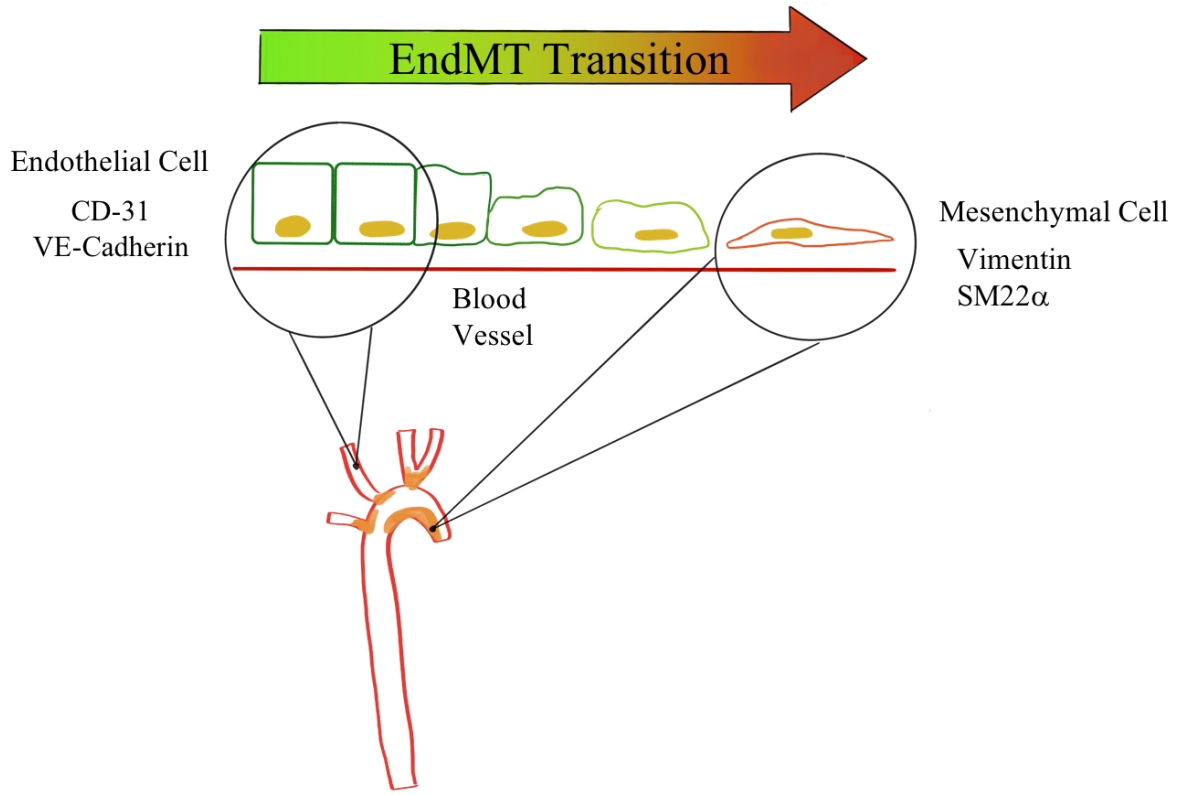

Figure 1. TGF-β induces EndMT. Endothelial cells experience EndMT in response to TGF-β signaling activation. As cells lose endothelial-specific and gain mesenchymal markers, the organization of the endothelium is disrupted and vessel permeability is increased.

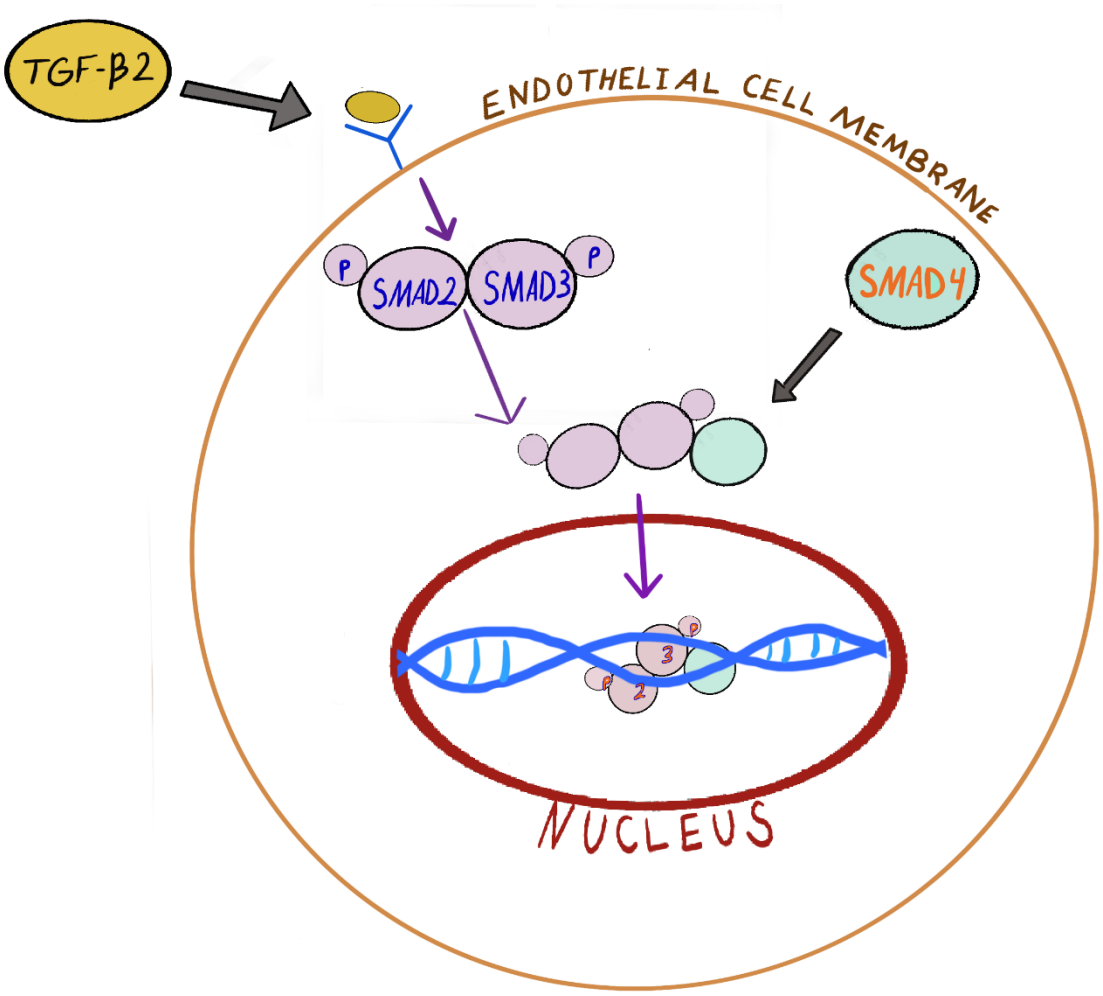

Figure 2. TGF-β signaling pathway. TGF-β is part of the cytokine family that promotes EndMT in endothelial cells. TGF-β activates the phosphorylation of Smad2/3 that will regulate gene expression. The binding of pSmad2/3 to DNA will enhance the expression of mesenchymal markers such as SM22α, Vimentin, and αSMA. In turn, the expression of endothelial markers, such as CD31 and VE-Cadherin, is downregulated.

CHAPTER II

METHODS

Cell culture

Frozen HUVEC cells were thawed at 37°C in a heated water bath and plated on a fibronectin-coated 10cm plate in complete media with 2% serum. Cells were then incubated at 37°C with 5% CO_2. Media was changed the next day and then every 48 hours until 90% confluence. Regents used are listed in Appendix A.

TGF-β stimulation

HUVEC cells were plated at a density of 15 x 10^4cells/cm^2 onto fibronectin-coated 6-well plates in complete media. After 24 hours, cells were either left untreated or treated with 10ng/mL of TGF-β2 in 0.2% serum starvation media (Figure 3). The media was changed every 24 hours until 48 hours (Table 1). Regents used are listed in Appendix A.

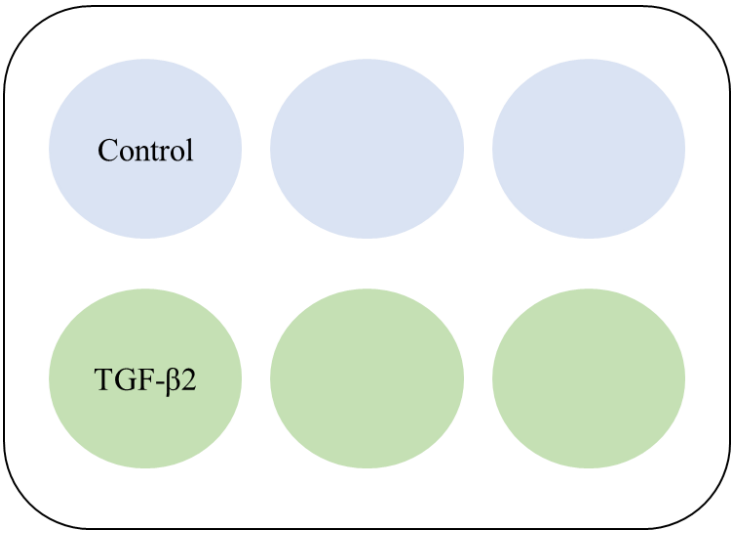

Figure 3. Experimental design using 6-well plates for TGF-β2 treatment. In one 6-well plate, 3 wells were left untreated as a control, and 3 wells were treated with 10 ng/mL TGF-β2 every 24 hours.

Table 1. Timeline of protocols performed for 48 hour treatment of TGF-β.

Time Interval	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
48 hours	Plate cells onto 10cm plate	Change media (CM)	Pass cells onto 6-well plates	Add TGF-β2 to half of wells	Add TGF-β2 to half of wells	Collect cell lysate

siRNA knockdown

HUVEC cells were passed at 20 x 10^4cells/cm^2 onto fibronectin-coated 6-well plates in complete media. The media was then replaced with antibiotic-free media after 24 hours to begin the transfection treatment. Dilutions of siRNA and basal media were prepared by combining diluted siRNA buffer with diluted DharmaFECT (Table 2). The mixtures were added into each well and the cells were incubated with the siRNA at 37°C for 6 hours. The antibiotic-free complete media was then replaced with complete media. Cells were then collected after 24 hours and passed at 10 x 10^4cells/cm^2 onto fibronectin-coated 6-well plates for TGF-β treatment (Table 3).

Table 2. Dilutions preparation of siRNA and antibiotic-free complete media.

Plating Format	Surface Area (cm^2/well)	Tube 1: diluted siRNA (µl/well)		Tube 2: diluted DharmaFECT (µl/well)	
		Volume of 5uM siRNA (µl)	Serum-free Medium (µl)	Volume of DharamaFECT4 reagent (µl)	Basal Medium (µl)
6-well plate siNck	10	Nck1: 20 Nck2: 20	160	4	196
6-well plate siScr	10	10	190	4	196

Table 3. Timeline of protocols performed for transfection followed by TGF-β treatment.

Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Plate cells onto 10cm plate	Change media (CM)	Pass cells onto 6-well plates	siRNA transfection	Pass cells onto 6-well plates	Add TGF-β2 to half of wells	Add TGF-β2 to half of wells	Collect cell lysate

Western blot

SDS-PAGE was used to separate the proteins based on their molecular weight. The proteins were transferred to a 0.2μm pore nitrocellulose membrane. The membrane was blocked with either nonfat dry milk or bovine serum albumin for one hour at room temperature, incubated at 4°C overnight with the primary antibody, and incubated with the secondary antibody for one hour at room temperature. The membrane was then imaged by chemiluminesence using an ImageQuant 4000. Western blots conditions are listed in Appendix B.

CHAPTER III

RESULTS

TGF-β2 induces EndMT in HUVEC

After treating HUVEC with TGF-β2 for 48 hours there was evidence that cells experienced EndMT. A change in cellular morphology was a major difference between the control and treatment group (Figure 4). For the control group, cells are tightly packed together and presented a cobblestone morphology (Fig. 1 A). As for the treatment group, cells were sparser and presented spindled shaped morphologies (Fig. 1 B). Cells extracts were obtained and analyzed to determine expression of endothelial and mesenchymal markers by western blotting. The western blot analysis shows that there was an upregulation of mesenchymal cell marker, vimentin, and downregulation of endothelial cell markers, VE-Cadherin and CD31, in response to TGF-β treatment (Figure 5). Total Smad2/3 and phospho-Smad2/3 (pSmad2/3) were used to determine TGF-β2 signaling activation (Figure 5). TGF-β2 induced EndMT is pSmad2/3 which is the indicator for TGF-β pathway activation. There is a more prominent signal in the treatment group for pSmad2/3 which suggests that TGF-β2 did cause the HUVEC to undergo EndMT.

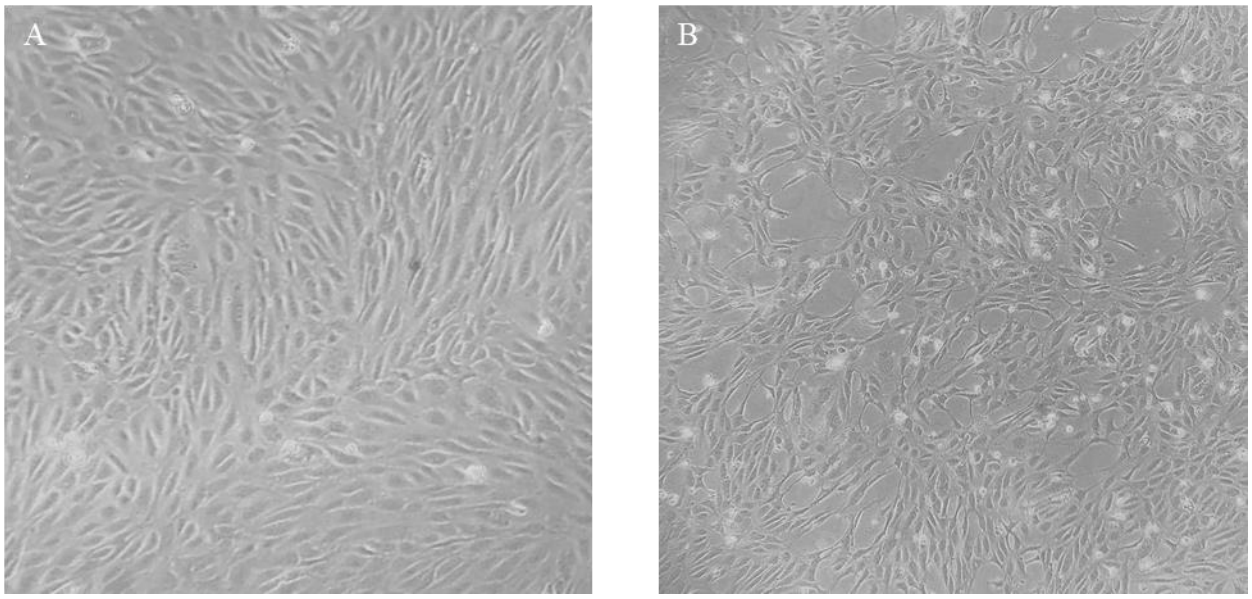

Figure 4. TGF-β2 treatment alters cell morphology after 48 hours. The cell morphology after treating with 0.2% serum starvation media only **(A),** and 10ng/mL of TGF-β2 in 0.2% serum starvation media **(B).**

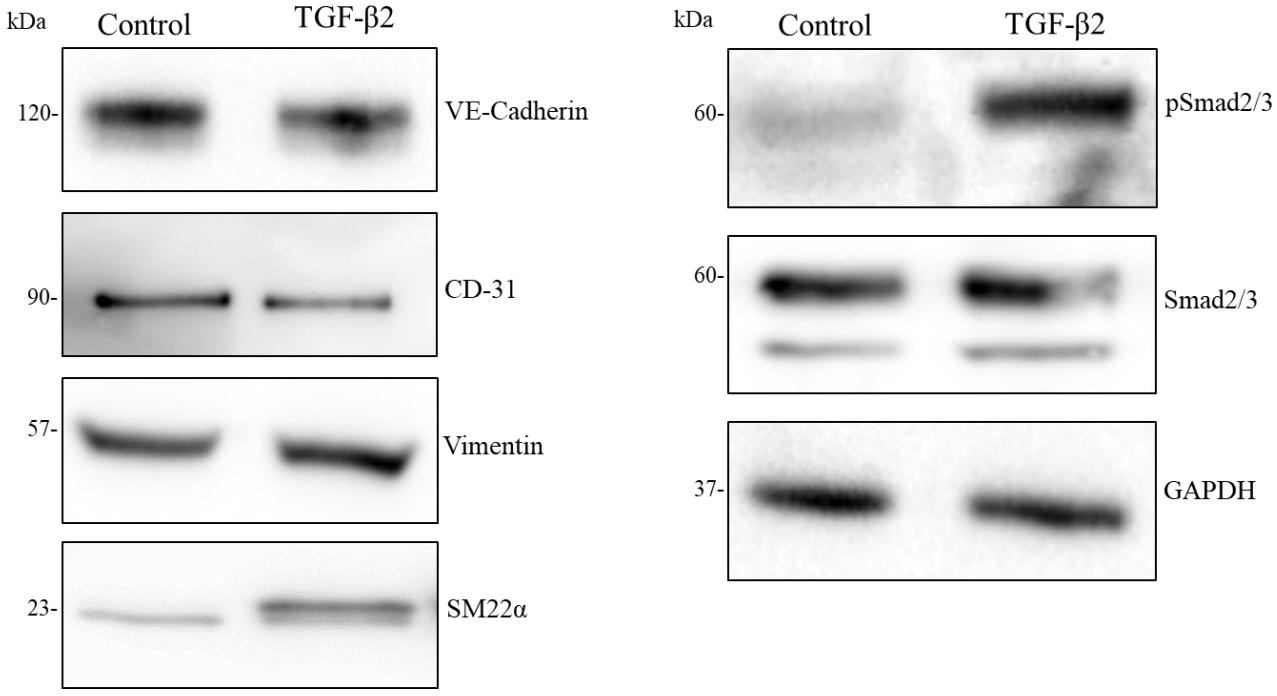

Figure 5. TGF-β2 induces EndMT in HUVEC. Western blots analysis of HUVECs 48 hours control and treatment group. The endothelial cell markers, VE-Cadherin and CD31, presented a downregulation of signaling. The mesenchymal cell marker, Vimentin, presented an upregulation of signaling. Phosphorylated Smad2/3 was used as an indicator of TGF-β pathway activation. GAPDH was used a loading control.

Role of Nck in TGF-β2-induced EndMT

We performed protein silencing using non-targeting (siScr) or Nck-targeting (siNck) oligonucleotides. Based on the results, siNck reduced the expression of both endothelial cell markers, VE-Cadherin and CD31, and mesenchymal cell marker, Vimentin (Figure 6). When compared to siNck, the protein bands of the control and treatment group of siScr presented a less dramatic change. Basically, the change of protein expression for siNck was more intense than siScr, which presented a slight change of intensity. Intensities are indicated by the lightness and darkness of bands. For the siNck group, there was a downregulation of Nck when compared to the siScr group, which indicated the successful transfection of knocking down Nck from the DNA of the HUVEC cells. With the successful knockdown of Nck and the equal loading indicated from GAPDH, it can be inferred that silencing of Nck results in downregulation of both endothelial and mesenchymal cell markers.

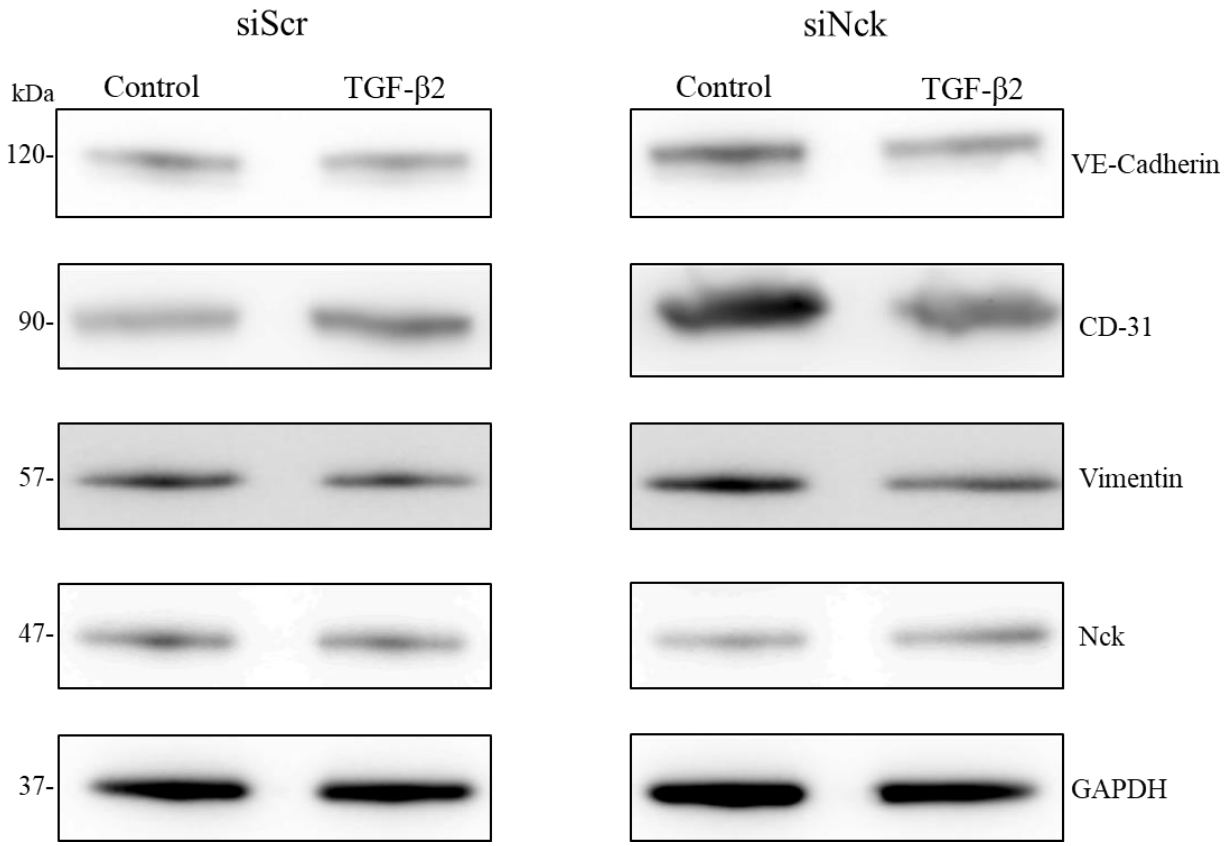

Figure 6. siNck reduces effects of TGF-β induced EndMT. Western blot analysis comparing siScr and siNck. The endothelial cell markers, VE-Cadherin and CD31, presented a downregulation of signaling in both groups. The mesenchymal cell marker, Vimentin, presented an upregulation of signaling for siScr and a downregulation of signaling for siNck. Nck was an indicator for the effectiveness of siNck treatment. GAPDH was used a loading control.

CHAPTER IV

CONCLUSION

Effect of Nck adaptor proteins

The results demonstrate a significant reduction in the progression of EndMT in siNck HUVEC cells. There was a downregulation in both endothelial and mesenchymal cell markers for siNck, while siScr presented the normal progression of EndMT, which had a downregulation of endothelial cell markers and an upregulation of mesenchymal cell markers. These results suggest that Nck plays an important role in the progression of TGF-β induced EndMT.

Future Research

From the studies of the role of Nck in TGF-β induced EndMT, future studies will revolve around the signaling mechanisms modulated by Nck in TGF-β-stimulated cells. In addition to finding the signaling mechanisms, time intervals, such as 48 or 96 hours, would be tested to see the effects of TGF-β on HUVEC cells. We hypothesize that the Nck family drives EndMT in response to activation of TGF-β signaling.

REFERENCES

Aman, J., Weijers, E. M., van Nieuw Amerongen, G. P., Malik, A. B., & van Hinsbergh, V. W. (2016). Using cultured endothelial cells to study endothelial barrier dysfunction: Challenges and opportunities. American journal of physiology. *Lung cellular and molecular physiology, 311*(2), L453-66.

Cho, J. G., Lee, A., Chang, W., Lee, M. S., & Kim, J. (2018). Endothelial to Mesenchymal Transition Represents a Key Link in the Interaction between Inflammation and Endothelial Dysfunction. *Frontiers in immunology*, *9*, 294. doi:10.3389/fimmu.2018.00294

Clouthier, D. L., Harris, C. N., Harris, R. A., Martin, C. E., Puri, M. C., & Jones, N. (2015). Requisite role for Nck adaptors in cardiovascular development, endothelial-to-mesenchymal transition, and directed cell migration. *Molecular and cellular biology*, *35*(9), 1573-87.

Dejana, E. & Lampugnani M. G. (2018). Endothelial cell transitions. *Science, 362*(6416), 746-747. doi:10.1126/science.aas9432

Heldin, C. H., & Moustakas, A. (2016). Signaling Receptors for TGF-β Family Members. *Cold Spring Harbor perspectives in biology*, *8*(8), a022053. doi:10.1101/cshperspect.a022053

Lettau, M., Pieper, J., & Janssen, O. (2009). Nck adapter proteins: functional versatility in T cells. *Cell Communication and Signaling*, *7*(1). doi:10.1186/1478-811x-7-1

Morris, D. C., Popp, J. L., Tang, L. K., Gibbs, H. C., Schmitt, E., Chaki, S. P., Bywaters, B. C., Yeh, A. T., Porter, W. W., Burghardt, R. C., Barhoumi, R., … Rivera, G. M. (2017). Nck deficiency is associated with delayed breast carcinoma progression and reduced metastasis. *Molecular biology of the cell*, *28*(24), 3500-3516.

Schwartz, M. A., Vestweber, D., & Simons, M. (2018). A unifying concept in vascular health and disease. *Science (New York, N.Y.)*, *360*(6386), 270–271. doi:10.1126/science.aat3470

Yau, J. W., Teoh, H., & Verma, S. (2015). Endothelial cell control of thrombosis. *BMC cardiovascular disorders*, *15*, 130. doi:10.1186/s12872-015-0124-z

APPENDIX A

REAGENTS

Table 4. Reagents used to culture and harvest cells and perform Western blots

Process	Reagent	Vendor	Catalogue number
Cell Culture and Passing	EGM2 Bullet kit (EBM2 Basal Media + growth factors)	Lonza	CC-3162
	HUVEC	Lonza	CC-2519
	Trypsin/EDTA (0.25mg/mL)	Lonza	CC-5012
	FBS	Gemini	100-106
	PBS	Gibco	10010-031
	Fibronectin	Calbiochem	341631-5MG
Transfection	5X siRNA buffer	Thermo Fisher	002000-UB-100
	DharmaFECT4	GE Dharmacon	T2004-02
	ON-TARGETplus Nck1 siRNA	GE Dharmacon	J-006354-06-0002
	ON-TARGETplus Nck2 siRNA	GE Dharmacon	J-019547-05-0002
	ON-TARGETplus Non-targeting Pool	GE Dharmacon	D-001810-10-05
Harvesting Cells	1M Tris, pH 7.4	Sigma	T1503-1KG
	5M NaCl	EMD	SX0420-5
	0.5M EDTA	Sigma	E5134
	Triton X-100	VWR	9002-93-1
	1M β-glycerophosphate	Sigma	G9422
	Glycerol	J.T. Baker	4043-00
	0.5M phenylmethylsulfonyl fluoride in DMSO	Sigma	P-7626
	Aprotinin, from Bovine Lung	Sigma	A6279-5ML
	100mM Sodium Orthovanadate (Na_3VO_4)	Alfa Aesar	81104
Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis	30% Acrylamide Solution	Protogel	EC890
	1M Tris, pH 8.8	Sigma	T1503-1KG
	1M Tris, pH 6.8		
	10% Sodium dodecyl sulfate	J.T. Baker	4095-02

(SDS-PAGE) Gel Casting	10% Ammonium persulfate (APS)	J.T. Baker	4030-04
	TEMED	Thermo Scientific™	17919
Precast Gels	NuPAGE™ 10% Bis-Tris Protein Gels, 1.0 mm, 10-well	Novex™, NuPAGE®	NP0301PK2
	NuPAGE™ MOPS SDS Running Buffer (20X)	NuPAGE®	NP0001
	NuPAGE™ Antioxidant	NuPAGE®	NP0005
SDS-PAGE Protein Transfer to Nitrocellulose	Nitrocellulose Membrane	BioTrace™	NT 66485
	Whatman™ Chromatography Paper	Whatman™	3030-6188
Detection of Western blot Signal	Western Lightning Plus-ECL, Enhanced Chemiluminescence Substrate	PerkinElmer	NEL103E001EA

APPENDIX B

WESTERN BLOT CONDITIONS FOR ALL ANTIBODIES USED

To block non-specific binding on the membrane, Non-Fat Dried Milk (NFDM) or Bovine Serum Albumin (BSA) were diluted in 1X Tris-Buffered Saline and Tween 20 (TBST). The membranes were blocked for one hour at room temperature on the Belly Dancer and placed in 1X TBST upon completion. The membranes were then incubated in the primary antibody overnight at 4°C on an orbital shaker and then washed 5 times for 5 minutes with TBST (Table 5). The secondary antibodies were incubated for an hour at room temperature on the Belly Dancer and washed with TBST 5 times for 5 minutes (Table 6). To detect signals, the Western Lightening Plus-ECL kit was used and the membranes were imaged by ImageQuant 4000.

Table 5. Western blot conditions for primary antibodies used.

Primary Antibody	Blocking Solution	Antibody Concentration	Primary Dilution
CD31	3% NFDM	1/4000	3% NFDM
GAPDH		1/5000	
Nck		1/4000	
VE-Cadherin		1/1000	
Vimentin		1/2000	
pSmad2/3		1/500	5% BSA
Smad2/3	3% BSA	1/1000	

Table 6. Western blot conditions for secondary antibodies used.

Secondary Antibody	Primary Antibodies Targeted	Antibody Concentration	Secondary Dilution
Goat anti-Mouse IgG	GAPDH	1/1000	3% NFDM
	Nck		
	VE-Cadherin		
	Vimentin		
Goat anti-Rabbit IgG	CD31	1/5000	
	Smad2/3		
	pSmad2/3		