ROLE OF THE MECHANOSENSITIVE ION CHANNEL TRPV4 IN ANGIOGENESIS

A dissertation submitted to Kent State University in collaboration with Northeast Ohio Medical University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

By
Roslin J. Thoppil

May 2015
© Copyright
All rights reserved
Except of previously published materials
Dissertation written by:

Roslin J. Thoppil

Ph.D. Kent State University, 2015
B.S., Sathyabama University, India, 2009

Approved by

_________________________,
Charles K. Thodeti, Assistant Professor, Ph.D., Integrative Medical Sciences, Doctoral Advisor

_________________________,
J. Gary Meszaros, Associate Professor, Ph.D., Integrative Medical Sciences

_________________________,
Priya Raman, Assistant Professor, Ph.D., Integrative Medical Sciences

_________________________,
Moses O. Oyewumi, Assistant Professor, Ph.D., Pharmaceutical Sciences

_________________________,
Gail C. Fraizer, Associate Professor, M.P.H., Ph.D., Biological Sciences

_________________________,
Altaf Darvesh, Assistant Professor, M.Pharm., Ph.D., Pharmaceutical Sciences

Accepted by

_______________________,
Eric M. Mintz, Professor, Ph.D., Director, School of Biomedical Sciences

_______________________,
James L. Blank, Ph.D., Dean, College of Arts and Sciences
# TABLE OF CONTENTS

LIST OF FIGURES .................................................................................................................. iv

LIST OF TABLES .................................................................................................................... viii

LIST OF ABBREVIATIONS ..................................................................................................... ix

ACKNOWLEDGEMENTS ......................................................................................................... xii

CHAPTER I ............................................................................................................................. 1

Introduction

CHAPTER II ............................................................................................................................. 30

Materials and Methods

CHAPTER III .......................................................................................................................... 43

The Functional Significance of the Mechansensitive Ion Channel TRPV4 in Tumor Angiogenesis.

CHAPTER IV .......................................................................................................................... 64

TRPV4 Channels Regulate TEC Proliferation via Modulation of ERK1/2.

CHAPTER V ............................................................................................................................ 83

TRPV4 is a Negative Regulator of Angiogenesis.

SUMMARY AND CONCLUSION ............................................................................................ 111

BIBLIOGRAPHY ..................................................................................................................... 113
LIST OF FIGURES

FIGURE 1.1 ................................................................................................................................. 3
Vasculogenesis and angiogenesis.

FIGURE 1.2 .................................................................................................................................. 9
Mechanotransduction in endothelial cells.

FIGURE 1.3 .................................................................................................................................. 12
Schematic model of TRPV4 structure.

FIGURE 1.4 .................................................................................................................................. 24
Concept of vascular normalization.

FIGURE 1.5 .................................................................................................................................. 26
TRPV4 mediated mechanotransduction in EC reorientation.

FIGURE 1.6 .................................................................................................................................. 28
Endothelial cell reorientation and basal Rho-activity levels in normal and tumor CE (capillary endothelial cells) in response to mechanical stretch.

FIGURE 3.1 .................................................................................................................................. 48
TRPV4 expression and functional activity is decreased in TEC.

FIGURE 3.2 .................................................................................................................................. 51
TRPV4 overexpression increases calcium influx and restores TEC mechanosensitivity to ECM elasticity.
FIGURE 3.3
TRPV4 overexpression or activation normalizes TEC angiogenesis in vitro.

FIGURE 3.4
TRPV4 overexpression or pharmacological activation decreases abnormal Rho activity in TEC.

FIGURE 3.5
Activation of TRPV4 with GSK1016790A restores pericyte coverage and vessel maturity.

FIGURE 3.6
TRPV4 activation with a specific agonist GSK1016790A in conjunction with Cisplatin reduces tumor growth in vivo.

FIGURE 4.1
TEC exhibit increased proliferation in vitro.

FIGURE 4.2
TRPV4 activation or overexpression decreases abnormal TEC proliferation.

FIGURE 4.3
Pharmacological activation of TRPV4 decreases TEC proliferation via modulation of ERK1/2 but not AKT pathway.

FIGURE 4.4
TEC express increased levels of proliferation-associated genes.
Pharmacological activation of TRPV4 inhibits TEC proliferation \textit{in vivo}.

Proposed Mechanism by which TRPV4 regulates TEC proliferation.

Tumor growth is increased in TRPV4KO mice.

TRPV4KO tumors exhibit enhanced vessel malformations.

Microvessel density is increased in tumors from TRPV4KO mice.

Angiogenic sprouting from aortic rings isolated from WT and TRPV4KO mice.

\textit{In vivo} Matrigel plug angiogenesis assay showing increased angiogenesis in TRPV4KO mice.

Characterization of EC isolated from WT and TRPV4KO aortic sprouts.

Absence of TRPV4 enhances EC proliferation through ERK1/2 activation.

TRPV4 deletion induces abnormal EC migration.
FIGURE 5.9 ......................................................................................................................... 101

Absence of TRPV4 results in abnormal angiogenesis in vitro.

FIGURE 5.10 ......................................................................................................................... 103

TRPV4KO EC exhibit increased basal Rho-activity and inhibition of ROCK (Rho kinase) normalizes angiogenesis in TRPV4KO EC.

FIGURE 5.11 ......................................................................................................................... 107

ROCK inhibitor, Y-27632, in conjunction with Cisplatin reduces tumor growth in vivo in TRPV4KO mice.
LIST OF TABLES

TABLE 1 ................................................................................................................................. 42

List of Primers
LIST OF ABBREVIATIONS

AA = Arachadonic Acid
ACh = Acetylcholine
Ang-1 = Angiopoietin-1
Ang-2 = Angiopoietin-2
ANK = Ankyrins
ANOVA = Analysis of variance
ARD = Ankyrin rich domain
BCE = Bovine capillary endothelial cells
BM = Basement membrane
BrdU = Bromodeoxyuridine
EC = Endothelial cells
ECM = Extracellular matrix
EET = Epoxyeicosatrienoic acids
EGFP = Enhanced green fluorescent protein
ERK1/2 = Extracellular regulated Kinase 1/2
eNOS = Endothelial nitric oxide synthase
FAK = Focal adhesion kinase
FGF = Fibroblast growth factor
FN = Fibronectin
GAP = GTPase activating protein
G-CSF = Granulocyte-colony stimulating factor
GEF = Guanine nucleotide exchange factor
HER-2 = Human epidermal growth factor receptor-2
HUVEC = Human umbilical vein endothelial cell
i.p. = Intraperitoneal
ICAM-1 = Intercellular adhesion molecule 1
IFP = Interstitial fluid pressure
KO = Knock out
LLC = Lewis lung carcinoma
MAPKs = Mitogen-activated protein kinases
MLC = Myosin light chain
NEC = Normal endothelial cells
NO = Nitric oxide
OTRPC4 = Osm-9-like TRP channel 4
PAI-1 = Plasminogen activator inhibitor-1
PCNA = Proliferating cell nuclear antigen
PDGF = Platelet-derived growth factor
PI3K = Phosphatidylinositol 3-kinase
PIGF = Placental growth factor
PKC = Protein kinase C
PRD = Proline rich domain
ROCK = Rho kinase
RhoGAP = Rho GTPase activating protein
RTK = Receptor tyrosine kinase
RTKI = Receptor tyrosine kinase inhibitor
SA = Stretch-activated channels
SMA = Smooth muscle actin
SMC = Smooth muscle cell
TEC = tumor endothelial cells
TEM = Tumor endothelial markers
TIMP = Tissue inhibitors of metalloproteinases
TRAMP = Transgenic adenocarcinoma of the mouse prostate
TRP = Transient receptor potential
TRPC = Transient receptor potential canonical
TRPM = Transient receptor potential melastatin
TRPV = Transient receptor potential vanilloid
TRPV4 = Transient receptor potential vanilloid 4
VE-cadherin = Vascular endothelial- cadherin
VEGF = Vascular endothelial growth factor
VEGFR2 = VEGF receptor 2
VRL-2 = Vanilloid receptor-like channel 2
VR-OAC = Vanilloid receptor-related osmotically activated channel
vWF = Von Wildebrand Factor
WT = Wild type
Y-27 = Y-27632
ACKNOWLEDGEMENTS

I would like to thank the Kent State Biomedical Science Program and NEOMED Integrative Medical Science Department for giving me the opportunity to complete my Ph.D. To my mentor Dr. Charles Thodeti, I am immensely grateful for always putting the highest confidence in me, instilling in me the passion for research, giving all of us in the lab the freedom to explore and think big, Thank you for all the encouragement and support you have given me. To my committee members, Dr. Meszaros, thank you for your guidance and support over the years and to Dr. Raman, Dr. Oweyumi and Dr. Fraizer and for taking the time out to read, critique and provide insight into my thesis. I also would like to thank Dr. Altaf Darvesh for being an excellent moderator and friend to me.

A big thank you to the people at the IMS department and NEOMED, our chair Dr. Chilian who gave every graduate student an opportunity to shine and better themselves and learn a lot of things that being in the lab alone doesn’t teach us. To all the administrative and research staff, especially Dr. Walter Horton, Dr. Eric Mintz, Karen Greene, Carolyn Miller, Deb Enos, Sharon Usip, Ruby Pahls, Dori Parker, Corey Robinson, Margaret Weakland, Cheryl Hodnichak, Judy Wearden (KSU)- thank you for all your help, encouragement and for all the fun banter! A special mention of thanks to Drs. Sailaja Paruchuri and Liya Yin and Suzanna Logan, for inspiring me and making me believe that you can be an awesome mom and scientist at the same time, in short – wonder women!
To my parents, papa and mummy- thank you for all that you have done. This work is dedicated to the sacrifices you made to see me succeed. For the love, acceptance and freedom you have given me, I am eternally grateful and also thank you to my brothers, for their unconditional support and love and wise words shared with me throughout these years.

To the most important person of my life, my best friend and better half, Matthew Earich, thank you for the best 6 years of my life, for every up and down we have shared throughout graduate school and life outside, I have learnt so much and become a better person because of it. To dad (Scott), mom (Leslie), Robert, Michelle, Curt, Carol, Kelly, Tony, Andrew and Hannah, thank you for letting me be part of your family, there are no words to describe how much all of you mean to me and I am grateful for everything each of you have done for me. You all provided me with ‘a home away from home’.

Special thanks to TTLH&C, BMMPD, and all their cousins for providing me with an alternate universe of absolute silliness and complete joy, where all my problems somehow cease to exist.

Last but not the least, to all my friends I know and here at NEOMED; specially Nikky Thomas whom I have known for 23 years, what an amazing friendship we have, thank you. Ravi Adapala, my brother and friend, you not only taught me everything in lab, but you were there for me in all the toughest times of my graduate life, outside of lab and in general being there (no questions asked) whenever I needed you. To my self-appointed PA, Holly Cappelli, I have no words to describe what a miracle you are! You are one of the finest people I am fortunate to know, and being your friend is truly an honor, thank you for all your help and encouragement throughout. To Danielle Janota,
Ritu-Ayan, Shomu (Sahuji, Sumi), Suzanna Logan, Ajay Donepudi, Ernest Duah, Vinay Kondeti, Preeti Pathak and Farai – you guys have made my time memorable at NEOMED. We have learnt so much from each other, shared so much science, so many frustrations and tonnes of fun, it has been incredible knowing all of you, and I hope we will always remain good friends.
CHAPTER I

Introduction

1.1 Blood Vessel Formation: Vasculogenesis and Angiogenesis

Blood vessels are the primary organs that develop within the embryo that form the largest structural network in the adult human. The formation and function of both the blood and lymphatic vasculature is not only critical for supporting the growth and adequate functioning of organ systems during development and early postnatal life, but is also necessary for defending the body from diseases, maintaining body temperature and general homeostasis in adults. The very first growth and expansion of the vasculature is known as vasculogenesis (Fig. 1.1 A) which involves the formation of the primary network.

of vessels within the embryo which includes the aorta, veins and a plexus that connect these major vessels (Cai et al., 2009; Djonov et al., 2000). This process is further modified and refined through angiogenic remodeling where pruning and vessel enlargement give rise to the mature vasculature.

Defined as a process in which new blood vessels arise from pre-existing ones, angiogenesis can be further broken down, into two major processes: sprouting and splitting/intussusceptive angiogenesis; the underlying difference between the two being that sprouting angiogenesis forms entirely new vessels as opposed to the latter process which promotes splitting existing vessels (Burri et al., 2004; Patan et al., 1996) (Fig. 1.1 B). Establishment of an adequate vascular system within the growing embryo and reparative wound healing processes represent most physiological angiogenesis (Folkman and Hanahan, 1991; Moses et al., 1995). However, insufficient or excessive angiogenesis has been documented to be the primary cause of various pathological conditions such as, cancer, age-related macular degeneration, stroke, arthritis, infertility and diabetic retinopathy (Carmeliet, 2003; Folkman, 2007).
Figure 1.1. Vasculogenesis and angiogenesis. A. During development, mesodermal cells differentiate into hemangioblasts, which further develop into angioblast, the precursors of endothelial cells. Vasculogenesis involves the differentiation of EC from these angioblast cells to form a primitive vascular plexus. B. This rudimentary vessel structure undergoes extensive growth, remodeling and pruning by two specific angiogenic processes. In sprouting angiogenesis, endothelial cells proliferate behind the tip cell of a growing branch in response to growth factors and lumens can form by vacuole fusion. Both forms of angiogenesis require the recruitment of smooth muscle cells (SMCs) or pericytes to stabilize the newly formed vessels. Intussusceptive angiogenesis involves the insertion of tissue columns into the lumen of preexisting vessels to partition the vessel lumen.
1.2 Endothelial Cells (EC) and the Angiogenic Process

The endothelium is a unique and dynamic structure that serves not only as a barrier between blood and tissue, but is an essential component required for maintaining vessel homeostasis. EC, depending on their location in the vascular network, regulate several processes; for e.g. macrovascular EC are involved in the modulation of vascular tone and blood flow regulation while microvascular EC are required for controlling vascular permeation of gases and other metabolites. No matter the location, all EC respond to mitogenic factors and mechanical stresses, participate in platelet activation, coagulation, wound healing and angiogenesis (Li et al., 2005). The angiogenic process is complex but tightly regulated and requires the precise control and coordination of EC behavior. In adults, EC form a quiescent monolayer that is structured in a streamlined manner, covered with pericytes and interconnected by means of junctional proteins such as VE-cadherins and claudins, that permit the laminar flow of blood (Lampugnani et al., 1995).

The cascade of events in angiogenesis begins with an angiogenic stimulus that initiates the production and release of angiogenic growth factors such as VEGF and fibroblast growth factor (FGF), which bind to EC receptors on the quiescent vessel to activate the endothelium. The first response is the detachment of the pericytes from the vascular wall and their release from the BM, by means of proteolytic degradation. More pro-angiogenic molecules are released which causes loosening of tight junctions and increases the permeability of the EC layer, to lay the foundation for an interim extracellular
matrix (ECM). The EC then acquire significant invasive and motile properties, enabling their migration toward the ECM; while the ECM is remodeled to enable vessel sprouting, tube formation and ultimately blood perfusion. It becomes pertinent to understand that only a certain fraction of EC that sense these angiogenic signals are selected to guide the formation of the new blood vessels. These ‘tip’ cells, equipped with many filopodial extensions, sense and respond appropriately to the various angiogenic stimuli within the microenvironment to lead the formation of new vessels. Meanwhile, the neighboring EC, referred to as ‘stalk’ cells, are less motile than the tip cells but are involved in supporting the formation of the newly sprouted vessels, proliferating and extending the stalk to create the lumen. EC sprouting continues in a directional manner until the tip cells come into contact with neighboring vessels and undergo anastomosis or vessel fusion. Upon contact with EC from adjacent vessels, the tip cells reestablish EC-EC junctions and fuse with the preexisting vessel to form a continuous lumen that permits blood flow. The blood vessel networks are formed through these loops and the newly formed vessels become functional and mature once the EC return to their quiescent state and are stabilized by pericytes and smooth muscle cells (SMC). Additionally protease inhibitors, mainly tissue inhibitors of metalloproteinases (TIMP) and plasminogen activator inhibitor-1 (PAI-1) facilitate the deposition of a BM and the strengthening of tight-junctions that allow optimal blood flow. This process of angiogenesis continues, to promote the further expansion of the vasculature with subsequent pruning and vessel
regression until the formation of a large and stable network of blood vessels (Ausprunk and Folkman, 1977; Eilken and Adams, 2010; Risau, 1997)

1.3 Factors Regulating Angiogenesis

1.3.1 Growth Factor Based Regulation of Angiogenesis

Numerous endogenous cytokines participate in the angiogenic process (Folkman, 1997). Possibly the most well characterized pro-angiogenic molecule is VEGF, which binds to receptor VEGFR2 (also known as KDR or Flk-1), to stimulate a variety of responses that mediate angiogenesis such as EC proliferation, migration, apoptosis and increase in EC permeability. Furthermore, FGF, working in tandem with VEGF, helps promote EC proliferation, migration, and the production of matrix proteases. Platelet-derived growth factor (PDGF) which is secreted by the endothelium, binds to receptors on the surrounding SMC to stimulate proliferation (D’Amore and Smith, 1993; Ferrara, 2000a; Ferrara, 2000b) and strengthen capillary walls (Leveen et al., 1994) aiding in the maturation of blood vessels. Lastly, angiopoietins bind to the Tie-2 receptor where Ang-1 aids in maintenance of quiescent endothelium while Ang-2 is involved in promoting vessel sprouting (Saharinen et al., 2010).

1.3.2 Mechanical Regulation of Angiogenesis

Mechanotransduction

Although angiogenesis has primarily been studied as being controlled by a balance of soluble factors and their corresponding receptors, an emerging
field of study has highlighted the role of cell mechanics and mechanotransduction in this process. Mechanotransduction refers to the conversion of mechanical forces into biochemical signals, a process achieved by cells, through means of diverse mechanosensory machinery such as integrins, cytoskeleton, cell-cell adhesion molecules and ECM. Cells utilize this process as a way to sense their physical surroundings and activate various intracellular signaling pathways that ultimately decide cell fate and survival (Huang and Ingber, 1999; Ingber, 2004).

The endothelium is an example of a tissue subjected to constant mechanical stimulation, in the form of hemodynamic forces, namely shear stress and cyclic strain (Davies et al., 1995; Davies et al., 2005). These hemodynamic forces modulate the structure and function of EC in blood vessels and under normal conditions, these modulating influences allow the vascular wall to adjust to changes in pressure and flow for ideal functioning (Chien, 2007).

In EC, external mechanical stimuli by shear flow/stretch or variations in ECM elasticity cause changes in intracellular mechanics and mechanotransduction (Fig. 1.2). Here, mechanical forces, generated within the cell by cytoskeletal actin-myosin interactions, are balanced by integrin adhesions to the ECM, cadherin adhesions to neighbouring cells and internal cytoskeletal structures such as microtubules. Balancing these forces permit cell stability and tissue structure (Ingber, 2004). Cells sense changes in ECM stiffness through cell adhesion integrin receptors, which transduce these mechanical signals into the cell through the actin cytoskeleton. Molecules including actin, Rho, ROCK and p190RhoGAP are involved in cytoskeletal tension generation that contribute
to mechanical signaling. Fluid shear stress, cyclic strain, as well as the binding of growth factors and ECM ligands to their respective cell surface receptors, can also alter cell signaling and gene expression (Wang et al., 2009).
Figure 1.2. Mechanotransduction in endothelial cells. Mechanical forces within the cell, generated by cytoskeletal actin-myosin interactions are balanced by integrins, cadherins and microtubules. Any alteration in this force balance between the ECM, adjacent cells and cytoskeletal components have shown to influence biochemical signaling pathways and regulate gene expression (Ingber, 2006; Wang et al., 1993). Molecules such as actin, myosin II, Rho and ROCK, are important participants in generating cytoskeletal tension and can regulate mechanical signaling. External forces such as fluid shear stress, cyclic strain and changes in substrate rigidity can also alter cell signaling and gene expression.
Defects in mechanotransduction signaling have been reported to be the basis of diverse pathological conditions including cancer (Huang and Ingber, 2005; Suresh, 2007). Different studies have described a balance of underlying mechanical forces as driving factors in sensitizing capillary EC to angiogenic growth factors, while a spatial and temporal variation of these forces climaxes in vascular development (Ingber and Folkman, 1989; Matsumoto et al., 2007). These studies together highlight the importance of mechanical forces in regulating the angiogenic process.

**TRPV4 and Mechanotransduction**

A growing body of evidence has identified members of the transient receptor potential (TRP) family including TRPC (canonical), TRPM (melastatin) and TRPV (vanilloid) to be key regulators of mechanotransduction (Christensen and Corey, 2007; Liedtke and Kim, 2005; O'Neil and Heller, 2005; Pedersen and Nilius, 2007). In recent years, TRPV4 has emerged to be a mechanosensor contributing significantly to the process of mechanotransduction. TRPV4, a non-specific Ca\(^{2+}\) permeable channel has been shown to be activated by a variety of physical and chemical stimuli such as temperature, hypotonicity, phorbol esters, endocannabinoids, arachidonic acid (AA), and epoxyeicosatrienoic acids (EETs) (Vriens et al., 2004). The TRPV4 protein (Fig. 1.2 B) is composed of a cytosolic N-terminal region and six transmembrane domains (green), including the pore region and an intracellular C-terminal tail. The N-terminal region contains the ankyrin repeat domain (ARD), which consists of six ankyrin repeats (ANK) (ANK1–6) (yellow) (Nilius and Owsianik, 2011; Owsianik et al., 2006; Phelps et
al., 2008). A proline-rich domain (PRD) that has been implicated in the mechanosensitivity of the TRPV4 channel precedes the first ANK. Within this PRD, proline residue at positions 142, 143, and 144, interacts with pacsin 3, a protein implicated in vesicular membrane transport, endocytosis and cytoskeleton reorganization (Cuajungco et al., 2006; D’Hoedt et al., 2008). The TRPV4 C-terminal tail contains additional functional domains such as a TRP box, a calmodulin-binding site and a binding site for cytoskeletal proteins such as MAP7, actin and tubulin (Strotmann et al., 2003; Suzuki et al., 2003a) (Fig. 1.3).
Figure 1.3. Schematic model of TRPV4 structure. The TRPV4 protein is composed of a cytosolic N-terminal region and six transmembrane domains, including the pore region and an intracellular C-terminal tail. The N-terminal region contains six ankyrin repeat domains.
Expressed in a variety of tissues, TRPV4 channels have garnered significant attention especially due to their implications in mechanotransduction. TRPV channels were first recognized as important in mechanotransduction when studies by Colbert and Bargmann (1995),(1997) identified the osm-9 gene in a screen for mutants defective in olfaction in *Caenorhabditis elegans* (*C. elegans*). Osm-9 plays an important role for various sensory functions such as chemosensation and osmosensation; TRPV4 channels were first found by screening expressed tag databases for sequences similar to the *C. elegans* TRPV isoform osm-9. Cloned from the kidney, auditory epithelium and hypothalamus, TRPV4 is known by a number of other names: Osm-9-like TRP channel 4 (OTRPC4) (Strotmann et al., 2000), vanilloid receptor-related osmotically activated channel (VR-OAC) (Liedtke et al., 2000) and vanilloid receptor-like channel 2 (VRL-2) (Delany et al., 2001). Changes in the extracellular osmolarity were one of the first physiological activities found to be modulated by TRPV4. Increases in osmolarity from 300 mosmol/l reduced TRPV4 activity whereas reductions (hypotonic solutions) led to an increase in activity (Nilius et al., 2001; Strotmann et al., 2000; Wissenbach et al., 2000). In *C. elegans*, Liedtke et al. (2003) found that expression of mammalian TRPV4 (VR-OAC) cDNA in sensory neurons of *C. elegans* directs behavioral responses to osmotic and mechanical stimuli, providing evidence that TRPV4 can function as a component of an osmotic/mechanical sensor *in vivo*.

The functional significance of TRPV4 in lung mechanotransduction has been elucidated in a number of studies. TRPV4 is expressed abundantly in
mammalian airways and pulmonary blood vessels including lung epithelium, endothelium, fibroblasts and macrophages (Alvarez et al., 2006). These channels have been shown to contribute to bronchoconstriction upon hypotonic swelling, a phenomenon similar to stretch-induced activation of TRPV4 in airway SMC of the lung (Yin and Kuebler, 2009). A major mediator of injury to pulmonary microvascular endothelium (Yin et al., 2008), TRPV4 activation and calcium influx occurs with increases in pulmonary vascular or airway pressures, which induce an increase in vascular permeability caused by high vascular and airway pressures (Hamanaka et al., 2007; Jian et al., 2008).

It is evident that TRPV4 plays an important role in bone homeostasis from the observation that various skeletal defects occur from mutations within the TRPV4 gene. TRPV4 has been shown to be involved in the differentiation and migration of osteoclasts; TRPV4 has also been demonstrated to stimulate chondrogenesis and exhibit a chondroprotective role by mediating responses to hypo-osmotic stimuli (Lieben and Carmeliet, 2012). Recently, Suzuki and co-workers (2013) showed that TRPV4 expression is upregulated in osteoblast differentiation and is directly involved in flow-induced calcium signaling in osteoblasts.

Among the various TRP channels, TRPV4 has been proposed as a potential candidate for the mechanosensitive channel(s) in the endothelium through which shear stress is transduced into Ca\(^{2+}\) signaling and then flow-mediated dilation. Köhler and colleagues (2006) demonstrated that the mechanical activation of TRPV4 by shear stress or the activation of TRPV4
pharmacologically by 4-α-PDD triggered endothelial Ca\(^{2+}\) influx and subsequent nitric oxide (NO) mediated vasodilation in the rat carotid artery. A number of studies have shown that in both conduit and small arteries from TRPV4\(^{-/-}\) mice compared with wild-type mice, flow-induced dilation was decreased (Hartmannsgruber et al., 2007; Loot et al., 2008). We and others have also shown PKC\(\alpha\) mediates the vasoactive agonist acetylcholine (ACh)-induced activation of TRPV4-dependent calcium influx and NO production in EC (Adapala et al., 2011; Earley et al., 2009; Zhang et al., 2009). These studies together demonstrate that TRPV4 plays an important role in the mechanical force and agonist-induced regulation of vascular tone. TRPV4 is also expressed in SMC in the mesenteric and cerebral vascular beds (Earley et al., 2005; Marrelli et al., 2007) and in aortic myocytes (Tanaka et al., 2008). These channels have recently been found to be important mediators of vasodilation induced by EET's in vascular SMC and TRPV4 activity has also been shown to contribute to the regulation of peripheral vascular resistance in vascular myocytes \textit{in vivo} (Earley et al., 2009) as well as in blood pressure regulation (Gao et al., 2009). Similar to EC, flow-induced Ca\(^{2+}\) entry via TRPV4 has been shown in renal tubular epithelial cells (Taniguchi et al., 2007; Wu et al., 2007).

1.4 Tumor Angiogenesis and Tumor Vasculature: A Target for Cancer Therapy

Tightly regulated, both at a molecular and mechanical level (Carmeliet and Jain, 2011; Jain, 2003), physiological angiogenesis meets the optimal nutrient and oxygen supply demands of the various organ systems in the body,
by generating a well-organized and highly structured network of blood vessels. In contrast, the persistent neovascularization occurring within a tumor, to serve its unending demand for nutrient supply, results in the generation of blood vessels that are abnormal in both structure and function (Fukumura et al., 2010; Nagy et al., 2009). As the tumor grows, insufficient nutrient supply and reduced waste clearance generates an environment of hypoxia (Folkman, 1971) that induces VEGF and other growth factor overexpression leading to the growth of new vessels (Nagy et al., 2002). In addition to hypoxia, angiogenesis in solid tumors is propelled by multiple factors including acidosis, inflammatory cytokines as well as the activation of oncogenes that occur at the cellular level, upstream of VEGF and tumor-promoting hormones (Carmeliet and Jain, 2011; Ferrara, 2005; Vogelstein and Kinzler, 2004). Structurally the tumor microvasculature is tortuous, dilated, and hyper-permeable and contains regions of heterogeneity with areas within the tumor being ‘hyper’ or ‘hypo’ vascular (Kim et al., 2010; Wojciak-Stothard and Ridley, 2003). Pro-angiogenic factors, at the cellular level, induce the weakening of the VE-cadherin-mediated EC junctions and thereby increased EC migration resulting in distortion of the vessel wall structure (Hashizume et al., 2000). In addition, the perivascular cells comprising of pericytes and vascular SMC are found to be less in number and loosely attached to EC making vessels immature and leaky. It has previously been demonstrated that pro-angiogenic factors such as VEGF can impede the adherence of pericytes to EC (Abramsson et al., 2003; Greenberg et al., 2008; Jain, 1988; Morikawa et al., 2002). These structural abnormalities result in dysfunctional
vessels that promote erratic blood flow and hyper-permeability, due to reduced pericyte coverage, that allows protein and fluid extravasation into the extracellular space resulting in increased matrix deposition. Within the tumors, the lack of a functional lymphatic vessel system increases interstitial fluid pressure (IFP) within the tumor that ultimately can result in reduced transvascular blood flow, which can impede drug delivery (Baluk et al., 2003; Boucher et al., 1990; Inai et al., 2004; Jain, 1988; Less et al., 1991; Leunig et al., 1992). While the tumor continues to grow, the expanding tumor mass can often lead to compression of the vessels which finally collapse and further diminishes blood supply, increasing hypoxic and acidic conditions within the tumor (Fukumura and Jain, 2007; Jain, 1988; Padera et al., 2004). Such an environment within the tumor reduces tumor cell sensitivity to radiation as well as chemotherapy (Teicher, 1996). These factors altogether, generate tumor cells that demonstrate a more a hostile phenotype that are highly resistant to treatment and have high metastatic potential (DeClerck and Elble, 2010). The structural and functional aberrations of the tumor vasculature not only disable the efficient distribution of systemically administered chemotherapeutic drugs but also promote drastic genetic and morphological changes within the cells, which further fuel tumor growth and compromise therapeutic intervention (Bottaro and Liotta, 2003).

1.5 Tumor Endothelial Cells (TEC)

Considering the abnormalities of the tumor vasculature, it becomes pertinent to address the complexities associated with the EC that form this vasculature. It is now known to a large extent that the tumor endothelium is
defective with TEC showing distinct irregularities with respect to shape and size, when compared to normal endothelial cells (NEC). These cells tend to have long cytoplasmic projections that can extend across the lumen, with the tips of some TEC protruding into the lumen, creating intercellular gaps within the vessel wall that result in the leakage or pooling of blood (Dudley, 2012). Di Tomasi and colleagues (2005) have additionally demonstrated that the EC lining the tumor blood vessels often lack endothelial CD31 and CD105 expression, describing these vessels to be “mosaic”. This “mosaic” property of the tumor blood vessels are attributed to individual TECs lacking these EC markers at certain regions while at other regions, simply gaps or the absence of these cells.

At the molecular level, the tumor endothelium, express a host of genes recently identified as transmembrane proteins called tumor endothelial markers (TEM), that are only found under circumstances of physiological angiogenesis (Nanda et al., 2004; St Croix et al., 2000). In-depth studies on TEC isolated from different carcinomas found that these cells do not undergo senescence, are resistant to serum starvation and apoptosis, and are structurally abnormal compared to NEC. TEC isolated from mice xenograft tumors were found to have variable DNA content, not only between its normal counterpart cell types but between individual cells, indicating heterogeneity among TEC. These cells have larger nuclei and exhibit characteristic cytogenic and structural abnormalities such as aneuploidy and chromosomal aberrations including deletions, non-reciprocal translocations and abnormal centrosomes (Allport and Weissleder, 2003; Bussolati et al., 2003; Hida et al., 2004; Hida and Klagsbrun, 2005).
Furthermore, TEC isolated from mouse prostate tumors were found to express both hematopoietic and mesenchymal stem cell markers, verifying the heterogeneity of these cells. Additionally, it was found that these cells were also able to undergo unusual mesenchymal differentiation into cartilage and bone-like tissues in conditioned medium, confirming the ability of TEC to adapt to their surrounding environment (Dudley et al., 2008). Based on these reports, it can be interpreted that the cellular and molecular aberrations of TEC can invariably contribute to the abnormal angiogenic process and ultimately tumor growth and metastasis. However, when grown in defined endothelial medium, these TEC (derived from mouse prostate tumors) express endothelial markers and show morphological features similar to NEC (Dudley et al., 2008). Interestingly, we have demonstrated that TEC show aberrant mechanosensing and abnormal angiogenesis in vitro, suggesting deregulation of mechanosensing mechanisms in these cells (Ghosh et al., 2008). A fascinating question, that has yet to be considerably studied, pertains to the origin of these TEC. An interesting question that is yet to be extensively studied pertains to the origin of these TEC, as there has been limited progress in conclusively identifying the source of these cells, making it difficult to develop any specific targets as part of an anti-angiogenic strategy.

1.6 Anti-Angiogenic Strategies and Current Challenges

Since first described by Dr. Folkman in (1971), the concept of angiogenesis and anti-angiogenic therapies has revolutionized the way cancer has been studied and clinically treated. Inhibition of angiogenic activators such
as VEGF, placental growth factor (PIGF), FGF (Khoury and Ziyadeh, 2011) and associated growth factor signaling mechanisms, have provided new avenues in successfully treating as well as studying tumor angiogenesis. Anti-VEGF therapies, including anti-VEGF neutralizing monoclonal antibodies and receptor tyrosine kinase inhibitors (RTKI) dominate current approaches in treating malignant tumors (Kim et al., 1992; Kim et al., 1993), initially achieving great success. However, since its clinical debut over a decade ago, anti-angiogenic therapies are plagued by significant challenges over the long run, with multiple anti-angiogenic drugs demonstrating lower clinical efficacy than that was observed in preclinical trials (Ebos et al., 2009). One of the reasons for this effect pertains to the fact that angiogenesis is promoted by other pro-angiogenic factors, such as PIGF, FGF, and interleukins (Ebos and Kerbel, 2011; Li et al., 2011; Loges et al., 2009). As indicated by clinical trials, treating patients with selective VEGF inhibitors or VEGF receptor blockers can result in the development of resistance mechanisms, wherein tumors begin to utilize VEGF-independent growth factor pathways to promote tumor growth. Recent observations have also suggested that resistance towards anti-VEGF drugs can also be due to the fact that various cancers can modify vascularization methods, either choosing to co-opt vessels with tumors growing around pre-formed vessels, or take part in vascular mimicry – wherein tumor cells replace EC, or even promote vasculogenesis (Kirschmann et al., 2012; Ricci-Vitiani et al., 2010; Wang et al., 2010).
Aside from these challenges, various preclinical studies (Ebos and Kerbel, 2011; Ebos et al., 2009; Paez-Ribes et al., 2009) although some being debatable, have pointed out that inhibition of VEGF in several cancer models promote invasiveness and tumor malignancy. VEGF inhibitors have also demonstrated several side effects such as hypertension, impaired wound healing, renal toxicity and other symptoms as a result of depriving dormant/quiescent EC of VEGF’s pro-survival effect. Anti-angiogenic drugs have increasingly being reported to stimulate and promote a surge in proinflammatory and proangiogenic response systemically by upregulating factors such as PIGF, granulocyte-colony stimulating factor (G-CSF), and osteopontin, which can then induce the mobilization of resistance-conferring myeloid-derived suppressor cells (MDSC) (Ebos et al., 2007; Shojaei, 2012; Shojaei et al., 2009). In short, many anti-angiogenic therapies are redundant, ineffective and in some instances detrimental to the treatment of cancer due to inherent or acquired drug resistance, potential metastatic abilities and the general absence of predictive markers to monitor tumor responses in select patient population.

1.7 Vascular Normalization

The disruption of the tumor vasculature through the use of anti-angiogenic drugs, though intended, has paradoxically disabled the delivery of other chemotherapeutic drugs to the tumor (Fenton et al., 2004). The lack of an organized vessel structure that is immature and unstable in a hypoxic microenvironment impedes the homogenous distribution of chemotherapeutic
drugs to the tumor, rendering radiation therapies ineffective. However, an interesting observation was made when results from phase 3 clinical trials demonstrated that the combined treatment of Bevacizumab (anti-VEGF antibody) with systemic chemotherapy greatly increased patient survival and better therapeutic outcomes than anti-VEGF therapy alone (Hurwitz et al., 2004; Saltz et al., 2008). These results seemed puzzling due to the fact that, for the systemic and homogeneous delivery of chemotherapeutic drugs to the tumor, there needs to be a good vascular network, and anti-VEGF therapies were originally tested and administered to promote vessel disruption so as to cut off blood supply to the tumor. These confounding results were later attributed to the understanding that anti-VEGF drugs could transiently induce tumor ‘vascular normalization’, hence the improved delivery of chemotherapeutic agents. The principle behind vascular normalization is to restore the balance between pro- and anti-angiogenic factors that control vessel growth and maturity (Jain, 2005) (Figure 1.4). In recent years, a large body of pre-clinical and clinical evidence has validated many direct and indirect angiogenesis inhibitors to transiently promote the normalization of the tumor vasculature, fundamentally restoring the properties of the normal vasculature enough to enable uniform drug delivery to the tumor.

Most direct angiogenesis inhibitors that have been assessed for vessel normalization properties mainly target the VEGF pathway. Antibodies targeting VEGF or its receptor (VEGFR2) have been shown, in different tumor models, to improve the vasculature of the tumors with vessels exhibiting increased maturity, decreased permeability, uniform diameter as well as improved oxygenation
(Tong et al., 2004; Winkler et al., 2004; Yuan et al., 1996). Direct angiogenesis inhibitors are those that target VEGF-dependent pathways either upstream or downstream, such as inhibitors of oncogenes. Endocrine therapies for sex hormone-dependent tumors have also shown similar normalization effects; Izumi and colleagues (2002), observed blocking the human epidermal growth factor receptor (HER)-2 signaling pathway using a specific antibody Herceptin in tumors expressing HER2, not only decreased the expression of various pro-angiogenic factors such as ANG-1 and VEGF, but consequently induced vascular remodeling and normalization within these tumors.

It is obvious from the aforementioned studies and vast array of pre-clinical and clinical data that is available, that vascular normalization therapies provide a novel avenue towards treating cancers and other non-malignant vascular diseases. Although this therapy based on anti-VEGF-mediated approaches holds promise, it has a narrow window in terms of the transient nature of the resulting ‘normal’ vessel. Studies have shown the continuous blockade of VEGF ultimately cause excess regression or pruning, rendering the vessel to be functionally inadequate; additionally, development of resistance towards these drugs, reverts the ‘normalized’ vessel back to being abnormal (Winkler et al., 2004). The challenges associated with these current normalization methods make it pertinent to explore growth factor-independent pathways that will eventually result in a more stable and sustained ‘normal’ vessel.
Figure 1.4. Concept of vascular normalization. The normal vasculature consists of a structurally and functionally organized network of blood vessels that is uniquely regulated by a balance between different pro- and anti-angiogenic factors. In the tumor vasculature, this balance is tipped to favor the sustained activity of pro-angiogenic factors, leading to uncontrolled angiogenesis. This results in the development of abnormally dilated, tortuous, poorly perfused and leaky vessels that transform the uniformly normoxic microenvironment to varying regions of hypoxia, hindering drug delivery and radiation therapy. Anti-VEGF drugs, when used judiciously, have recently been found to induce a transient normalization of the tumor vasculature wherein there is a reestablishment of the balance between the pro- and anti-angiogenic factors. This balance promotes a restoration of the properties of the normal vasculature and generates a normoxic microenvironment surrounding the vessels, ultimately enabling the efficient delivery of chemotherapeutic drugs while increasing the success of radiation therapies at the same time.
1.8 Significance of Study

Currently soluble factor targets, such as VEGF, FGF and other growth factors, dominate the field of clinically approved therapies targeted against tumor angiogenesis. However, these therapies have failed to permanently abolish tumor growth and are ridden with limitations. In the angiogenic process, key events such as EC activation, proliferation and its subsequent migration occurs in response to the aforementioned indispensable growth factors, which are in turn directed by underlying micromechanical forces such as ECM-mediated substrate elasticity and hemodynamic forces generated by blood flow. These mechanical forces, as previously explained, are sensed by EC through the process of mechanotransduction.

Our studies have identified TRPV4 channels as the specific stretch-activated (SA) channel in EC that is activated when mechanical strain is applied on the integrin-ECM adhesions (Thodeti et al., 2009). The resulting influx of $\text{Ca}^{2+}$ was demonstrated to promote cellular reorientation by activating phosphatidylinositol 3-kinase (PI3K) which further stimulates the activation of additional $\beta 1$ integrins that may regulate Rho/Rac signaling required for the reorganization of the actin cytoskeleton and EC reorientation. We further showed that mechanical strain application on $\beta 1$ integrins rapidly activates TRPV4 through its interaction with a transmembrane CD98 protein located in focal adhesions (Matthews et al., 2010) (Fig. 1.5).
Figure 1.5. TRPV4 mediated mechanotransduction in EC reorientation. A model showing TRPV4-dependent mechanical signaling in endothelial cells. Application of mechanical force (cyclic stretch) to integrins activates ultra-rapid calcium influx through TRPV4 via interaction with a transmembrane protein CD98. The released Ca$^{2+}$ activates additional integrins via PI3K (Matthews et al., 2010; Thodeti et al., 2009). This integrin-to-integrin signaling further regulates downstream Rho/Rac pathways necessary for reorganization of the actin cytoskeleton and reorientation of EC.
Further, our work (Ghosh et al., 2008) on TEC revealed a clear phenotypic distinction to normal EC, wherein these cells failed to reorient its actin cytoskeleton following mechanical stimulation and exhibited increased baseline Rho-activity (Fig. 1.6). Furthermore, these TEC also exhibited an abnormal mechanosensitive response to substrate elasticity, increased contractility and finally abnormal in vitro angiogenesis. Altogether, it is very likely that the anomalous behavior exhibited by TEC, with respect to its mechanosensing abilities, could be the consequence of an alteration in TRPV4-mediated signaling and this underlying phenomenon may be a causative or contributory factor for the abnormal nature of the tumor vasculature. If this indeed is the case, TRPV4 channels provides a novel avenue for studying and treating tumor angiogenesis, one that can be a necessary alternative to the existing but inadequate growth-factor therapies; or at the least, a supporting therapeutic target that, together with existing therapies, can effectively surmount cancer.
Figure 1.6. Endothelial cell reorientation and basal Rho-activity levels in normal and tumor capillary endothelial cells (CE) in response to mechanical stretch. *Figure from Ghosh et al. 2008.*

**A.** TCE are shown to reorient abnormally in response to uniaxial cyclic strain. Representative fluorescent images show NCE and TCE grown on fibronectin-coated flexible silicone substrates that were subjected to either no strain (Control) or 10% uniaxial cyclic strain for 18 h at 1 Hz; the arrow indicates the direction of applied strain. Computerized morphometric quantitation of the reorientation response in cells cultured in the absence or presence of 10% uniaxial cyclic strain. Data are presented as the percentage cells that reoriented 90° ± 30° relative to the direction of applied strain; **, *P < 0.001*).

**B.** TCE exhibit increased baseline Rho activity than NCE. NCE and TCE were grown on fibronectin-coated flexible silicone substrates and subjected to either no strain (Control) or 10% uniaxial cyclic strain for 2 h, and Rho activity was analyzed by using the Rhotekin-RBD binding assay. Representative western blot demonstrates the levels of active GTP-Rho and total Rho for NCE and TCE in the presence or absence of applied strain. Relative changes in Rho activity under the various experimental conditions. GTP-Rho levels were measured as a percentage of total Rho levels and normalized to basal GTP-Rho levels in normal CE cells; **, *P < 0.002* for comparison of baseline Rho levels in normal versus tumor CE cells).
This dissertation provides insight into the role of the mechanosensitive TRPV4 channel in tumor angiogenesis, its significance in normal angiogenesis and the underlying molecular mechanism that regulates both these processes. To effectively delineate the importance of this unique ion channel in the equally important process of angiogenesis, this dissertation is divided into three aims that consist of the following chapters:

Chapter III

**Aim 1: The Functional Significance of the Mechanosensitive Ion Channel TRPV4 in Tumor Angiogenesis.**

*Hypothesis:* Alterations in TRPV4 expression and/or function leads to abnormal TEC mechanosensing and targeting TRPV4 may restore mechanosensitivity and induce tumor vascular normalization.

Chapter IV

**Aim 2: TRPV4 Channels Regulate TEC Proliferation via Modulation of ERK1/2.**

*Hypothesis:* Targeting TRPV4 decreases TEC proliferation possibly through modulation of ERK1/2.

Chapter V

**Aim 3: TRPV4 is a Negative Regulator of Angiogenesis.**

*Hypothesis 1:* Absence of TRPV4 enhances physiological and pathological (tumor) angiogenesis in TRPV4KO mice.

*Hypothesis 2:* Inhibition of Rho Kinase, the downstream effector of Rho (TRPV4), normalizes the tumor vasculature in TRPV4KO mice.
CHAPTER II

Materials and Methods

Cell culture

Normal and tumor EC were obtained from a transgenic adenocarcinoma mouse of the prostate (TRAMP) model, as previously described (Dudley et al., 2008; Ghosh et al., 2008). Cells were plated on fibronectin (FN) or gelatin-coated tissue culture dishes and grown in a defined medium composed of low glucose DMEM, 10% fetal bovine serum (FBS), 10% Nu Serum IV, VEGF(1ng/ml) basic fibroblast growth factor (bFGF) (3 ng/ml), heparin salt (0.1 mg/ml), 1% insulin-transferrin-selenium and antibiotic/mycotic mix. Cells were cultured in a 37°C, 5%CO₂ incubator, split at ~90-95% confluence, and used between passages 11-22. The cells were characterized for the presence of endothelial markers and function. We found that both cell types expressed endothelial markers including CD31(PECAM-1), VE-cadherin, Von Wilebrand Factor (vWF), endothelial nitric oxide synthase (eNOS) and bind to isolectin-IB4 as measured by FACS, Western blotting, qPCR and immunocytochemistry. In contrast, these cells do not express
mesenchymal markers, α-SMA and PDGFR-β receptor (Dudley et al., 2008). Cells from WT-C57BL/6 (WT) and TRPV4KO mice were isolated using the aortic explant method as described previously (Mahabeleshwar et al., 2006). Briefly, aortic rings were placed in Matrigel for 7 days. The aortic rings were carefully removed, and EC were isolated, washed, and plated on gelatin (0.1%)-coated dishes. The isolated primary EC were cultured in a defined media and used between passages 1-8.

**SDS-PAGE and Western blot Analysis**

Control and treated cells were washed with PBS and lysed in RIPA buffer [50 mM Tris·HCl at pH 7.4, 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS with protease, and phosphatase inhibitor cocktail (Boston Bioproducts). Lysates were incubated at 4°C and then centrifuged at 14,000xg for 20 min. Protein concentration was measured using a Coomassie (Bradford) Protein Assay Kit (Thermo Scientific) and equal amounts of protein were aliquoted and laemmli sample buffer (Boston Bio Products) was added and then heated to 95°C for 5 min. Proteins were kept on ice until loading onto 8% or 12% Acrylamide gels for SDS-PAGE. Proteins were transferred onto a PVDF membrane and the membrane was blocked in 5% milk in TBS with 0.05% Tween-20 (TBS-Tw) for 1 h. The blot were incubated with primary antibody: anti-TRPV4 (1:300) (alomone labs), phospho-ERK1/2 (1:1000) (Cell Signaling); total ERK1/2 (1:1000) (Cell Signaling); phospho AKT (1:1000) (Cell Signaling), total AKT (1:1000) (Cell Signaling), anti- PCNA (1:1000) (abcam), anti-actin (1:1000),
tubulin (1:5000) (abcam) overnight and then rinsed three times with TBS-Tw and incubated with the appropriate secondary antibody goat anti- mouse (1:20000) or goat anti-rabbit (1:20000) conjugated with horseradish peroxidase (Jackson labs). Signals were detected with the use of chemiluminescent substrate (Thermo Scientific) and a FluorChem M Simple Imager (Protein Simple).

**Ex vivo aortic ring angiogenesis assay**

Isolated aortic rings from WT and TRPV4KO mice as previously described (Baker et al., 2012), were carefully placed between 2 layers of Matrigel (BD Biosciences) and supplemented with EC media. Aortic explants were monitored and imaged from day 0 to day 7 for vessel sprouting. The average number of sprouting vessels was calculated for day 6 and data are expressed as fold increase from WT explants.

**qPCR**

RNA isolation of cells was performed using Qiagen kit and measured using the NanoDrop 2000 UV-Vis Spectrophotometer). cDNA synthesis was done using qscript cDNA SuperMix (Quanta Biosciences) and qPCR analysis was performed using Fast SYBR green master mix (Applied Biosystems) method on the Fast Real-Time PCR system (Applied Biosystems). Real time PCR was performed using real time cyclin D1, cyclin A, cyclin E1, CDK 1, CDK2, CDK 4, CDK 6, TRPV4 and GAPDH primers obtained from IDT technologies. The levels of all genes relative to GAPDH levels were analyzed and the ΔΔCT values are expressed as fold change.
**Transfection**

Cells were transfected with TRPV4-EGFP (kind gift of Dr. Jendrach, Germany) or EGFP constructs using targefect (targetingsystems) (Adapala et al., 2011). The transfection efficiency was found to be 80-90%. The expression of EGFP-TRPV4 in EC was visualized using a Nikon Eclipse TE 2000-E microscope (Nikon, Japan) fitted with a CoolSnap HQ digital camera (Photometrics) or Olympus IX72 fluorescence microscope (Olympus, Japan).

**Cell spreading on flexible substrates**

Transglutaminase-crosslinked gelatin hydrogels of increasing stiffness were prepared using 5 and 10% (w/v) final gelatin concentration and incubated at 4°C overnight to stabilize crosslinking. Cells in regular culture medium were plated at low density (to minimize cell-cell interactions) and allowed to spread for 6 h. For cell spreading studies, TEC cell areas were measured by tracing cell perimeter and normalized as described previously (Ghosh et al., 2008). The cells spread on flexible gelatin hydrogels were fixed with 4% paraformaldehyde while cells forming tubular structures were left untreated, and samples from both studies were imaged using a Nikon Diaphot 300 phase contrast microscope (Nikon, Japan) fitted with a Hamamatsu digital camera (Hamamatsu Photonics, Japan) or Olympus IX72 fluorescence microscope (Olympus, Japan). Image analyses were performed using ImageJ software (NIH). At least 30 cells were evaluated.
All data are expressed as mean ± SEM and evaluated for differences using student’s t-test and/or one-way ANOVA.

**Rho activation assay**

Rho activity was determined using the Rhotekin-RBD affinity precipitation assay as described previously (Ghosh et al., 2008; Thodeti et al., 2002). Briefly, cells were lysed in 1% Triton X-100 buffer and centrifuged at 12000X g for 15 min. Equal volumes of clarified lysate were incubated with GST-Rhotekin-RBD beads (Cytoskeleton Inc.), for 1 h at 4ºC. The beads were collected by centrifugation and washed 3 times with wash buffer. The bound GTP-Rho was extracted with SDS-sample buffer and was detected using Rho mAb (SantaCruz) on a Western blot. GTP-Rho levels were calculated from the densitometric analyses of Western blot and normalized to the levels of total-Rho and presented as relative Rho activity.

**Calcium imaging**

TEC overexpressing TRPV4-EGFP or EGFP alone or NEC were cultured on MatTek glass bottom dishes and loaded with Fluo-4/AM (1-4 μM) for 30 min, washed 3 times in calcium medium (136 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.1 mM CaCl2, 1.2 mM KH2PO4, 5 mM NaHCO3, 5.5mM glucose, and 20 mM Hepes. pH 7.4). Cells were stimulated with TRPV4 activator GSK1016790A (100 nM) in calcium medium (Adapala et al., 2011; Matthews et al., 2010; Thodeti et al., 2009). Calcium imaging was performed on Leica SP2 Confocal Microscope.
or Olympus FluoView 300 confocal microscope and analyzed using Leica/Olympus software and Microsoft Excel. We have previously confirmed that TRPV4 channels are expressed in WT and NEC and that TRPV4 activators 4-α-PDD (10 μM) or GSK1016790A (100 nM) specifically induce calcium influx in TRPV4 expressing but not TRPV4KO EC in the calcium containing media (Adapala et al., 2011).

**In vitro angiogenesis assays**

Growth factor-reduced Matrigel® (Matrigel) was plated on 48 well plates and kept at 37°C in an incubator for 30 min. Cells overexpressing TRPV4-EGFP or EGFP alone or WT and TRPV4KO EC (2-8 X 10⁴ cells/well) were plated on Matrigel and incubated at 37°C for 6-12h (Ghosh et al., 2008). For 2D assays performed in the presence or absence of TRPV4-specific agonist or ROCK inhibitor, cells were treated with GSK1016790A (100nM) or Y-27632 (10μM) prior to plating. Images were obtained and tube length was measured using NIH Image J.

**Immunofluorescence assay**

Normal and tumor EC were cultured and fixed for 20 min with 4% PFA (paraformaldehyde) upon attaining 75% confluency. Cells were washed with PBS and permeabilized for 15 min using 0.25% Triton X buffer. After PBS washes, permeabilized cells were blocked in serum containing media for 1h. Cells were incubated with primary anti-TRPV4 (1:200) (alomone labs), for 1h. Post-incubation cells were rinsed in PBS and incubated for 1h with secondary antibody conjugated to Alexa-fluor 594 or Alexa-fluor 488 (Invitrogen). Post-
washes, cells were mounted with DAPI (vector labs) to visualize nuclei. Images were taken at 40x or 60x magnification with Olympus Epifluorescence Microscope using QCapture Pro (QImaging) with standard FITC and TRITC filters.

**Syngeneic tumor model in mice and analysis of tumor growth, vascular malformation and angiogenesis**

All the experiments were performed according to approved protocol by Northeast Ohio Medical University, IACUC. The TRPV4 KO mice were procured from Dr. David Gutterman (Medical college of Wisconsin, WI) (Zhang et al., 2009) and rederived at Charles River Laboratories, Wilmington, MA.

Mouse Lewis lung carcinoma (LLC) cells (kind gift from Dr. Venkat Keshamouni, University of Michigan, Ann arbor, MI) (2 X 10^6) were subcutaneously injected in the flank region of WT C57BL/6 mice or TRPV4KO mice (Standiford et al., 2011). Tumor size was measured using calipers at 7, 14 and 21 days and tumor volume was calculated according to the formula \[ V = \frac{4}{3} \pi \frac{\text{Length} \times \text{Width}^2}{4} \]. At day 21, mice were euthanized and tumor tissues were collected and fixed for immunohistochemistry or stored at -80° C. To measure tumor angiogenesis, tumor tissue sections of 10 µm thicknesses were stained with anti-CD31 (PECAM-1) to visualize the microvessels, α-SMA to stain pericytes and DAPI to label the nuclei. Images were acquired using Olympus IX72 microscope.

For the *in vivo* drug experiments in WT animals, six to eight mice/group were used and the animals were divided in to four groups: 1) WT (control) 2) WT +
TRPV4 activator 3) WT + Cisplatin and 4) WT + TRPV4 activator+ Cisplatin.

Once the tumors were palpable (after 7 days), the mice were daily given an intraperitoneal (i.p.) injection of TRPV4 agonist GSK1016790A (10 µg/kg) to groups 2 and 4 until day 21. The anti-cancer drug Cisplatin (3 mg/kg/week) was given once/week to group 3, and in group 4 Cisplatin (3 mg/kg/week) was administered i.p. once/week, 2-4 days post treatment with TRPV4 activator, until day 21. The WT control received saline as a vehicle.

For the *in vivo* drug experiments in TRPV4KO animals, six to eight mice/group were used and the animals were divided in to four groups: 1) TRPV4KO (control) 2) TRPV4KO + ROCK inhibitor- Y-27632 3) TRPV4KO + Cisplatin and 4) TRPV4KO + Y-27632 + Cisplatin. Once the tumors were palpable (after 7 days), the mice were daily given an i.p. injection of Y-27632 (10 µg/kg) to groups 2 and 4 until day 21. The anti-cancer drug Cisplatin (3 mg/kg/week) was given once/week to group 3, and in group 4 Cisplatin (3 mg/kg/week) was administered i.p. once/week, 2-4 days post treatment with ROCK inhibitor, until day 21. The KO control received saline as a vehicle.
**Immunohistochemistry**

Tumor tissues isolated 21 days post tumor implantation in WT and TRPV4KO mice were fixed in OCT (Tissue Tek) and cryosectioned to 10μm thickness on coated slides. Frozen sections were thawed at room temp for 10 min and were fixed in acetone for 15 min and washed with PBS for 3x for 5 min. Sections were circled using a hydrophobic pap pen and blocked with serum containing media for 1h. Sections were incubated with rat probe (Biocare Medical) for 1h. The sections were then incubated overnight with rat anti-CD31 (invitrogen), mouse monoclonal α-SMA (invitrogen). For in vivo proliferation assays, sections were incubated rat-anti CD31 (Invitrogen) and anti-rabbit ki-67 (proliferation marker) (abcam) overnight. Post-incubation, sections were washed with 3x PBS and incubated for 1h with appropriate secondary antibody conjugated with FITC (Invitrogen) and Alexa-fluor 594 (Invitrogen); finally the sections were washed and then mounted with DAPI (vector labs). Images were taken at 10x or 20x or 40x magnification with Olympus Epifluorescent Microscope using QCapture Pro (QImaging) with standard FITC and TRITC filters and quantified using image J.
**Matrigel plug assay**

Mice were anesthetized with ketamine/xylazine. Growth factor-reduced Matrigel (Becton Dickinson, Mountain View, CA) supplemented with basic fibroblast growth factor (bFGF) (R&D biosystems) (125 ng/plug) and heparin sulphate (sigma Aldrich) (15μg/plug) was injected subcutaneously into the lower abdominal region of each of WT and TRPV4KO mice. Plugs were excised and imaged using Olympus IX72 microscope (40x), 2 weeks post implantation. Matrigel plugs were then fixed in 10% PFA and cryosectioned for immunohistochemistry analysis.

**BrdU proliferation assay**

Cells were counted and plated equally at low density on cover glasses and cultured for 24 h. Post 24 h, cells were treated with 10μM BrdU (abcam) for 2 h. The cells were fixed with 4% paraformaldehyde, washed with PBS and incubated for 10 min with 1N HCL on ice. The fixed cells were then incubated in 2N HCL at room temp for 10 min and moved to the incubator (37°C) for 20 min. Cells were washed with PBS containing 1% TritonX100 (3x 5 min) at room temp and fixed cells were blocked with PBST solution containing 1M glycine and 5 % fetal bovine serum (FBS) for 1 h and then incubated with anti-BrdU primary antibody (1:100) (abcam) overnight at room temperature. Following incubation, cells were washed 3X with PBST and incubated with secondary antibody (protocol modified from Abcam), Alexa Fluor-488 (Invitrogen) and mounted with DAPI (vector labs). Images were captured using an Olympus IX71-fluorescence microscope. Image
analyses were performed by using ImageJ software (National Institutes of Health).

**Cell proliferation assay**

Cell Viability was determined using a calcein assay kit (Enzo biosciences) which is based on the conversion of the cell permeant non-fluorescent calcein AM dye to the fluorescent calcein dye by intracellular esterase activity in live cells. Cultured ECs were trypsinized, counted and plated in a 96-well plate (2000 cells/well). Twenty four hours post plating, media was removed and cells were treated with GSK1016790A (GSK) (100 nM) in serum free (SF) media for 24 hours. Cells were washed with 1X PBS and calcein dye (3uM) diluted in 200ul of warm 1X PBS was added to the cells and plated was incubated for 30min-2h. As controls, wells containing no cells with Calcein dye were used. Absorbance was read at 485-535nm.

**Scratch-wound migration assay**

Cells were plated on gelatin-coated 6-well plates and grown to 95% confluence after which they were washed with serum free media to remove traces of growth factors. The scratch was made using a 200μl tip end and images captured at 0h and 12h using an Olympus IX51 bright-field microscope and percent migration was quantified using the formula ((area of scratch at 0h- area of scratch at 12h)/area of scratch 0h)*100).
**Statistical analysis**

All the data shown is mean ± SEM from at least three independent experiments. Significance was determined using Student’s *t* test and one-way ANOVA and significance was set at *p* ≤ 0.05.
Table 1: List of Primers

<table>
<thead>
<tr>
<th>GENES</th>
<th>FORWARD PRIMER</th>
<th>REVERSE PRIMER</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPV4-Set1</td>
<td>GCCTGTGTATTCTTCTCTTAC</td>
<td>GGTTCTCGATCTTGCTGTT</td>
</tr>
<tr>
<td>TRPV4-Set2</td>
<td>ATGAGGGAGGCTACTTCTACTT</td>
<td>CTTTCTTGTAGGGTTCTCTGT</td>
</tr>
<tr>
<td>CD31</td>
<td>GGTTGTCAAGCGAAGGATAGA</td>
<td>GGCAGCGAAACACTTAAAC</td>
</tr>
<tr>
<td>Cyclin A</td>
<td>GGTTGAGGTTGGAAGAAGATATAA</td>
<td>CCCCCTAAACACACAGACATGGAG</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>CTAAGATGAAGGAGACCATTCC</td>
<td>ACCAGAGGAGGATTCCATT</td>
</tr>
<tr>
<td>Cyclin E1</td>
<td>GGATGTGTTGGCTGTTAGAA</td>
<td>ATGTCCGACCACCTGATAAC</td>
</tr>
<tr>
<td>CDK1</td>
<td>ACGAGAACGGCTTGATTTT</td>
<td>TAAATAGGGTGGCTCCCAG</td>
</tr>
<tr>
<td>CDK2</td>
<td>AAGATGGAGGGACCGTGGTTAC</td>
<td>GGCACCTGGAAGTTGATAC</td>
</tr>
<tr>
<td>CDK4</td>
<td>TCAAGATGTGGAGGGTTGACGTT</td>
<td>GACATCCATCACCCGTACAA</td>
</tr>
<tr>
<td>CDK6</td>
<td>CTGGATGGGTGGACGAGATT</td>
<td>GTTGAGACGAGTGATACAG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CTCCCACCTCTCCACCATCG</td>
<td>CACCACCCCTGTTGCTGAG</td>
</tr>
</tbody>
</table>
CHAPTER III

The Functional Significance of the Mechanosensitive Ion Channel TRPV4 in Tumor Angiogenesis

Introduction

Angiogenesis, the formation of new blood vessels from pre-existing capillaries, is critical for solid tumor maintenance, growth and progression as it ensures proper oxygen and nutrient delivery (Folkman, 2002; Folkman, 2006). However, the tumor vasculature is structurally and functionally abnormal, as characterized by its high tortuosity, non-uniform pericyte coverage and hyper-permeability (Fukumura et al., 2010; Jain, 2008). These abnormal vessels cause irregular blood flow and distribution, impairing oxygen delivery and impeding immune cell function.

---

2 The information in this chapter was published in: Adapala RK#, Thoppil RJ#, Ghosh KG, Cappelli HC, Dudley AC, Paruchuri S, Keshamouni V, Klagsbrun M, Meszaros JG, Chilian WM, Ingber DE, and Thodeti CK (2015). Activation of mechanosensitive ion channel TRPV4 normalizes tumor angiogenesis and improves cancer therapy. Oncogene. doi: 10.1038/onc.2015.83. #Equally contributed to this work.
Altogether, these effects make the delivery of anti-cancer agents inefficient and overall, can lead to tumor cell resistance to radiation and chemotherapies (Abdollahi and Folkman, 2010; Bergers and Hanahan, 2008).

Current anti-angiogenic therapies focus on either neutralizing the effect of soluble growth factors such as vascular endothelial growth factor (VEGF) using specific antibodies or inhibiting VEGFR kinase activity (Carmeliet and Jain, 2011; Duda et al., 2007). However, these conventional anti-angiogenic strategies have shown only modest success in clinical trials due to the development of resistance (evasive or intrinsic) as TEC became refractory to anti-VEGF therapy over time (Bergers and Hanahan, 2008; Shojaei, 2012; Shojaei and Ferrara, 2008). These findings led to the emergence of a new concept called “vascular normalization”, where the goal of the therapy is to reestablish the balance between pro- and anti-angiogenic factors that control vessel growth and maturity, thereby restoring the properties of the normal vasculature so as to enable uniform drug delivery to the tumors. (Jain, 2001; Jain, 2005). Although vascular normalization therapies provide a novel avenue towards treating cancers and other non-malignant vascular diseases, it has a narrow window in terms of the transient nature of the resulting ‘normal’ vessel. The continuous blockade of VEGF results in excess regression or pruning while the development of resistance towards these drugs, reverts the ‘normalized’ vessel back to being abnormal (Winkler et al., 2004). These challenges therefore make it pertinent to explore growth-factor independent pathways that will eventually result in a more stable and sustained ‘normal’ vessel.
Apart from soluble stimuli, such as VEGF and PDGF (platelet derived growth factor), mechanical forces, due to cyclic deformation of blood vessels and hemodynamic forces, are known to induce directional capillary growth and vascular remodeling in vitro and in vivo (Ingber, 1992; Ingber, 2002; Mammoto et al., 2009; Mammoto et al., 2008; Thodeti et al., 2009). Exogenous force stimulation such as matrix stiffening, enhances cytoskeletal actin-myosin contractility by activating Rho and its downstream target ROCK, which induces integrin clustering and focal adhesion assembly, that drives cell spreading and growth (Burridge and Wennerberg, 2004; Wozniak et al., 2003). Indeed, in tumors which are known to have stiffer matrices, basal Rho-activity is found to be higher, while the destabilization of adherens junctions by ROCK, has shown to promote tumor angiogenesis and invasiveness (Croft et al., 2004; Fritz et al., 1999). The mechanical properties of the ECM therefore have enormous implications in the angiogenic process and the exposure of EC to an atypical mechanical environment within the tumor has been suggested to be a causative factor of the aberrant nature of tumor vessels.

In addition to integrin receptors, stretch-activated (SA) ion channels have been shown as mechanotransducers that can convert mechanical forces into ion gradients at the cell membrane (Lansman et al., 1987; Matthews et al., 2006). These channels were shown to be activated upon EC exposure to cyclic stretch and the resulting calcium influx response through these channels were found to regulate cell shape and reorientation (Naruse et al., 1998). Recently, we identified TRPV4 channels as the specific SA channel, in EC, that are activated
when stress-induced force is applied on the integrin-ECM adhesions. The resulting influx of Ca$^{2+}$ was demonstrated to activate phosphatidylinositol 3-kinase (PI3K) dependent activation of additional β1 integrins which may then target downstream molecules such as Rho and Rac that is necessary for the reorientation response of EC. The importance of TRPV4 as a mechanosensor in EC was further validated when the siRNA-mediated knockdown of TRPV4, in NEC, resulted in the failure of these cells to reorient perpendicular to the direction of applied cyclic strain. In addition, TEC exhibit aberrant mechanosensitivity to ECM stiffness and undergo abnormal angiogenesis \textit{in vitro} that may be partly attributed to high basal active-Rho levels found in TEC (Ghosh et al., 2008; Thodeti et al., 2009). However, the upstream signaling molecule or the molecular mechanism(s) governing high Rho-mediated aberrant mechanosensitivity in TEC remains unknown.

Although tumor angiogenesis has been fundamentally studied as a growth factor dependent process, the mechanical regulation of this process remains to be studied. Here in this chapter, we determine if an alteration in TRPV4-dependent mechanosensing may be responsible for aberrant TEC mechanosensitivity and abnormal angiogenesis exhibited by TEC. We also explored whether modulation of TRPV4 activity can normalize TEC dysfunction and abnormal vasculature which improves cancer therapy.
Results

**TEC exhibit low levels of functionally active TRPV4 channels**

Our lab has previously (Thodeti et al., 2009) shown that mechanical force induced reorientation of EC is dependent on TRPV4 channels, therefore we investigated whether these channels contribute to the abnormal mechanosensitivity of TEC (Ghosh et al., 2008). We first compared the TRPV4 expression levels between normal and tumor EC using Western blot analysis. While NEC exhibited strong expression of TRPV4, (Adapala et al., 2011; Thodeti et al., 2009) it was significantly lower in TEC (Fig. 3.1 A). Further, we confirmed the reduced expression of TRPV4 channels in TEC via immunocytochemistry and qPCR analysis (Fig. 3.1. B,C). To determine the functional implications of lower TRPV4 expression in TEC, we measured Ca²⁺ influx in Fluo-4/AM loaded cells in response to the addition of specific TRPV4 activator, GSK1016790A and 4-α-PDD (figure not shown). By using calcium imaging, patch-clamp, siRNA knockdown and TRPV4 null cells, we have previously demonstrated that these compounds specifically activate TRPV4-mediated calcium influx in EC (Adapala et al., 2011; Thodeti et al., 2009). Here, we found that GSK1016790A (100 nM) induced Ca²⁺ influx in both NEC and TEC, but the influx in TEC was decreased by almost 40-50% compared to NEC ($p\leq0.001$) (Fig. 3.1. D). These results demonstrate that TRPV4 expression and function are reduced in TEC.
Figure 3.1. TRPV4 expression and functional activity is decreased in TEC. 
A) Western blot analysis of TRPV4 expression in NEC and TEC. Quantitative analysis of the Western blots showing significant (p≤0.05) reduction in TRPV4 expression in TEC. 
B) Representative immunofluorescence images (40X) showing reduced expression of TRPV4 in TEC. 
C) Relative gene expression analysis showing significantly (p≤0.01) reduced expression of TRPV4 in TEC. 
D) Representative traces showing relative changes in cytosolic calcium in response to a selective TRPV4 agonist, GSK1016790A (100 nM) in Fluo-4 loaded NEC and TEC (n=300). Arrow denotes the time when the cells were stimulated with the TRPV4 agonist. Quantitative analysis of cytosolic calcium influx induced by GSK1016790A in NEC and TEC. (F/F0 = ratio of normalized Fluo-4 fluorescence intensity relative to time 0). The results shown are mean ± SEM from 3 independent experiments. The significance was set at p≤0.05.
**TRPV4 overexpression restores mechanosensitivity towards substrate stiffness**

The above findings suggest that lower levels of TRPV4, a known mechanosensor in EC, may contribute to the previously reported aberrant mechanosensitivity of TEC in response to cyclic strain and substrate elasticity (Ghosh et al., 2008; Liedtke, 2005; Thodeti et al., 2009). Since tumors are often distinguished by a rigid and/or stiff ECM (Paszek et al., 2005), which can ultimately influence TEC spreading and tube formation, we investigated if overexpression of TRPV4 might rescue abnormal mechanosensitivity. To achieve this, we expressed a human TRPV4-EGFP construct in TEC; EGFP fluorescence revealed that more than 80% cells were transfected with TRPV4-EGFP (Fig. 3.2 A). Overexpression of TRPV4 increased GSK-induced calcium influx in TEC by almost 4-fold \((p \leq 0.01)\) compared to EGFP-alone transfected cells (Fig. 3.2 B). Next, we compared the degree of cell spreading between TRPV4-overexpressing TEC to control EGFP-expressing cells by plating them on transglutaminase linked gelatin of varying stiffness substrates (370 and 2278 Pa) for 6h (Fig. 3.2 C). Previously, NEC and TEC spread similarly on low stiffness (98 Pa) gels, however, while spreading of NEC reached optimal levels on the intermediate stiffness gel (370 Pa), TEC continued to spread on the stiffest substrates (Ghosh et al., 2008). Interestingly, in the present study, TEC expressing TRPV4-EGFP exhibited reduced spreading on gels of intermediate and high stiffness, suggesting that TRPV4 overexpression restored substrate mechanosensitivity in these cells (Fig. 3.2 C). In contrast, NEC-EGFP spread maximally at 370 Pa and reached a plateau at
2278 Pa while TEC-EGFP increased their degree of spreading with increasing gel stiffness (Fig. 3.2 C).
Figure 3.2. TRPV4 overexpression increases calcium influx and restores TEC mechanosensitivity to ECM elasticity. A) Representative phase contrast and fluorescence images showing the expression of TRPV4-EGFP in TEC. B) Representative traces showing increases in the cytosolic calcium in response to a selective TRPV4 agonist, GSK1016790A (100nM) in Fluo-4 loaded TEC overexpressing TRPV4-EGFP or EGFP alone. Arrow denotes the time when the cells were stimulated with the TRPV4 agonist. Quantitative analysis of the relative changes in the GSK1016790A-induced cytosolic calcium influx. The results shown are mean ± SEM from 3 independent experiments. The significance was set at p≤0.05. C) Representative cell spreading images of EGFP-transfected NEC, TEC and TRPV4-overexpressing TEC at 6h. Quantitative analysis of projected cell areas of TEC (expressing EGFP alone) and TEC+TRPV4 (expressing TRPV4-EGFP) on gelatin gels of intermediate and high stiffness (370-2278 Pa). The results shown are mean ± SEM from 3 independent experiments. The significance was set at p≤0.05.
**TRPV4 overexpression or pharmacological activation normalizes abnormal angiogenesis in vitro via modulation of Rho activity.**

Since TRPV4 overexpression restored TEC mechanosensitivity to ECM stiffness, we explored whether TRPV4 expression normalizes vessel formation *in vitro* and if so, further determine the molecular mechanisms that underlie this process. First, TEC expressing TRPV4-EGFP or EGFP alone were tested for their ability to form capillary networks using a Matrigel based *in vitro* 2D and 3D angiogenesis assays. We have previously shown that TEC form robust tubes when plated at low density (2 x 10^4 cells/ well) on Matrigel (2D) but at high density (8 x 10^4 cells/ well), these cells form tubes and then undergo multicellular retraction with disruption of tubular networks (Ghosh et al., 2008). Subsequently, it was demonstrated that this could be due to high contractile/tensional forces that are triggered by high basal Rho activity (Paszek et al., 2005). To determine if TRPV4 overexpression (which reduced high Rho activity) could normalize abnormal tube formation by TEC, we plated TEC expressing EGFP alone or overexpressing TRPV4 (expressing TRPV4-EGFP) at low and high density (2 and 8 x 10^4 cells/ well) for 2D assays. As expected, TEC expressing EGFP alone formed tubes at lower density (2 x 10^4 cells/ well) but these formed tubes that collapsed at high density (8 x 10^4 cells/ well) (Fig. 3.3 A) while TEC overexpressing TRPV4 formed a robust tubular network. Interestingly, overexpression of TRPV4 in NEC did not change tube formation in 2D. To examine if pharmacological activation of TRPV4 also normalizes abnormal tube formation *in vitro*, we activated TRPV4 in TEC using GSK (100 nM). We found
that, similar to TRPV4 overexpression, TEC treated with GSK also formed robust tubes at high plating density ($8 \times 10^4$ cells/well) (Fig. 3.3 B). These results demonstrate that TRPV4 overexpression or pharmacological activation by GSK normalizes abnormal angiogenesis exhibited by TEC through the restoration of mechanosensitivity towards ECM stiffness and thus identifies TRPV4 as a critical determinant of vessel formation and patterning.

To understand the molecular mechanism downstream of TRPV4 that mediates normalization of angiogenesis, we focused on Rho which regulates endothelial contraction that is required for partial rounding of endothelial cells during tube formation (Wojciak-Stothard and Ridley, 2003). Our results confirmed previous findings by Ghosh et al. (2008) that TEC express high basal Rho activity; upon overexpression of TRPV4, there was a significant reduction in Rho activity compared to EGFP-expressing control TEC (approximately $\frac{1}{2}$-fold lower; $p \leq 0.001$), suggesting that TRPV4 is a critical modulator of Rho activity (Fig. 3.4 A). Next, we examined if pharmacological activation of TRPV4 with GSK1016790A also inhibits Rho activity in TEC. Similar to our overexpression studies, we found a decrease in active-Rho levels in TEC treated with GSK (Fig. 3.4 B).
Figure 3.3. TRPV4 overexpression or activation normalizes TEC angiogenesis in vitro. Phase contrast micrographs showing the normalizing effects of TRPV4 overexpression (A) or pharmacological activation with GSK1016790A (100 nM) (B) on TEC angiogenic behavior when plated on 2D Matrigel (at high densities; 8 × 10⁴ cells/ well that cause collapse of tubular networks). NEC-EGFP (NEC), TEC-EGFP (TEC) and TEC-TRPV4-EGFP (TEC+TRPV4) cells were plated and cultured on the surface of Matrigel (2D). Bar graph in (A) shows that overexpression of TRPV4 in TEC restored tube formation on 2D Matrigel. The results shown are mean ± SEM from 3 independent experiments. The significance was set at p≤0.05.
Figure 3.4. TRPV4 overexpression or pharmacological activation decreases abnormal Rho activity in TEC. A) Representative Western blot showing the levels of active-Rho and total Rho for TEC and TEC+TRPV4. Rho activity was analyzed in TEC (EGFP) and TEC+TRPV4 cells cultured under regular growth conditions using the Rhotekin-RBD binding assay. Densitometric analysis of relative changes in Rho activity. Rho activity levels were measured by normalizing the levels of active Rho with that of total Rho. B) Representative Western blot showing the levels of active-Rho and total Rho for TEC and TEC treated with TRPV4 activator GSK1016790A. Densitometric analysis of changes in Rho activity which was quantified by normalizing the levels of active Rho with that of total Rho. The results shown are mean ± SEM from 3 independent experiments. The significance was set at p≤0.05.
**TRPV4 activation with GSK1016790A normalizes tumor vasculature in vivo and reduces tumor growth in conjunction with Cisplatin**

Finally, we explored if pharmacological activation of TRPV4 induces tumor vascular normalization and/or maturation *in vivo* and improves delivery of the chemotherapeutic drug, Cisplatin. To achieve this, we injected TRPV4 activator GSK1016790A intraperitoneally (i.p.) every day for 14 days in WT mice that had developed palpable tumors (after 7 days). Cisplatin was given once per week starting 2-4 days after GSK treatment and tumor growth was monitored every week until day 21 (Fig. 3.5. A). First, we examined vessel maturity in tumors by staining for pericyte coverage. We observed that the vessels in GSK and GSK-Cisplatin-treated tumors, but not in control or Cisplatin treated ones, showed increased pericyte coverage (Fig. 3.5 B) suggesting that TRPV4 activation normalized tumor angiogenesis and induced vessel maturation which may help efficient delivery of Cisplatin. Consistent with this observation, we found that tumor growth was markedly reduced in GSK-Cisplatin treated animals (Fig. 3.6), but not in control or Cisplatin treated mice, suggesting the improved delivery of Cisplatin due to the normalization of vessels by TRPV4 activation.
Figure 3.5. Activation of TRPV4 with GSK1016790A restores pericyte coverage and vessel maturity in WT tumors. A) Schematic of treatment in WT (C57BL/6) mice injected with syngeneic tumors (LLC), injected in the flank region of WT mice. Tumor growth was monitored for 21 days. TRPV4 activator, GSK1016790A (GSK) was injected (i.p.) everyday starting from day 7 (when tumors were palpable) until day 21. Cisplatin was injected i.p. (once/week) 3 days after the injection of GSK1016790A. B) Frozen sections of tumors (10 μm thickness; from 21 day) were stained with CD31 (PECAM-1; green) and α-SMA (red) to measure pericyte coverage (matured vessels). Quantitative analysis of pericyte covered microvessels in tumors from control, Cisplatin, GSK and GSK+ Cisplatin treated mice. The results shown are mean ± SEM from 3 independent experiments. The significance was set at p≤ 0.05.
A

Inject LLC cells (2×10^6/100 μl)

GSK (10 μg/kg/day)

Cisplatin (3 mg/kg/week)

B

Control | GSK | Cis | GSK+ Cis

| CD31 | | | |

| α-SMA | | | |

| CD31/α-SMA | | | |

% Pericyte Coverage

CON | GSK | CIS | CIS+GSK
Figure 3.6. TRPV4 activation with a specific agonist GSK1016790A in conjunction with Cisplatin reduces tumor growth \textit{in vivo}. Tumor volumes between mice groups. Tumor growth was reduced in GSK + Cisplatin (\*) treated mice, however, treatment with either of drug alone did not inhibit tumor growth, indicating that GSK treatment improved Cisplatin delivery possibly through the normalization of the abnormal tumor vasculature. The data shown are ± SEM of three independent experiments (n=8-10 mice/group) and significance was set at p≤0.05.
Discussion

Although growth factors are critical for angiogenesis, emerging studies have proposed that in an environment saturated with these factors, mechanical forces may drive capillary EC responses to angiogenic mitogens (Ingber, 1992; Ingber, 2002; Mammoto et al., 2009; Mammoto et al., 2008; Thodeti et al., 2009). In the current study, we have demonstrated, for the first time, that TEC express lower levels of the mechanosensitive ion channel TRPV4, a key upstream signaling molecule that regulates TEC mechanosensitivity and tumor angiogenesis in vitro. Importantly, we demonstrate that overexpression of TRPV4 normalizes aberrant TEC mechanosensitivity and angiogenesis through modulation of Rho activity. Further, we show that in the presence of a specific TRPV4 activator, GSK1016790A (GSK), TEC mechanosensitivity is restored on high stiffness substrate compared to the untreated controls. Finally, we demonstrate that administration of the specific small molecule TRPV4 activator, GSK1016790A induced vessel maturation and, in combination with a chemotherapeutic drug, Cisplatin, reduced tumor growth in WT mice.

Endothelial cells are constantly exposed to hemodynamic force stimulation such as shear stress and cyclic strain. The resulting mechanotransduction promotes EC reorientation, either parallel or perpendicular to the applied stress (Matthews et al., 2010; Thodeti et al., 2009; Tzima et al.,
In this regard we and others have shown that EC express functional TRPV4 channels, and act as mechanosensors of flow and mechanical strain (Baylie and Brayden, 2011; Hartmannsgruber et al., 2007; Liedtke, 2005; Matthews et al., 2010; Mendoza et al., 2010; Thodeti et al., 2009). In bovine capillary endothelial cells (BCE), strain application on ECM-integrin adhesions triggers the influx of Ca\textsuperscript{2+} into the cell by activating TRPV4. This Ca\textsuperscript{2+} influx was found to be necessary to induce cytoskeletal remodeling, directional cell migration and reorientation (Thodeti et al., 2009). However, within the tumor, the abnormal vasculature promotes turbulent blood flow and extravascular leakage of plasma proteins, resulting in increased matrix deposition (matrix rigidity) and creating an abnormal mechanical microenvironment. Previous work by Ghosh and colleagues (2008) found that TEC exhibit aberrant mechanosensitivity i.e. they not only fail to reorient to cyclic strain, but also exhibit enhanced spreading with increase in substrate rigidity. This prompted us to explore TRPV4 expression levels between NEC and TEC. Our results showed TEC express low levels of functionally active TRPV4 channels, implicating an alteration in TRPV4-dependent signaling in TEC may be the underlying reason that these cells exhibit aberrant mechanosensitivity towards substrate stiffness.

Variations in ECM stiffness have shown to alter cell form and function by modulating cell shape, and this has been indicated to be an indirect measure of how well cells sense and respond to changes in ECM elasticity (Chicurel et al., 1998). In this study, we prepared gelatin gels of ~2kPa, to mimic the stiffness of tumor stroma (Paszek et al., 2005), in order to determine EC spreading.
Previously, studies have demonstrated that in tumor epithelial cells (Paszek and Weaver, 2004), an increase in ECM stiffness feeds back to enhance integrin-mediated Rho/ROCK activity and cell contraction. Our current findings demonstrate that TEC exhibit increased cell spreading with increasing ECM stiffness (370-2278 Pa), compared to NEC. This enhanced spreading was inhibited when TRPV4 was overexpressed in TEC, suggesting that TRPV4 is an important molecule that regulates mechanosensitivity. Our present and past studies have shown that TEC exhibits high basal Rho-activity that contributes, in part, to their inability to respond correctly to mechanical strain and to exhibit abnormal angiogenesis in vitro (Ghosh et al., 2008). However, the governing molecular mechanism upstream of Rho is not known. Here, we demonstrate that TRPV4 overexpression and pharmacological activation significantly inhibited high basal Rho activity in TEC and normalized tube formation in 2D Matrigel assays. This reduction in basal Rho activity could possibly inhibit the high basal contraction of TEC, which may restore mechanosensitivity of these cells towards substrate stiffness and normalization of angiogenesis in vitro. In contrast to the present work, a recently published study showed EC derived from breast tumors express significantly higher TRPV4 levels compared to normal human microvascular endothelial (HMVE) cells (Fiorio Pla et al., 2012). However, this work did not examine the role of TRPV4 in angiogenesis in vitro or in vivo. Recent pre-clinical and clinical evidence has demonstrated that, anti-VEGF drugs as well as many other direct and indirect angiogenesis inhibitors can transiently promote the normalization of the tumor vasculature. Although VEGF is required
for normal proliferation and migration of EC, it is important to emphasize that the tumor vasculature in certain cancers is largely refractory to therapies designed to alter VEGF signaling, as growth factors are redundant for angiogenesis (Ellis and Hicklin, 2008). Nevertheless, current vascular normalization therapies still focus on targeting soluble growth factors such as VEGF and PDGF. Our findings show that the combination of GSK and Cisplatin, but not each them alone, inhibited tumor growth, suggesting that TRPV4 activation induced vascular maturation improved the efficacy of Cisplatin.

Our present study therefore demonstrates TRPV4 to play a critical role in the regulation of tumor angiogenesis and tumor vessel maturation, possibly through modulation of Rho-dependent mechanosensitivity of TEC. Our finding also identifies TRPV4 as a novel mechanotransduction based therapeutic target for vascular normalization in cancer and other angiogenic disorders such as age-related macular degeneration and diabetic retinopathy.
CHAPTER IV

TRPV4 Channels Regulate TEC Proliferation via Modulation of ERK1/2

Introduction

The proliferation of EC is a critical event in angiogenesis, regulated by both soluble factors and mechanical forces. Little is understood about the proliferation of TEC in tumor angiogenesis, although these events have been extensively studied in tumor cells. While EC require soluble factors such as VEGF, they are also dependent on mechanical and hemodynamic forces generated by the ECM and blood flow respectively, for organized growth and differentiation (Heil and Schaper, 2004; Huang and Ingber, 1999; Ingber and Folkman, 1989). Matrix rigidity has been shown to influence cellular behavior, and cells grown on rigid matrices (such as within a tumor), in comparison to softer matrices, can trigger proliferative and survival pathways through alterations in gene expression (Paszek et al., 2005; Provenzano et al., 2009; Wozniak et al., 2003). Several labs have reported EC isolated from different tumors display
unique genetic markers as well as exhibit structural and functional characteristics not typical of EC that make up the normal blood vessel (Ghilardi et al., 2008; Wu et al., 2008). In fact, TEC that make up the tumor vasculature, fail to sense these mechanical cues accurately, resulting in malformed blood vessels (Ghosh et al., 2008). Thus, EC isolated from tumors exhibit abnormal structural and functional characteristics and therefore requires anti-angiogenic therapies that are specifically designed to target EC in tumors, but not normal endothelial cells such as HUVECs.

Although not largely pursued as a potential anti-angiogenic strategy, the ubiquitous but critical role of intracellular calcium has been demonstrated in the regulation of angiogenesis, including cell proliferation, survival and migration (Monteith et al., 2012; Roderick and Cook, 2008). Two potent pro-angiogenic molecules, VEGF and bFGF are known to stimulate Ca^{2+} permeable channels on the EC plasma membrane, including members of the TRP family (Koch et al., 2011). Previously, we have shown that TRPV4 channels are activated by cyclic strain and mediate EC reorientation, via activation of integrin to integrin signaling (Thodeti et al., 2009). We have also shown TEC to exhibit high ERK1/2 activity, although the underlying cause and downstream signaling were not elucidated in this study (Ghosh et al., 2008). Another important event that takes place during neovascularization pertains to the directional migration of capillary EC towards angiogenic stimuli and TRP channels have been previously implicated in EC migration (Fiorio Pla and Gkika, 2013; Naruse et al., 1998). In this regard, our lab has shown TRPV4 to be involved in EC reorientation and cytoskeletal
remodeling, a process necessary for cell migration and tube formation (Thodeti et al., 2009). Our latest findings have shown that TEC express reduced levels of TRPV4 compared to NEC and exhibit abnormal Rho-dependent mechanosensing and angiogenesis, which were restored upon overexpression or pharmacological activation of TRPV4. However, it is not known if TRPV4 expression also modulates key events in angiogenesis such as EC proliferation.

In this chapter, we investigated how decreased functional activity and expression of TRPV4 affect TEC proliferation, an important process in angiogenesis. Since members of the mitogen-activated protein kinase (MAPK/ERK) family are critical regulators of cell proliferation, and mechanical signaling is known to stimulate ERK1/2 (Ikeda et al., 1999; Traub and Berk, 1998; Xu et al., 1996; Yamazaki et al., 1993), we investigated if TRPV4 regulates TEC proliferation via modulation of the ERK1/2 pathway.
Results

TEC exhibit increased proliferation levels compared to NEC

The cellular and molecular aberrations of TEC can invariably contribute to the abnormal angiogenic process and ultimately tumor growth (Allport and Weissleder, 2003; Hida et al., 2004; Hida and Klagsbrun, 2005). As the growth of EC is a critical event in angiogenesis, we explored if proliferation levels are altered in TEC compared to normal endothelial cells (NEC), which may be a contributing factor in the abnormal structure of tumor vessels. Our results showed that in comparison to normal EC, tumor EC exhibit increased protein levels of proliferating cell nuclear antigen (PCNA) with quantitative analysis data revealing a significant (p≤0.05) upregulation of PCNA expression in TEC (Fig. 4.1 A). We then measured Bromodeoxyuridine (BrdU) incorporation into EC which unequivocally confirms cell proliferation. We found that TEC showed significantly (p≤0.05) higher numbers of BrdU positive cells compared to NEC, further confirming that cell proliferation is higher in TEC (Fig. 4.1 B).
Figure 4.1. TEC exhibit increased proliferation levels  

A) Western blot analysis of PCNA expression in normal (NEC) and tumor endothelial cells (TEC). Quantitative analysis of the Western blots showing significant (p≤0.05) increase in PCNA expression in TEC. 

B) Representative images showing nuclear colocalization of BrdU and DAPI in NEC and TEC (n≥1000). Quantitative analysis of BrdU positive cells in NEC and TEC. The results shown are mean ± SEM from 3 independent experiments. The significance was set at p≤0.05.
**TRPV4 overexpression or pharmacological activation by GSK1016790A decreases TEC proliferation**

The growth-factor induced influx of \([Ca^{2+}]\) or \([Mg^{2+}]\) through TRP channels as well as the differential expression of these channels in various cancers have been implicated in tumor progression [reviewed by Fiorio Pla and Gkika (2013)]. In certain cases, this disparity in TRP channel expression have been shown to mediate EC proliferation, enhance vascular permeability and boost apoptosis evasion, thereby promoting angiogenesis [Yao and Garland, 2005; Pocock et al. 2004; Antioniotti et al. 2002; Zhang et al. 2006; Cheng et al. 2006]. Since TEC express low levels of functional TRPV4, we investigated if these altered levels of TRPV4 may be involved in the increased proliferation of TEC during tumor angiogenesis. To determine this, we examined if pharmacological activation or overexpression of TRPV4 modulates the increased TEC proliferation. TRPV4 activation using a small molecule activator GSK1016790A (GSK; 100nM) markedly decreased TEC proliferation, as evidenced by the reduced incorporation of BrdU in TEC, compared to untreated controls (Fig. 4.2 A). Further, when proliferation was measured using the fluorophore Calcein, GSK treatment reduced TEC proliferation in a concentration dependent manner. In contrast, GSK treatment did not inhibit proliferation of NEC, indicating the specific anti-proliferative effect of GSK on TEC (Fig. 4.2 B). Furthermore, we found that this reduction in TEC proliferation was also correlated with reduced PCNA protein expression (Fig. 4.2 C). Finally, overexpression of TRPV4 in TEC but not in NEC, significantly reduced PCNA protein levels confirming that TRPV4
regulates TEC proliferation (Fig. 4.2 D). Together, our results demonstrate TEC proliferate at higher rate than NEC and that TRPV4 activation or overexpression decreases this abnormal proliferation of TEC.
Figure 4.2. TRPV4 activation or overexpression decreases abnormal TEC proliferation

A) Representative images showing nuclear colocalization of BrdU and DAPI in control and GSK-treated TEC. Quantitative analysis indicating significant decrease in BrdU expression in TEC post treatment with GSK. The significance was set at p≤0.05, (n>1000).

B) Representative graph showing absolute changes in calcein fluorescence (indicating live cells) in response to increasing concentrations of the selective TRPV4 agonist, GSK, in NEC and TEC (n=6). Significance was set at p≤0.05 and compares NEC to TEC at each concentration.

C) Western blot depicting PCNA protein expression in control and GSK-treated TEC. Quantitative analysis of the Western blots showing significant (p≤0.05) reduction in PCNA expression in TEC. The results shown are mean ± SEM from 3 independent experiments. Results are shown as mean ± SEM of 3 independent experiments. The significance was set at p≤0.05.

D) Western blot analysis of PCNA protein levels in TEC expressing EGFP alone (TEC) and TRPV4-EGFP (TEC+TRPV4). Quantitative analysis of the Western blots showing reduction of PCNA expression levels in TEC (n=1).
**TRPV4 regulates TEC proliferation via modulation of ERK1/2 but not AKT**

Since TRPV4 activation normalizes TEC proliferation, we next investigated the molecular mechanisms by which TRPV4 mediates these effects. We have previously showed that ERK1/2 and AKT are phosphorylated in response to mechanical stretch downstream of TRPV4 in EC. Here, we examined if TRPV4-mediated decrease in proliferation is dependent on modulating the ERK1/2 proliferation pathway and/or the AKT-PI3K survival pathway. Consistent with reports (Ghosh et al., 2008), we found significantly (p≤0.05) higher basal phosphorylation of ERK1/2 (active) in TEC when compared to NEC (Fig. 4.3 A). However, the total ERK1/2 levels were comparable in both NEC and TEC. Importantly, this upregulation of ERK1/2 phosphorylation was inhibited when TEC were treated with GSK; however active ERK1/2 levels remained unchanged in NEC after GSK treatment (Fig. 4.3 A). Further, overexpression of TRPV4 also reduced high basal ERK1/2 phosphorylation (Fig. 4.3 B). We next examined AKT phosphorylation in TEC, as many tumor cells exhibit increased survival rates and evade apoptosis possibly through mechanisms that upregulate PI3K-AKT [reviewed by Bergers and Hanahan (2008); (Hanahan and Weinberg, 2011)]. We found that basal AKT phosphorylation (p-AKT at ser-473) was lower in TEC than NEC (Fig. 4.3. C) Subsequently, we measured AKT phosphorylation in response to TRPV4 activator and found that GSK treatment markedly reduced p-AKT levels in NEC but slightly increased AKT phosphorylation in TEC (Fig. 4.3 C). These findings suggest that TRPV4 activation or overexpression reduces TEC proliferation via modulation of ERK1/2 but not AKT pathway.
Figure 4.3. Pharmacological activation of TRPV4 decreases TEC proliferation via modulation of ERK1/2 but not AKT pathway

A) Representative Western blots showing ERK1/2 phosphorylation in control and GSK1016790A treated NEC and TEC. Densitometric analysis of the Western blots showing decrease in ERK1/2 phosphorylation in TEC treated with GSK. Significance was set at (p≤0.05) (n=5). B) Representative western blots showing phospho- and total-ERK1/2 protein levels in TEC expressing EGFP (TEC) or overexpressing TRPV4-EGFP (TEC+TRPV4). Densitometric analysis revealing significant (p<0.05) decrease in ERK1/2 activity in TEC overexpressing TRPV4 (n=2). C) Representative Western blots depicting AKT phosphorylation in NEC and TEC treated or untreated with GSK (100 nM). Densitometric analysis of the Western blots showing changes in AKT phosphorylation (n=5). Results are shown as mean ± SEM of 5 independent experiments. The significance was set at p≤0.05.
**Increased TEC proliferation is associated with enhanced expression of cell cycle genes**

The activation of the ERK signaling pathway as well as the ECM/integrin/receptor tyrosine kinase signaling has been demonstrated to induce cyclin D1 mRNA expression thereby allowing entry of cells into S-phase [reviewed by (Roovers and Assoian, 2000)]. We therefore measured gene expression levels of several cell cycle regulators (G1 and S phase) to determine if increased ERK1/2 phosphorylation in TEC (due to decreased TRPV4 expression) may induce upregulation of these genes. Our results revealed that when compared to NEC, there was a significant (p≤0.05) increase in mRNA expression levels of several G1-S phase genes including cyclin A, D1, E1 and cyclin-dependent kinases (CDK) 1 and 6 (Fig. 4.4). These results suggest that TRPV4 deficiency in TEC may increase cell proliferation via ERK1/2 dependent modulation of cell cycle genes.
Figure 4.4. TEC express increased levels of proliferation-associated genes. Relative gene expression of cell-cycle associated genes in NEC and TEC. The data shown are mean ± SEM of 3 independent experiments. The significance (*) was set at p≤0.05.
Pharmacological activation of TRPV4 attenuated TEC proliferation in tumors in vivo.

Finally, to determine the functional significance of TRPV4 in the regulation TEC proliferation in vivo, we used frozen sections from tumors harvested from WT and GSK treated mice (described in Chapter III), to measure TEC proliferation. The proliferation of EC within the tumors was determined by means of immunohistochemical staining with a) the specific EC marker CD31 (red) and b) the proliferation marker ki-67 (green) and c) nuclei marker DAPI (blue). The proliferating TEC were assigned by visualizing the co-localization (yellow) of these two markers (Fig. 4.5 A). Consistent with our findings on tumor growth and angiogenesis (Chapter III), we found increased proliferation of TEC in WT control tumors. However, we found a marked reduction in TEC proliferation in the tumors that were treated with the specific TRPV4 activator GSK1016790A. Quantitative analysis revealed a significant reduction in the number of proliferating TEC in tumors from GSK-treated mice (WT-GSK) compared to tumors from saline injected control mice (WT-Control). Together, these results provide strong evidence that TRPV4 regulates tumor angiogenesis and growth via modulation of TEC proliferation.
Figure 4.5. Pharmacological activation of TRPV4 inhibits TEC proliferation in vivo. A) Mouse Lewis Lung Carcinoma (LLC) cells \( (2 \times 10^6) \) were subcutaneously injected into wild type C57BL/6J mice (WT). TRPV4 activator, GSK1016790A was injected (i.p.) to WT animals alone, everyday starting from day 7 (after palpable tumors were observed) until 21 days as described in Chapter III. Representative images (20x) show the immunohistochemical analysis of frozen sections of tumors (10 µm thickness) obtained from the WT mice that are treated with saline (WT-control) or GSK1016790A (WT-GSK); sections were stained with CD31 (red), ki-67 (green) to quantify the proliferation of TEC. Arrows indicate proliferating TEC. Quantitative analysis demonstrating significant \( (p<0.05) \) decrease in the percentage of proliferating TEC in tumors treated with GSK1016790A compared to control tumors. The data shown are mean ± SEM of 3 independent experiments. The significance (*) was set at \( p \leq 0.05 \).
Figure 4.6. Proposed Mechanism by which TRPV4 regulates TEC proliferation. TEC exhibit high basal levels of active ERK1/2, decreased phosphorylation (ser473) of the survival protein AKT and upregulation of several cell cycle genes. TRPV4 activation via specific small molecule activator GSK demonstrated no change in levels of AKT phosphorylation but inhibited high active ERK1/2 leading to reduced TEC proliferation and decreased angiogenesis. This decrease in abnormal TEC proliferation may be through mechanisms that involve modulating the expression of cell cycle genes.
Discussion

In the present study, we demonstrate that the altered levels of the mechanosensitive ion channel TRPV4 induces increased proliferation of TEC, which may lead to abnormal tumor angiogenesis. Specifically, we show the pharmacological activation of TRPV4 decreases TEC proliferation in vitro and in vivo. The molecular mechanism behind this abnormal proliferation in TEC, we found to be mediated via ERK1/2 phosphorylation which is attenuated by pharmacological activation or overexpression of TRPV4. Finally, we demonstrate that TEC proliferation is higher in tumors from in WT mice which were significantly inhibited by treatment with a small molecule activator of TRPV4, GSK1016790A.

Several studies have indicated different TRP channels, specifically TRPC1, -C6, -C3, -M6 and -M7 to influence the angiogenic process through multiple mechanisms [reviewed by (Antoniotti et al., 2002; Fiorio Pla and Gkika, 2013; Yao and Garland, 2005)]. A few of the mechanisms pertain to mitogenic factors activating these TRP channels to stimulate calcium influx or vice versa. Although these findings offer information for the substantial contribution of these channels to angiogenesis, there is no direct evidence linking endothelial TRP channels to stimulate or suppress tumor angiogenesis or TEC growth and function. Previously, we demonstrated for the first time, mechanosensitive TRPV4 channels regulate tumor angiogenesis by modulating Rho-dependent EC
mechanosensitivity. Specifically, we have shown that the pharmacological activation of TRPV4 with GSK1016790A normalizes tumor vasculature and in combination with Cisplatin reduces tumor growth in WT mice and Chapter III].

TRPV4 has been previously shown to increase normal EC proliferation *in vitro* and in response to shear stress *in vivo* (Troidl et al., 2010; Troidl et al., 2009). Further, Hatano et al. (2013) demonstrated TRPV4 activation with GSK and 4-α-PDD induces hyper proliferation of human brain EC. However, there are no studies on the role of TRP channels in general and TRPV4 in specific on TEC proliferation *in vitro* or *in vivo*.

In the previous chapter we have shown that the low levels of functional TRPV4 leads to abnormal mechanosensing in TEC, resulting in enhanced cell spreading on high stiffness ECM substrates. It has been shown that the ECM plays a pivotal role in regulation of cell proliferation; controlling cell shape or cell spreading which is a critical modulator of EC proliferation and cell cycle progression. An increase in cell spreading was shown to promote the increased progression of EC from G1 into the S phase by upregulating cyclin D1 gene expression (Assoian and Zhu, 1997; Chen et al., 1994; Dike and Ingber, 1996; Huang et al., 1998; Pardee, 1989). The activation of the MAPK (ERK) pathway, is usually associated with the induction of cyclin D mRNA expression. Interestingly, an early report from Ingber’s lab (Huang et al., 1998) has shown that in addition to ERK activation and integrin signaling, cell cycle progression and ultimately cell proliferation relies on ‘tension-dependent changes in cell shape and cytoskeletal structure’. TRPV4, in this regard, has been shown to be
an important EC mechanosensor that is important for reorientation, EC spreading and cytoskeletal remodeling (Adapala et al., 2015; Thodeti et al., 2009) and therefore represents a novel regulator of TEC proliferation. Indeed, our results have shown TEC exhibit increased proliferation, as evidenced by increased levels of the S phase protein, PCNA. In tumors, we saw the increased co-localization of the proliferative marker ki-67 and endothelial marker CD31, confirming the increased growth of TEC in tumors, while TRPV4 activation with GSK inhibited this enhanced proliferation. Additionally, TEC also exhibited increased ERK1/2 activity which induced the downstream expression of several G1 and S phase regulatory genes, including cyclins A, D1, E1 and their associated kinases CDK 1 and 6. Taken together, our data confirms significant role for the mechanosensitive TRPV4 channel in regulating TEC proliferation via modulation of the ERK pathway (Fig. 4.6).

We have demonstrated that pharmacological activation of TRPV4 induces vascular normalization in tumors by modulating Rho activity which improved chemotherapy and reduce tumor growth (Adapala et al., 2015). Our current study presents an additional role for the mechanosensitive ion channel TRPV4 in the proliferation of TEC, which is a key event in tumor angiogenesis. Understanding the functional significance and molecular signaling behind TRPV4 dependent regulation of TEC proliferation and tumor angiogenesis, can provide a novel avenue for therapeutic intervention.
CHAPTER V

TRPV4 is a Negative Regulator of Angiogenesis

Introduction

Although blood vessel formation can occur de novo by means of vasculogenesis (Djonov et al., 2000), this process only leads to the development of a rudimentary and inadequate vascular structure. In the adult human, angiogenesis constitutes the main process by which blood vessels are established. Angiogenesis is required for normal function of the cardiovascular system; however, excessive or insufficient angiogenesis can lead to cardiovascular diseases. Therefore, angiogenesis can be an ideal target for therapeutic intervention in a variety of vascular diseases, including angiogenesis-dependent cancers. Despite the complexities associated with the angiogenic process, significant progress has been made to identify numerous growth-factor dependent (specifically VEGF-dependent) signaling pathways that are involved in angiogenesis, leading to the development of several anti-angiogenic drugs [reviewed by (Quesada et al., 2006)]. However, challenges linked with current anti-VEGF therapies (Shojaei and Ferrara, 2008), have lately resulted in the
exploration of new avenues in studying and treating angiogenesis. Recent advances, especially in the field of mechanotransduction, have brought to light the importance of mechanical forces in regulating the angiogenic process. Cell and ECM-generated mechanical stimuli have shown to be involved in complex, yet largely unidentified, signaling pathways that control several angiogenic processes including EC growth, migration and survival (Chen et al., 1997; Ingber, 1992; Ingber, 2002; Ingber and Folkman, 1989; Mammoto et al., 2009; Mammoto et al., 2008).

In recent findings, TRPV4 has been identified to be a mechanosensitive ion channel that may serve as a candidate involved in facilitating the mechanical regulation of angiogenesis. We and others have demonstrated that TRPV4 plays an important role in regulating normal EC physiology via mechanotransduction (Baylie and Brayden, 2011; Hartmanngrubber et al., 2007; Liedtke, 2005; Martinac, 2004; Matthews et al., 2010; Mendoza et al., 2010; Thodeti et al., 2009). Specifically, we have shown that TEC do not realign themselves when exposed to mechanical stimulation, a phenomenon akin to that observed in NEC when TRPV4 was knocked down (Thodeti et al., 2009). In addition to this important finding, TEC demonstrated impaired mechanosensitivity to matrix rigidity as well as exhibited increased proliferation, migration and abnormal angiogenesis in-vitro (Adapala et al., 2015; Ghosh et al., 2008). Our latest findings revealed these aberrant properties of TEC are attributed to decreased functional levels of TRPV4, which can be rescued by overexpression or activation of TRPV4. Further, we show that pharmacological activation of TRPV4
with specific agonist GSK1016790A can normalize the abnormal tumor vasculature, promoting efficient chemotherapeutic drug delivery to reduce tumor growth (Adapala et al., 2015). Taken together, these findings provide compelling evidence as to the functional significance of TRPV4 in the tumor vasculature.

TRPV4 deletion in mice has shown to induce hearing loss, diminished sensation to temperature, pressure and osmotic regulation (Liedtke and Friedman, 2003; Mizuno et al., 2003; Suzuki et al., 2003b; Tabuchi et al., 2005). Surprisingly, TRPV4KO mice do not exhibit any obvious cardiovascular phenotype. However, the absence of TRPV4 appears to impair ACh-induced endothelium-dependent vasodilation and angiotensin II (Ang II)-induced endothelial dysfunction in vivo (Adapala et al., 2011; Nishijima et al., 2014; Zhang et al., 2009). In the current study, we investigated the role of TRPV4 in physiological and pathological angiogenesis using EC isolated from TRPV4 KO mice in vitro and using Matrigel plug angiogenesis assay and tumor implantation, in vivo.
Results

*Tumor angiogenesis and tumor growth are enhanced in TRPV4KO mice*

In previous chapters, we have demonstrated that TEC express low levels of functional TRPV4 which imparts aberrant mechanosensitivity and abnormal angiogenesis *in vitro*. Therefore, to determine if TRPV4 expression levels contributes to tumor angiogenesis *in vivo*, we induced tumors in TRPV4 knockout (TRPV4KO) and wild-type (WT) mice (C57BL/6) by subcutaneously injecting mouse Lewis lung carcinoma cells (LLC). We found that tumor growth was 2-3 times greater in TRPV4KO mice compared to WT mice at day 21 (Fig. 5.1 A, B). Importantly, immunohistochemical analysis revealed that tumors in TRPV4KO mice exhibited a greater fraction of hyper-dilated (malformed) vessels with significantly larger vessel diameters ($p \leq 0.05$) (Fig. 5.2 A). In contrast to tumor vessels in WT mice, those in TRPV4KO mice exhibited poor pericyte coverage, as determined by very weak α-SMA staining, indicating the immature nature of these vessels (Fig. 5.2 B). Additionally, tumors from the KO mice displayed increased micro-vessel density, suggesting increased angiogenesis (Fig. 5.3 A). Taken together, these results clearly suggest that TRPV4 plays a critical role in modulating tumor angiogenesis and the absence of TRPV4 can lead to abnormal tumor angiogenesis (immature vessels), possibly through altered mechanotransduction exhibited by EC.
Figure 5.1 Tumor growth is increased in TRPV4KO mice A) Mouse Lewis lung carcinoma (LLC) cells (2 X 10^6) were subcutaneously injected into WT C57BL/6 mice and TRPV4KO with a C57BL/6 background; images are representative of tumors isolated from both WT and TRPV4KO mice. B) Time-dependent growth of the tumors in WT and TRPV4KO mice; tumor growth was measured using calipers at indicated days. The data shown are ± SEM of three independent experiments (n=8-10 mice/group) and significance was set at p≤0.05.
Figure 5.2. TRPV4KO tumors exhibit enhanced vessel malformations  

A) Immunohistochemical analysis showing increased vessel diameter in tumors (21 days) from TRPV4KO compared to WT. Quantitative analysis of microvessels diameter in tumors from WT and TRPV4KO mice. B) Frozen sections of tumors (10 µm thickness) were stained with CD31 (PECAM-1; green) and α-SMA (red) to measure pericyte coverage (matured vessels). Quantitative analysis of pericyte covered microvessels in tumors from WT and TRPV4KO mice. The results shown are mean ± SEM from 3 independent experiments. The significance was set at p≤0.05.
Figure 5.3. Microvessel density is increased in tumors from TRPV4KO mice. A) Frozen sections of tumors (10 µm thickness) were stained with EC marker- CD31 to identify tumor micro-vessels (20x). Quantitative analysis of vessel density showing significantly (p≤0.05) increased number of vessels in TRPV4KO tumors (n=3).
Physiological angiogenesis is increased in the absence of TRPV4

Next, we asked if TRPV4 expression also regulates physiological angiogenesis. To achieve this, we cultured aortic rings from WT and TRPV4KO mice ex vivo in Matrigel as previously described (Mahabeleshwar et al., 2006). Although we observed vascular sprouting from both explants at day 3, we found that aortic rings from the TRPV4KO animals produced notably more sprouts compared to the WT (Fig. 5.4 A). This vascular sprouting was significantly (p<0.05) higher at day 5 from the TRPV4KO aortic rings (Fig. 5.4 A), compared to WT aortic rings. These findings suggest that absence of TRPV4 may enhance angiogenesis under physiological conditions. To further confirm our findings, we used Matrigel plug assays to monitor angiogenesis in vivo. Matrigel supplemented with VEGF and FGF was injected subcutaneously into the lower abdominal region of WT or TRPV4KO mice. After two weeks, the plugs were removed and imaged. We found that Matrigel plugs from TRPV4KO mice showed a visible increase in vascular and red blood cell content compared to the plugs isolated from WT mice (Fig. 5.5 A). Further, immunohistochemical analysis revealed an increase in the CD31 immunostaining in the TRPV4KO plugs (Fig. 5.5 B). Taken together, these data confirms that absence of TRPV4 increases physiological angiogenesis.
Figure 5.4. Angiogenic sprouting from aortic rings isolated from WT and TRPV4KO mice. A) Representative images (4x) comparing angiogenic growth from aortic explants from WT and TRPV4KO mice. Quantitative analysis showing significantly (p≤0.05) increased vascular sprouting in aortic explants from TRPV4KO mice (n=7).
Figure 5.5. *In vivo* Matrigel plug angiogenesis assay showing increased angiogenesis in TRPV4KO mice A) Representative images (20x) showing angiogenic growth in Matrigel plugs, two weeks post implantation in WT and TRPV4KO mice (n=2). B) Frozen Matrigel sections (10 µm thickness) were stained with CD31 (green; EC marker) and DAPI (blue) to identify micro vessels.
Absence of TRPV4 enhances EC proliferation via ERK1/2

Since TRPV4 negatively influences physiological angiogenesis, we next investigated the molecular mechanism underlying enhanced angiogenesis. First, we explored if the absence of TRPV4 increases EC proliferation, similar to that found in TRPV4 deficient TEC. To determine this, we isolated EC from vascular sprouts originating from aortic explants of WT and TRPV4KO mice cultured in Matrigel (Fig. 5.6). The EC were cultured in defined media and characterized by measuring the expression of endothelial markers, smooth muscle markers and TRPV4 using qPCR analysis, immunostaining and Western blot analysis (Fig. 5.6). EC from both WT and TRPV4KO explants exhibited a distinct endothelial phenotype in culture (Fig. 5.6 A) and expressed specific EC marker CD31 (Fig. 5.6 B). In contrast, these cells did not express smooth muscle marker, α-SMA (Fig. 5.6 E), confirming these are bonafide EC. Further, Western blot analysis revealed TRPV4 specific bands (two bands, one below and above 100 KDa) in WTEC which were totally absent in TRPV4KO EC. (Fig. 5.6 D). Since low levels of TRPV4 was shown to correlate with increased proliferation of TEC, we next measured proliferation of TRPV4KO EC using BrdU assays. Our results demonstrated a significant (p≤0.001) increase in BrdU incorporation in TRPV4KO EC compared to WT EC. Further, we also found TRPV4KO EC to express high levels of phospho-ERK1/2 (Fig. 5.7 B). In contrast to TEC, we found that AKT phosphorylation is increased in TRPV4KO EC (Fig. 5.7 C). Furthermore, our results demonstrated increased expression of several cell cycle genes including cyclin A and cyclin D, in TRPV4KO EC (Fig 5.7 D). Taken together, these
findings indicate TRPV4 negatively regulates angiogenesis via modulation of ERK1/2-dependent EC proliferation.
Figure 5.6. Characterization of EC isolated from WT and TRPV4KO aortic sprouts. A) Phase contrast images (4x) of primary EC isolated from aortic vascular sprouts cultured on gelatin coated dishes. B) qPCR analysis showing relative expression of endothelial cell marker CD31 in WT and TRPV4KO EC (n=2). C) Relative expression of TRPV4 mRNA in WT and TRPV4KO EC (n=2). D) Western blot showing absence of TRPV4 protein in TRPV4KO EC as opposed to WT EC. Quantification of TRPV4 protein levels in WT and TRPV4KO EC (n=3). The significance was set at p≤0.001. E) Representative fluorescence images (showing the absence of α-SMA (green) expression in isolated cultures of WT EC and TRPV4KO EC confirming the endothelial nature of the isolated cells. Note: isolated mouse fibroblasts served as a positive control for α-SMA. Nuclei were stained with DAPI (blue).
Figure 5.7. Absence of TRPV4 enhances EC proliferation and ERK1/2 phosphorylation

A) Fluorescent micrographs (10x) showing proliferation of WT EC and TRPV4KO EC. Quantitative analysis showing increased percentage of BrdU incorporation in TRPV4KO EC compared to WT EC. The results shown are mean ± SEM from 3 independent experiments (n≥1000). The significance was set at p≤0.001. B) Representative Western blot showing ERK1/2 phosphorylation. Quantitative analysis of immunoblotting experiments demonstrating significant (p≤0.05) upregulation of pERK1/2 in TRPV4KO EC. Results shown are mean ± SEM of 2 independent experiments. C) AKT expression and phosphorylation is decreased in WT EC and TRPV4KO EC. Quantitative analysis revealed increased AKT phosphorylation between WT and TRPV4KO EC. Results shown are mean ± SEM of 2 independent experiments, (p≤0.05). D) Relative expression of proliferation associated G1/S phase genes in WT and TRPV4KO EC (n=2) (*p≤0.05).
**TRPV4 deletion induces abnormal migration and in vitro angiogenesis in EC**

Our previous findings have demonstrated that low levels of TRPV4 induce anomalous TEC migration and abnormal angiogenesis in vitro. The ability of cells to migrate is reliant on the mechanosensing efficiency of the cell (Dvorak, 2003) and the lack of the mechanosensor TRPV4, we hypothesized would alter EC migration. Our scratch-wound migration assays confirmed our previous findings (not shown) that TEC migrate significantly (p≤0.05) more than NEC. Importantly, we found that TRPV4KO EC migrated significantly (p≤0.001) higher than TEC and NEC (Fig. 5.8 A). These results suggest that TRPV4 is an important regulator of EC migration and that alterations in TRPV4 expression can lead to increased migration in pathological angiogenesis.

Finally, we performed 2D angiogenesis assays to assess whether the inherent abnormalities associated with TRPV4KO EC (enhanced proliferation and migration) may also be translated in the way these cells form tubes. We found that when plated on Matrigels, WT EC formed tubular structures within 4 h and stabilized them until 8 h. In contrast, TRPV4KO EC though formed tubes within 3 h but these underwent multicellular retraction and tubular disruption to collapse after 6h (Fig 5.9 A). These findings are reminiscent of tube formation by TEC which we attributed to high basal Rho-mediated contraction (Chapter III; (Adapala et al., 2015). Further we found TRPV4KO EC induced the formation of abnormally dilated tubes, as opposed to NEC which formed uniformly shaped capillaries in 3D Matrigel assays (Fig. 5.9 B). Taken together, these findings
reveal that TRPV4 is an essential molecule for maintaining the normal progression of several key processes involved in angiogenesis, specifically proliferation and migration.
Figure 5.8. TRPV4 deletion induces abnormal EC migration A)
Representative brightfield images (4x) of scratch wounds taken at 0 and 12h. Quantitative analysis showing increased migration of TRPV4KO EC compared to NEC and TEC. Results shown are mean ± SEM of 3 independent experiments. The significance was set at p≤0.05.
Figure 5.9. Absence of TRPV4 results in abnormal angiogenesis in vitro

A) Phase contrast images (4x) taken at 4, 6 and 8h showing the angiogenic behavior of WTEC and TRPV4KO EC when plated on 2D Matrigel (at densities; 8 X 10^4/ well). Results shown are mean ± SEM of 3 independent experiments.

B) 3D angiogenesis assays (10x) where NEC and TRPV4KO EC were mixed in Matrigel and cultured for 7 days. Arrows (red) show the tubes formed by EC.
**TRPV4KO EC exhibit high basal Rho activity**

In chapter III, we have shown that increased baseline activity of Rho is the underlying cause for aberrant TEC mechanosensing (towards matrix rigidity) and abnormal angiogenesis. We further demonstrated that pharmacological activation or overexpression of TRPV4 normalized aberrant mechanosensing and abnormal angiogenesis via modulation of Rho activity in TEC. Therefore, we explored whether the complete deletion of TRPV4 also modulates EC function and angiogenesis via Rho (Fig. 5.10 A, B). Similar to TEC, we found that TRPV4KO EC showed significantly ($p \leq 0.05$) increased basal Rho activation compared to WT EC (Fig. 5.10 A).

Next, we asked if normalizing Rho or Rho kinase (ROCK) activity (downstream effector of Rho) would normalize angiogenesis in TRPV4KO EC. To achieve this we performed 2D angiogenesis experiments in the presence of the ROCK inhibitor Y-27632 (Y-27; 10 μM) (Fig. 5.10 B). We found that in the absence of Y-27, TRPV4KO EC formed tubes at the earlier time point which collapsed after 6h. However, in the presence of Y-27 these cells formed robust tubes which were found to be stabilized even after 12h (Fig. 5.10 B). These results suggests that TRPV4 regulate angiogenesis via modulation of Rho signaling and that absence of TRPV4 results in high Rho activity which can impart aberrant mechanosensing to EC leading to abnormal angiogenesis.
Figure 5.10. TRPV4KO EC exhibit increased basal Rho activity and inhibition of Rho kinase (ROCK) normalizes abnormal angiogenesis exhibited by TRPV4KO EC. A) Western blot showing the basal levels of active and total Rho for WTEC and TRPV4KO EC. Densitometric analysis of Rho activity (normalized with total Rho) showed increased basal Rho activity in TRPV4KO EC. The results shown are mean ± SEM from 3 independent experiments. B) Phase contrast micrographs showing the normalizing effects of Rho kinase inhibitor, Y-27632 (10 μM) on TRPV4KO EC angiogenic behavior when plated on 2D Matrigels (at high densities; 8 X 10^4 well that cause collapse of tubular networks). Representative images (4x) taken 8h post plating, showing the formation of stable tubes upon inhibition of the Rho pathway. Quantification of tube length revealed significant (p≤0.05) increase in tube length in TRPV4KO EC+Y-27 compared to untreated controls. The results shown are mean ± SEM from 3 independent experiments.
A small molecule inhibitor of ROCK in conjunction with Cisplatin reduces tumor growth in TRPV4KO mice

Rho pathway has been implicated as a central player in the regulation of angiogenesis (Bryan et al., 2010; Calvo et al., 2013; Takata et al., 2008). Our results clearly demonstrated that deficiency (TEC) or deletion of TRPV4 (TRPV4KO EC) results in increased Rho activation and angiogenesis. In fact, we have shown that both physiological (Matrigel Plug) and pathological (tumor) angiogenesis is increased in TRPV4KO mice. Further, our in vitro results showed that inhibition of Rho pathway with Y-27632 normalized abnormal EC function and angiogenesis in vitro. Based on these findings, we hypothesized that Y-27632 treatment may reduce tumor growth in TRPV4KO mice. To accomplish this, we generated LLC tumors in TRPV4KO mice; once the tumors became palpable (after 7 days), we injected ROCK inhibitor Y-27632 (i.p.) every day for 14 days and monitored tumor growth. Saline injected mice served as controls for these experiments. Additionally, to test if ROCK inhibition normalizes tumor vasculature and improves cancer therapy, we injected Cisplatin once per week starting 2-4 days after Y-27632 treatment (Fig. 5.11 A). We used TRPV4KO mice and divided them in to four groups 1) TRPV4 KO control 2) TRPV4 KO + Cisplatin 3) TRPV4 KO +Y-27632 and 4) TRPV4 KO+Y-27632 + Cisplatin. Tumor growth and angiogenesis was monitored as described in Chapter III. We found that time dependent tumor growth in control animals reached around 2000mm$^3$ at 21 days. Injection of Y-27632 or Cisplatin alone did not affect tumor growth in these mice. However, we found a significant reduction in tumor growth in mice.
that were given Y-27632 followed by Cisplatin. These results suggest that ROCK inhibition may have normalized the tumor vasculature which aided in the efficient and systemic delivery of Cisplatin (Fig. 5.11 B). Consistent with this observation, we found increased pericyte coverage in tumor vessels that were treated with Y-27632 (Y-27632 and Y-27632+Cisplatin), but not in the control, or Cisplatin alone treated mice (Fig. 5.11 C). Together, our data provides compelling evidence that Rho/ROCK pathway act downstream of TRPV4 and that TRPV4 modulates angiogenesis by maintaining optimal levels of Rho/ROCK. Our findings, thus, conclude that TRPV4 is an upstream regulator of Rho-dependent mechanical signaling in angiogenesis and reduction or deletion of TRPV4 results in abnormal Rho signaling that can lead to abnormal angiogenesis.
Figure 5.11. ROCK inhibitor, Y-27632, in conjunction with Cisplatin, reduces tumor growth in vivo in TRPV4KO mice. A) Schematic showing the drug treatment schedule. Syngeneic tumors (LLC) were injected in the back of TRPV4KO mice and tumor growth was monitored for 21 days. ROCK inhibitor, Y-27632 (Y-27) was injected (i.p.) (10mg/kg) everyday starting from day 7 until day 21. Cisplatin was injected (i.p.) (3mg/kg) once per week starting 3 days after the injection of Y-27632. B) Tumor growth in TRPV4KO mice is reduced with Y-27632+Cisplatin treatment. Time-dependent growth of tumors, in mice, injected with saline (CON) or Y-27632 or Cisplatin or Y-27632+Cisplatin. Tumor growth was significantly reduced in Y-27632+ Cisplatin (*) treated but not Y-27632 or cisplatin alone treated mice. The results shown are mean ± SEM from 3 independent experiments. The significance was set at p≤0.05. C) Tumor sections (10 μm) were stained with specific markers for endothelium (CD31) and pericyte (α-SMA). Quantitative analysis of pericyte covered micro-vessels in tumors from TRPV4KO control, Cisplatin, Y-27632 and Y-27632+ Cisplatin treated mice. The results shown are mean ± SEM from 3 independent experiments (p<0.05).
In the present study, we have demonstrated for the first time that TRPV4 negatively regulates angiogenesis i.e. absence of TRPV4 increases angiogenesis. Our findings clearly show that EC isolated from TRPV4KO mice exhibit increased proliferation, migration and enhanced angiogenesis in vitro. Using syngeneic tumor models, we further demonstrated that TRPV4KO mice exhibit severe tumor vessel malformations, characterized by increased vessel diameter and density, and enhanced tumor growth. Furthermore, we demonstrated that the absence of TRPV4 promotes enhanced tumor angiogenesis may be via up-regulation of the Rho/ROCK pathway. Finally, we show that inhibition of Rho/ROCK pathway with Y-27632 normalizes abnormal tumor vasculature and reduces tumor growth in conjunction with Cisplatin, in TRPV4KO mice. Taken together, it can be suggested that TRPV4 could be an important regulator of physiological angiogenesis, the loss or deficiency of which can negatively contribute to both physiological and pathological angiogenesis.

Mechanosensing and downstream mechanotransduction pathways have, in the recent past, identified to play a key role in maintaining cellular function and homeostasis. The cellular force balance that exists between the contractile forces generated by the cytoskeleton and the ECM, maintains cellular tensional homeostasis (Kumar et al., 2006). Any variation in this ECM-cell force balance, as a result of changes in the immediate microenvironment, has been
shown to downstream regulate cell shape, proliferation, migration and ultimately survival (Ingber, 2003). As described earlier, cellular tension is sustained and regulated, to a large extent by the Rho pathway (Kimura et al., 1996). With respect to mechanosensation, TRPV4 has of late recognized to be a ubiquitously expressed mechanosensor, especially vital in the vascular endothelium (Baylie and Brayden, 2011; Liedtke, 2005; Matthews et al., 2010; Thodeti et al., 2009). Specifically, in TEC, which exhibit low functional levels of TRPV4, we have shown Rho to be constitutively active; which can be suppressed via TRPV4 overexpression or activation. Further, we shown that activating TRPV4 can normalize angiogenesis in vitro and in vivo by restoring mechanosensitivity via Rho [Chapter III and (Adapala et al., 2015)] However, the role of TRPV4 in physiological angiogenesis and the in vivo contribution of Rho in tumor angiogenesis is not known.

Current therapies that are targeted against angiogenesis are largely based on inhibiting growth factors. However, angiogenic redundancy has been documented as the primary resistance mechanism identified, following anti-VEGF therapy, due to the availability of other pro-angiogenic factors that the tumors can utilize (Casanovas et al., 2005; Relf et al., 1997). Here, we find that in the absence of the mechanosensitive ion channel TRPV4, EC proliferation, migration and angiogenesis are enhanced, very similar to the effects that were observed with TEC (Adapala et al., 2015). Further our studies revealed that the mechanism underlying the aberrant effects exhibited by TRPV4KO EC, were similarly attributed to high basal levels of active ERK1/2 and active-Rho. Finally,
by inhibiting ROCK in tumors induced in TRPV4KO mice, we provide a proof of concept that TRPV4 mediated mechanosensing is dependent on Rho, confirming our prior findings (Adapala et al., 2015). Targeting TRPV4 in the tumor endothelium may thus provide a novel growth-factor independent therapy against tumor angiogenesis, without the possibility of encountering the limitations that are often linked with anti-VEGF therapies.
Summary and Conclusion

The general objective of this dissertation was to understand how the mechanosensitive ion channel TRPV4 contributes to the process of angiogenesis. The specific aims outlined in this dissertation described the functional significance of TRPV4 in normal and tumor angiogenesis and further addressed the mechanisms by which TRPV4 modulates TEC proliferation, and Rho-dependent mechanosensitivity to regulate tumor angiogenesis.

Chapter III found that decreased expression and function of TRPV4 in TEC contributes to abnormal Rho-mediated TEC mechanosensitivity and in vitro angiogenesis. We demonstrated for the first time that pharmacological activation of TRPV4 normalized tumor vasculature, and in combination with chemotherapeutic drug Cisplatin reduced tumor growth. The findings of this chapter identify a unique role of the mechanosensitive TRPV4 channel in the regulation of tumor angiogenesis and vessel maturity and have opened up an unexplored therapeutic target in vascular normalization strategies.

Chapter IV established that reduced expression of TRPV4 channels is an underlying factor that contributes to the enhanced proliferation of TEC. Further, TRPV4 activation via a small molecule activator GSK1016790A normalized the
anomalous growth of TEC in tumors. The results of this work contributes significantly to the existing knowledge that TEC are altered functionally, compared to their normal counterparts; and targeting TRPV4 to reduce abnormal proliferation of TEC may be beneficial in limiting tumor angiogenesis.

Chapter V demonstrated TRPV4 deletion negatively regulates physiological and pathological angiogenesis. Specifically, we demonstrated deregulation of key angiogenic events including EC proliferation, migration and tube formation that occur in the absence of TRPV4. This chapter concludes that TRPV4-dependent mechanotransduction in EC regulates angiogenesis via modulation of Rho/ROCK pathway and that deregulation of this pathway can lead to abnormal angiogenesis.

Taken together, the findings of this dissertation reveal that TRPV4 channels are a novel regulator of angiogenesis and therefore a potential target for developing therapeutics that can either inhibit or induce angiogenesis.


Greenberg, J. I., Shields, D. J., Barillas, S. G., Acevedo, L. M., Murphy, E., Huang, J., Scheppke, L., Stockmann, C., Johnson, R. S., Angle, N., and


endothelial growth factor autocrine mechanism in endothelial cells. Molecular cancer therapeutics 7, 1551-1561.


blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. Cancer research 64, 3731-3736.


