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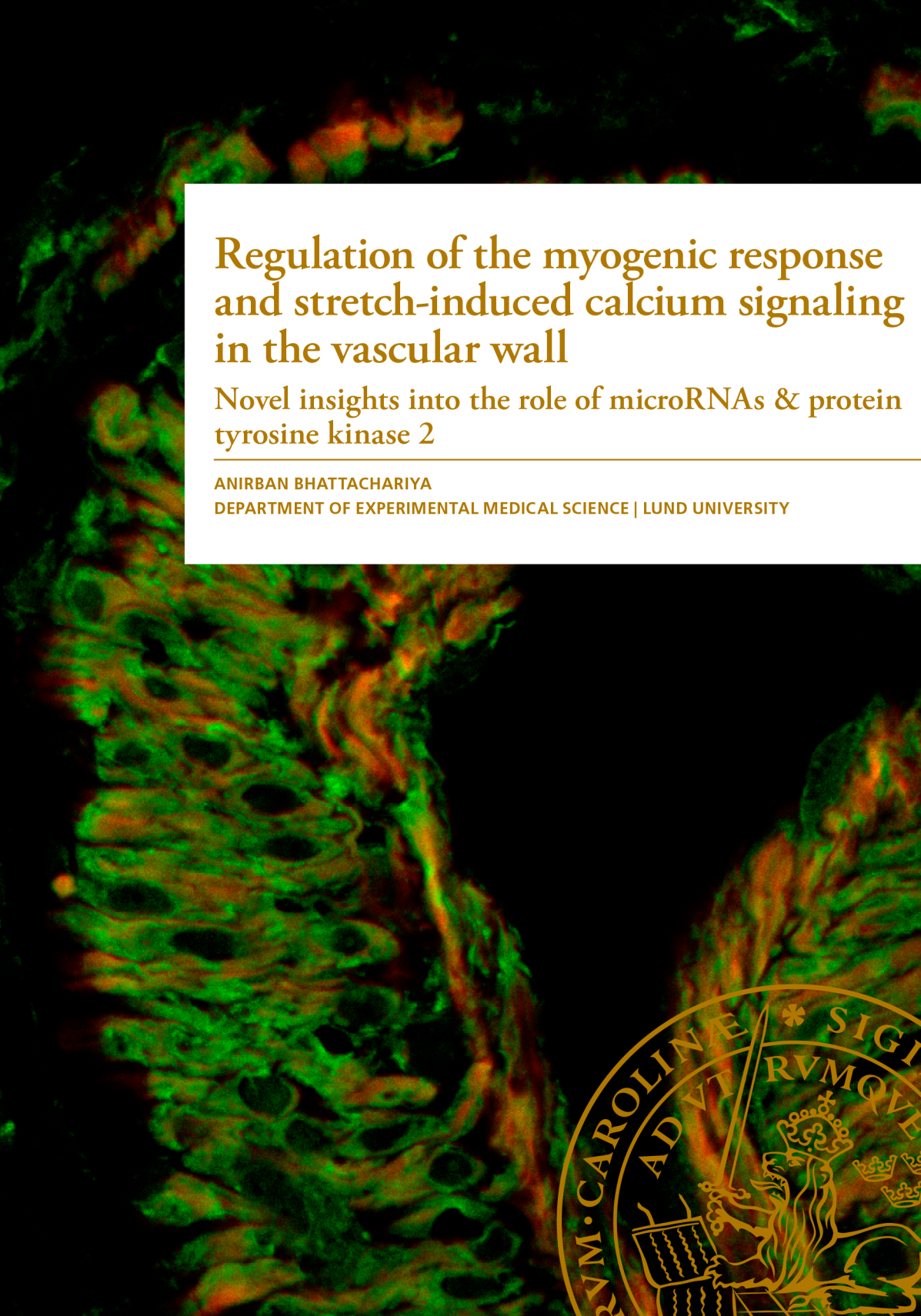
Bhattachariya, Anirban

2014



Regulation of the myogenic response and stretch-induced calcium signaling in the vascular wall

Novel insights into the role of microRNAs & protein tyrosine kinase 2

ANIRBAN BHATTACHARIYA
DEPARTMENT OF EXPERIMENTAL MEDICAL SCIENCE | LUND UNIVERSITY

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DOCTORAL DISSERTATION

The public defense of thesis for the degree of Doctor of Philosophy in Medicine, with due permission from the Faculty of Medicine, Lund University, Sweden will take place at Rune Grubb-salen, BMC, Sölvegatan 19, Lund on Friday the 26th of September 2014 at 9:00.

Faculty opponent

Professor Michael P. Walsh, PhD
Department of Biochemistry and Molecular Biology,
Faculty of Medicine, University of Calgary, Canada

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Title and subtitle: Regulation of the myogenic response and stretch-induced calcium signaling in the vascular wall: Novel insights into the role of microRNAs and protein tyrosine kinase 2

Abstract

Intraluminal pressure has a significant impact on vascular adaptability, phenotype and regulation of blood flow and pressure. On one hand, increased pressure/stretch for a prolonged time can cause structural changes in vessel wall; on the other hand, lack of pressure/stretch can promote a phenotype shift. This thesis investigates novel roles of microRNAs and protein tyrosine kinase 2 in pressure/stretch-induced signaling mechanisms in the vascular wall.

Using two different knockout mouse models, we uncovered a novel role of microRNAs in the pressure-induced myogenic response. We demonstrated that global deletion of smooth muscle-specific microRNAs causes a loss of pressure-induced contraction and that this likely involves diminished calcium influx due to reduced stretch-induced activation of the PI3K/Akt pathway. Similarly, global deletion of the smooth muscle enriched miRNA-143/145 also depleted myogenic responses but this effect could be due to several combined factors including loss of calcium influx and decreased expression of myosin light chain kinase. Furthermore portal veins of miRNA-143/145 KO mice exhibit lack of stretch-induced contractile differentiation, which may in part be due to a reduced expression of L-type calcium channels caused by an increased expression of the transcriptional repressor DREAM. Using a novel small molecule inhibitor of PYK2, we demonstrated that PYK2 could distinguish between non-voltage and voltage-dependent calcium pools to initiate signal transduction in the smooth muscle of portal vein. Inhibition of PYK2 can reduce phenotype modulation and apoptosis in balloon injured carotid arteries.

In conclusion, we have established an indispensable role of microRNAs in the presssure-induced myogenic response and maintainance of stretch-induced conctractile differentiation. Morover we have established that PYK2 is involved in stretch-induced calcium handling in spontaneously active portal vein and in phenotypic shift of smooth muscle cells following vascular injury.

Key words microRNA, calcium, myogenic tone, PYK2, resistance artery, portal vein.

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By

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Front cover image: Portal vein sections were stained for filamentous (F-actin) and globular (G-actin) with and Alexa Fluor 633-phalloidin (red) and Alexa Fluor 488-DNase I (green) respectively.

Back cover image: Influx of calcium following depolarization was measured in mouse small mesenteric arteries using calcium indicator Fluo-4 AM.

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Faculty of Medicine
Lund University, Sweden

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To my parents

for your unconditional support

"All the past we leave behind,
We debouch upon a newer mightier world, varied world,
Fresh and strong the world we seize, world of labor and the march,
Pioneers! O pioneers!

We detachments steady throwing,
Down the edges, through the passes, up the mountains steep,
Conquering, holding, daring, venturing as we go the unknown ways,
Pioneers! O pioneers!"

-Walt Whitman, Leaves of Grass (1865).

Contents

Peer reviewed articles and manuscripts

I. *Expression of microRNAs is essential for arterial myogenic tone and pressure-induced activation of the PI3-kinase/Akt pathway.* Bhattachariya A, Dahan D, Turczyńska KM, Swärd K, Hellstrand P, Albinsson S. Cardiovasc Res. 2014 Feb 1; 101(2):288-96. doi: 10.1093/cvr/cvt253.

II. *Reduced stretch-sensitivity and spontaneous activity in the vascular smooth muscle of miR-143/145 knockout mice*. Bhattachariya A, Dahan D, Ekman M, Boettger T, Braun T, Swärd K, Hellstrand P and Albinsson S. Submitted 2014.

III. *Expression of the miR-143/145 cluster is essential for myogenic responses in small mesenteric arteries*. Bhattachariya A, Dahan D, Ekman M, Boettger T, Braun T, Swärd K, Hellstrand P and Albinsson S. Manuscript 2014.

IV. *PYK2 selectively mediates signals for growth versus differentiation in response to stretch of spontaneously active vascular smooth muscle*. Bhattachariya A*, Turczyńska KM*, Grossi G, Nordström I, Buckbinder L, Albinsson S and Hellstrand P (* Equal contribution). Physiol Rep, 2 (7), 2014, e12080, doi: 10.14814/phy2.12080

V. *Effects of PYK2 inhibition on smooth muscle phenotype shift in the arterial wall.* Bhattachariya A, Turczyńska KM, Grossi M, Nordström I, Sebastian Albinsson and Hellstrand P. Manuscript 2014.

Additional peer reviewed articles not included in the thesis

I. *Stretch-sensitive down-regulation of the miR-144/451 cluster in vascular smooth muscle and its role in AMP-activated protein kinase signaling*. Turczyńska KM, Bhattachariya A, Säll J, Göransson O, Swärd K, Hellstrand P, Albinsson S. PLoS One. 2013 May 21;8(5):e65135. doi: 10.1371/journal.pone.0065135. Print 2013.

II. *Mir-29 repression in bladder outlet obstruction contributes to matrix remodeling and altered stiffness*. Ekman M, Bhattachariya A, Dahan D, Uvelius B, Albinsson S, Swärd K. PLoS One. 2013 Dec 10;8(12):e82308. doi: 10.1371/journal.pone.0082308. eCollection 2013. PubMed PMID: 24340017; PubMed Central PMCID: PMC3858279.

III. *Stretch-dependent smooth muscle differentiation in the portal vein-role of actin polymerization, calcium signaling, and microRNAs*. Albinsson S, Bhattachariya A, Hellstrand P. Microcirculation. 2014 Apr;21(3):230-8. doi:10.1111/micc.12106.

Abbreviations

ACE-1: angiotensin converting enzyme-1

Ang II: Angiotensin II

ANOVA: one-way analysis of variance

$Ca_v1.2$: L-type calcium channel α_{1c} subunit (protein)

CaMKII: Calmodulin kinase II

CAK-ß : Cell adhesion kinase-ß

CCD: Charge-coupled device

DREAM: Downstream Regulatory Element Antagonist Modulator

DTT: Dithiothreitol

ER^T: Estrogen receptor ligand binding domain

ERK: Extracellular signal-regulated kinase

FAK: Focal Adhesion Kinase

FCS: Fetal calf serum (FCS)

GPCR: G-protein-coupled receptors

GSK-3β : Glycogen synthase kinase-3β

HRP: Horseradish peroxidase

HSAEpC: Human small airway epithelial cells

KCl: Potassium chloride

KLF-4: Krüppel-like factor 4

KO: Knock out

LBD : Ligand binding domain

MAP: Mean arterial pressure

miRNA: MicroRNA

MRA : Mesenteric resistance arteries

MLC_{20}: Myosin regulatory light chain

MLCP :Myosin light chain phosphatase

MLCK: Myosin light chain kinase

MYPT1: Myosin phosphatase target subunit 1

MRTF: Myocardin-related transcription factors

ncRNA: Non-coding RNAs

nRTK: non-receptor tyrosine kinases

PABP: poly (A) binding protein

PDI: Protein disulfide isomerase

PDK1: Phosphoinositide-dependent kinase 1

PI3K: Phosphoinositide 3-kinase

PKB: Protein kinase B

PTEN: Phosphatase and tensin homolog (PTEN)

PYK2: Proline-rich tyrosine kinase 2

RhoA: Ras homolog gene family, member A

ROCK: Rho associated coiled-coil forming kinase

RTK: Receptor tyrosine kinases

SDS: Sodium dodecyl sulfate

SM-MHC : Smooth muscle myosin heavy chain

SRF: Serum response factor

TCA: Trichloroethanoic acid

TCF: Ternary complex factor

TEA: Tetraethylammonium chloride

TRP: Transient receptor potential

3'-UTR: 3'-untranslated region

VDCC: Voltage dependent calcium channels

1. Introduction

The main function of the cardiovascular system is the transportation and exchange of nutrients, oxygen, carbon dioxide, metabolic waste products and blood cells throughout the entire body in a rapid and efficient manner to maintain homeostasis, tissue viability, body temperature and pH.

The cardiovascular system is divided into two distinct circuits, the systemic and pulmonary circulation (Figure 1). Oxygenated and nutrient-rich blood is pumped from the left ventricle of the heart via the aorta and distributed throughout the body. After exchange, deoxygenated blood returns to the right atrium via the superior and inferior vena cava, both of which are part of the systemic circulation. In the pulmonary circulation, deoxygenated blood is then pumped from the right ventricle of the heart via the pulmonary artery to the lungs and after exchange, oxygen-rich blood is returned to the left atrium via the pulmonary veins.

Each blood vessel has a hollow lumen, which is surrounded by the vessel wall. A brief description of the structure and function of the vessel wall is given in the following section.

1.1 The vascular wall

1.1.1 Structure and function

Even though arteries and veins vary in their structure and function (see Table 1 for summary), they have several general features. The vascular wall is composed of three layers (Figure 2). The outermost layer is known as tunica externa or tunica adventitia and is a protective layer with no distinct outer border; it is composed of connective tissue (mainly the extracellular proteins collagen and elastin), perivascular nerves and in large vessels, small nutrient vessels called vasa vasorum. The main function of the adventitia is to tether the vessel to the surrounding tissue.

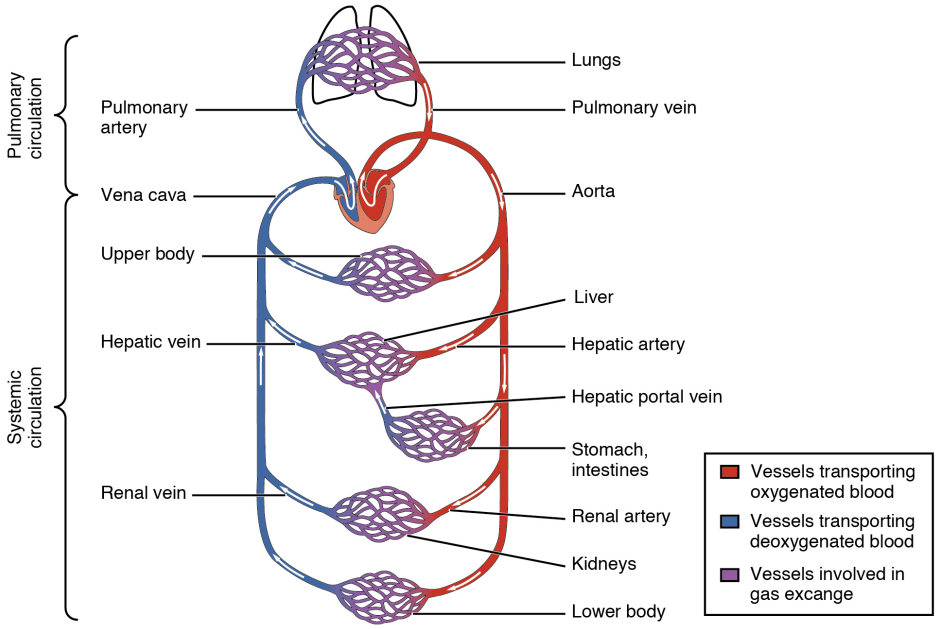

Figure 1: Overview of the cardiovascular system. Image is retrieved from OpenStax College. Structure and Function of Blood Vessels, OpenStax-CNX Web site. http://cnx.org/content/m46597/1.4/, Jun 28, 2013 and protected under a Creative Commons Attribution License CC-BY 3.

The innermost layer or tunica intima is mainly composed of flat structured cells called endothelium, which is next to the basement membrane or basal lamina that adheres the endothelium to the connective tissue. The endothelial layer is in direct contact with the blood flow and forms a barrier to keep cells and large molecules inside the lumen but, depending on its properties and anatomical location, allows restricted passage of water and small molecules across primarily the capillary wall.

The medial layer (tunica media) is the thickest layer in arteries and is mainly composed of spindle-shaped smooth muscle cells, embedded in a matrix of collagen and elastin and arranged mostly in a circular fashion. However, in the portal vein, a longitudinal layer of smooth muscle is dominant. Elastin sheets, called internal and external elastic lamina, mark the boundaries of the inner (endothelial) and outer (advential) sides of the medial layer, respectively. The external elastic lamina is present in large arteries but mostly absent in small arteries, arterioles, and veins. The main function of the medial layer is to give the vessel wall mechanical strength as well as the ability to contract and relax. One important task of the medial layer is to maintain

constant blood flow during changes in blood pressure, which is crucial for maintaining normal organ function.

	Artery	Vein
Anatomy	Thicker walls and smaller lumen. Thick and elastic smooth muscle layer can handle high blood pressure.	Thinner elastic walls but larger lumen. Veins are exposed to much lower blood pressure
Thickest layer	Media	Adventitia
Valves	Abscent	Valves are present to prevent blood flow in opposite direction due to gravity.
Blood flow direction	From heart	Towards heart
Oxygen concentration	Except the pulmonary and umbilical arteries, all arteries carry oxygenetated blood.	Except the pulmonary and umblical veins, all veins carry deoxygeneted blood.

Table 1: Comparison of common characteristics of artery and vein.

This is achieved by contraction and relaxation of the smooth muscle, which in turn regulates the diameter of the vessel and consequently regulates the flow of blood to the downstream capillary network. The contraction and relaxation of the smooth muscle is influenced by neural, endocrine, and local mechanisms, but myogenic contraction induced by an increase in pressure acting on the blood vessel itself is a critical property of small arteries and arterioles (so-called resistance vessels, see section 1.1.2). Myogenic properties of small arteries can be divided into myogenic tone, which is defined as tone at a constant pressure level, and the myogenic response, which is the alteration of tone in response to a change in pressure[1]. A brief introduction to the myogenic response is given in section 1.1.3.

1.1.2 Different classes of blood vessel

Blood vessels have the general task of carrying blood, but depending on their location, their structure has been adapted to specific demands on their functional properties.

Elastic artery (e.g., aorta): The main function of an elastic artery is to accommodate the stroke volume (volume of blood expelled by each heartbeat), maintain continuity of blood flow and, sustain relatively constant pressure of blood despite the fact that the heart only ejects blood during systole. The main components of the elastic artery are elastin and collagen. The presence of elastin allows the vessel

to expand by roughly 10% to accommodate the stroke volume and act as a temporary storage of ejected blood. The main function of collagen in elastic arteries is to limit over-distension when blood pressure is high.

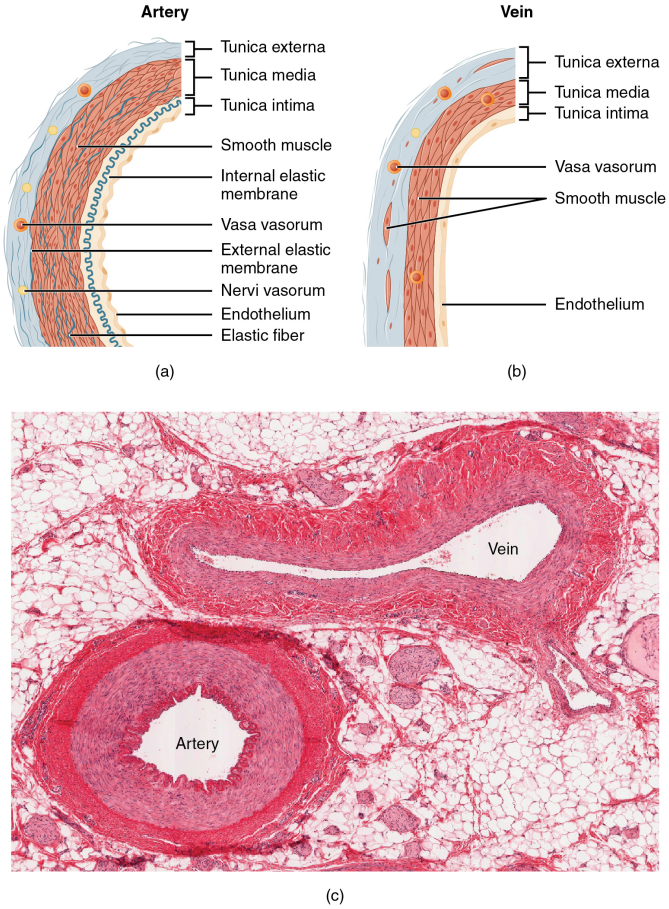

Figure 2: Arteries (a) and veins (b) share the same general features, but the walls of arteries are much thicker because of the higher pressure of the blood that flows through them. (Micrograph provided by the Regents of the University of Michigan Medical School © 2012). Image is retrieved from OpenStax College. Structure and Function of Blood Vessels, OpenStax-CNX Web site. http://cnx.org/content/m46597/1.4/, Jun 28, 2013 and protected under a Creative Commons Attribution License CC-BY 3.0

Conduit and muscular arteries: The main function of conduit arteries (such as the carotid artery) is to conduct flow from elastic arteries to resistance arteries. Conduit arteries have a thicker smooth muscle layer compare to elastic arteries.

Resistance vessels: These vessels consist of small arteries (100-300 μm) and arterioles (10-100 μm) and contribute to the overall resistance in the entire

circulation[2]. These blood vessels play a crucial role in keeping the blood flow to the capillaries relatively constant during fluctuations in blood pressure. More details on the importance of resistance vessels are given in section 1.4.

Capillaries or exchange vessels: Capillaries are about 4-7μm in diameter. Their walls lack both adventitia and media and consist of a single layer of endothelial cells. The main function of the capillaries is to participate in the exchange of oxygen, CO2, nutrients and fluids. Due to the large total cross sectional area of the capillary bed, the rate of blood flow is significantly lower than in larger vessels (Figure 3), which allows sufficient time for exchange.

1.2 Regulation of blood flow

Blood flow is regulated by two main elements: 1) blood pressure: the force which thrusts the blood within the blood vessel and 2) vascular resistance: the resistance of the vessels to the blood flow. A typical systemic arterial blood pressure of 120/80 mmHg (systolic/diastolic) corresponds to a mean arterial pressure (MAP) of about 93 mmHg. Average MAP is between 70 and 110 mmHg. MAP below 60 mmHg for a prolonged time can result in ischemia, or insufficient blood flow.

The cardiac output is around 5 l/min in an adult humans at rest and can increase ≈4-5 fold during heavy exercise[3]. According to Poiseuille's law, blood flow (Q) is determined by the following equation:

$$Q = \frac{\pi(P_i - P_o)\mathrm{r}^4}{8\eta l}$$

Hence, Q is linearly proportional to the difference in pressure (P_i=inflow pressure and P_O=outflow pressure) and the fourth power of the vessel radius (r), and inversely proportional to the vessel length (l) and the blood viscosity (η). Poiseuille's equation shows that small variations in vessel diameter significantly affect flow regulation. Vascular resistance (R) can be calculated using the same principle as Ohm's law, where resistance is expressed as the ratio of voltage drop and current flow. Rearranging Poiseuille's equation,

$$R = \frac{P_i - P_O}{Q} = \frac{8\eta l}{\pi \mathrm{r}^4}$$

Vascular resistance (R), defined as the ratio between pressure drop and flow, thus depends on the dimensions of the vessel and on the characteristics of the fluid. Small arteries and arterioles have much higher resistance than larger arteries, even over a

small length, but the total cross-sectional area of these vascular segments is larger[2]. Due to a graded diminution of intraluminal pressure along the vascular tree, the sensitivity and mechanism of the regulation may vary[4-6]. The relationship between vessel diameter, blood pressure and flow is depicted in Figure 3.

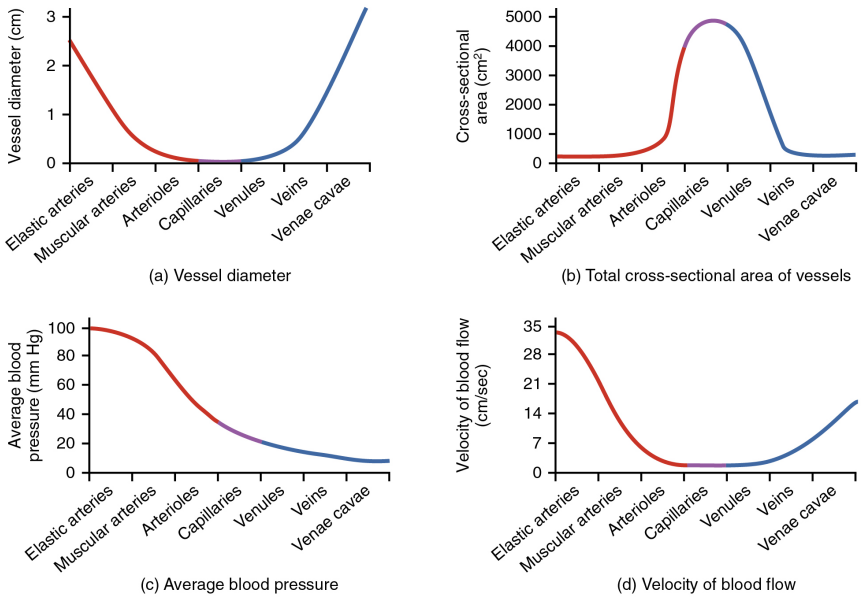

Figure 3: Relationship between vessel diameter, cross-sectional area, blood pressure and blood flow velocity. See text for details. Image is retrieved from OpenStax College, Anatomy & Physiology. OpenStax CNX.Jul 30, 2014 http://cnx.org/contents/14fb4ad7-39a1-4eee-ab6e- 3ef2482e3e22@6.27 and protected under a Creative Commons Attribution License CC-BY 3.

1.3 Vascular smooth muscle

During prolonged elevation of blood pressure (for example, hypertension), blood vessels undergo various transformations in structure. This is often referred to as "vascular remodeling". Small arteries and arterioles are most susceptible to this phenomenon, resulting in organ dysfunction and progression of vascular diseases. This is especially true for patients with hypertension and diabetes[7-10]. The smooth muscle layer of the vascular wall plays a key role in the remodeling process. In normal adult blood vessels, the smooth muscle cell is spindle shaped and has an extremely low level of migration and proliferation. This differentiated state is commonly referred to as the contractile phenotype. However, in certain conditions smooth muscle cells can

also display a so called "synthetic phenotype", which is characterized mainly by the following criteria: 1) increased rate of proliferation and migration, 2) augmented synthesis of extracellular matrix proteins such as collagen, elastin, and proteoglycans, 3) decreased expression of smooth muscle contractile marker proteins such as α-actin, myosin heavy chain and calponin 4) decreased contractile function[11-14]. The synthetic smooth muscle cells have long been thought to originate from phenotypically modified contractile cells but this well-established paradigm has recently been challenged by the controversial suggestion that synthetic smooth muscle cells in the vascular wall originate from so-called medial-derived multipotential vascular stem cells[15, 16]. Regardless of the origin, activation of the synthetic phenotype is an important mechanism in the vascular repair process.

Regulation of smooth muscle phenotype is a complex process involving multiple signaling pathways and environmental cues. One of the key modulators in vascular smooth muscle is mechanical stretch, which is exerted by the intraluminal blood pressure. We have suggested a role of microRNAs (miRNA) and proline-rich protein kinase 2 (PYK2) in stretch-dependent modulation of the smooth muscle phenotype. Brief accounts of smooth muscle contraction and differentiation are given in section 1.3.1 and 1.3.2; detailed molecular mechanisms are reviewed elsewhere[17-21].

1.3.1 Smooth muscle contraction and relaxation

A change in the intracellular calcium concentration is the key regulator of smooth muscle contraction and relaxation. There are two main sources of calcium: 1) influx from extracellular space, and 2) intracellular calcium stores in the sarcoplasmic reticulum (SR)[22]. Inside the cytoplasm, calcium binds to the ubiquitously expressed calcium-binding protein, calmodulin, which maximally binds 4 calcium ions. The calcium-calmodulin complex binds to the catalytic subunit of myosin light chain kinase (MLCK) and subsequently phosphorylates myosin regulatory light chain (MLC_{20}) at serine 19, which allows myosin ATPase to be activated by actin and to form actin-myosin cross bridges to elicit contraction[23]. In unstimulated smooth muscle cells, the average calcium concentration is 120-150 nM, and calcium then binds to calmodulin in maximally two sites. Upon stimulation, when the intracellular concentration is increased to 300-500 nM, all the four calcium binding sites in calmodulin are saturated, which drives a conformational change to phosphorylate MLCK and elicit contraction[24]. This phosphorylation is reversible by myosin light chain phosphatase (MLCP), which dephosphorylates the MLC_{20} and promotes relaxation[25]. MLCP is inhibited by Rho-kinase activation, which can result in an increased or maintained contraction despite reduced calcium levels. This phenomenon is often referred to as calcium sensitization[26]. In the context of

myogenic response, even though calcium entry is essential to elicit a contraction, increased constriction (with increase in pressure) is observed at a constant level of intracellular calcium, likely due to calcium sensitization[27, 28]. In other words, calcium sensitization can be defined as the activity ratio between MLCK and MLCP and is an essential mechanism to elicit a pressure/stretch-induced contraction.

1.3.2 Smooth muscle growth and differentiation

Regulation of smooth muscle phenotype is a fundamental mechanism in the vascular remodeling process that physiologically permits vascular repair and development. One of the key features of the contractile phenotype is the abundant expression of smooth muscle contractile marker proteins such as α-actin, calponin, SM22α, smooth muscle myosin heavy chain (SM-MHC), tropomyosin and desmin. All these genes are regulated by the transcription factor serum response factor (SRF)[29]. Although smooth muscle differentiation and proliferation are not mutually exclusive events, an antagonistic molecular mechanism exists for their regulation [30, 31]. On one hand, the myocardin family of transcription factors, comprising myocardin and myocardin-related transcription factors (MRTFs) A and B, can bind to SRF to promote differentiation and expression of smooth muscle contractile marker gene expression[32, 33]. On the other hand the ternary complex factor (TCF) family of ETS domain proteins can also bind to SRF to promote proliferation via expression of immediate-early genes[19, 34]. The myocardin and TCF families thus compete with each other to differentially regulate the transcription of SRF target genes via mutually exclusive binding to SRF[35]. Activation of the Rho/ROCK pathway is necessary to stimulate nuclear translocation of MRTFs to induce smooth muscle differentiation via increased actin polymerization[36, 37] and also increases myocardin expression[38], whereas the MAPK/Elk-1 pathway has been shown to activate TCFs[39, 40]. All SRF regulated genes contain a CArG box ([CC(A/T)$_6$GG]) DNA sequences in the promoter region for SRF to bind and induce transcription[41].

1.4 The myogenic response

The myogenic response can be defined as the inherent capability of small arteries and arterioles to constrict and decrease their diameter in response to augmented intraluminal pressure. This phenomenon was originally discovered by Bayliss (1902)[42]. Nearly four decades later, Folkow demonstrated that the myogenic response in small arteries is not neurogenic and depends on variations in intraluminal

pressure[43]. Mellander and co-workers further showed that capillary hydrostatic pressure in the intact vascular bed is dependent on pre-capillary myogenic response[44, 45]. Subsequent studies using isolated vessel preparations established that the myogenic response is solely dependent on a change in intravascular pressure and ensuing stretch of the vessel wall and distinctive from any influence of metabolites, neural factors or blood flow[1, 46, 47]. Myogenic tone acts as a 'rapid response' to stabilize fluctuations in systemic pressure thus preventing tissue damage and fluid leakage. Myogenic responses are present in almost all tissues but most pronounced in brain, kidney, intestine, heart and spleen[47, 48].

A key question in understanding how the myogenic response is regulated is how the primary stimulus is sensed in the membrane, which subsequently leads to membrane depolarization and influx of calcium ions. Proposed candidates are stretch-sensitive cation channels, interactions between extracellular matrix proteins, cell surface integrins and the cytoskeleton and mechanosensitive enzymes within the plasma membrane [1, 49, 50].

Pressurization of small cerebral arteries leads to depolarization, activation of voltage-dependent calcium channels, increase of intracellular calcium, and contraction[51]. The most accepted hypothesis concerning the mechanism of depolarization is the involvement of mechanosensitive ion channels. Davis and colleagues have shown the presence of a non-selective, stretch-activated, cation channel, which carries an inward current, resulting in depolarization of the vascular smooth muscle[52]. Welsh et al. (2002) suggested the involvement of transient receptor potential (TRP) channels in the depolarization of pressurized cerebral arteries[53]. However, Mederos y Schnitzler et al. (2008) suggested that TRP channel activation by stretch is indirect, being mediated by $G_{q/11}$-coupled receptors functioning as sensors[54]. Additionally it has been shown that gating properties of both BK_{Ca} and L-type voltage gated Ca^{2+} channels can be altered due to stretch, modulating membrane potential[55, 56]. The involvement of integrin activation in the myogenic response is supported by studies showing that pressure-induced constriction is reduced by antibodies directed against specific integrins or integrin subunits or by integrin recognition peptides (e.g. RGD)[57]. Integrin activation is also known to regulate various ion channels including non-selective cationic channels and L-type voltage gated Ca^{2+} channels [58].

1.5 Protein tyrosine kinases

A protein kinase is a type of enzyme that transfers phosphate groups from adenosine triphosphate to the side chains of specific amino acids (most commonly tyrosine,

serine, threonine or histidine) of the target protein to alter their function. It has been suggested that about 30% of the human proteins can be regulated by kinase activity and since this process is essential in signal transduction, it needs to be finely tuned[59]. Based on their specific catalytic targets, kinases can be subdivided into tyrosine kinase (act on tyrosine), serine/threonine kinases (act on serine and threonine) and dual-specificity kinases (act on tyrosine, serine and threonine). Broadly, there are two distinct classes of tyrosine kinases based on their localization and topology: Receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (nRTKs)[60]. RTKs have an extracellular domain for sensing a signal, a transmembrane domain, and an intracellular domain with kinase activity, whereas nRTKs lack extracellular and transmembrane domains. Tyrosine kinases have a wide range of functions including the transmission of signals, including those elicited by mechanical stimuli, from the extracellular space and cell membrane to the nucleus to influence cell-cycle control and the function of transcription factors. Hence they are essential in the cellular response to stretch.

1.5.1 PYK2 is a non-receptor tyrosine kinase

Most commonly, nRTKs are localized in the cytoplasm but they can also be tethered to or in near proximity to the plasma membrane due to post-transcriptional modification in the N-terminus. So far 32 genes have been found to encode nRTKs in humans and they are divided into 11 subfamilies. It has been suggested that receptors, which lack kinase activity can recruit nRTKs to the plasma membrane and thus initiate a signaling cascade[61].

Recently much focus has been attributed exploring the role of PYK2 in the vascular wall[40, 62, 63]. PYK2 belongs to the Focal Adhesion Kinase (FAK) family of nRTKs, of which FAK is the only other member.

FAK and PYK2 are structurally closely related with 65% similarity in amino acid sequence. Both kinases share a very similar centrally located catalytic protein tyrosine kinase domain flanked by a non-catalytic amino (N-) terminal region and two proline-rich regions at the carboxyl (C-) terminal (Figure 3). It is worth mentioning that neither member of the FAK family nRTKs has SH2 or SH3 (Src Homology) domains, which are otherwise common in nRTKs.

The amino (N-) terminal domain of PYK2 has a tyrosine (Y) residue at position 402; it is not only a major autophosphorylation site but also a binding site for SH2. Phosphorylation of PYK2 at Y402 leads to binding of Src via its SH2 domain, which is essential not only for activation of PYK2 itself but also for its association with the signal transduction adaptor proteins, paxillin and p130CAS[64]. It has been suggested

that the N-terminus might act as a link between transmembrane receptors and PYK2[65].

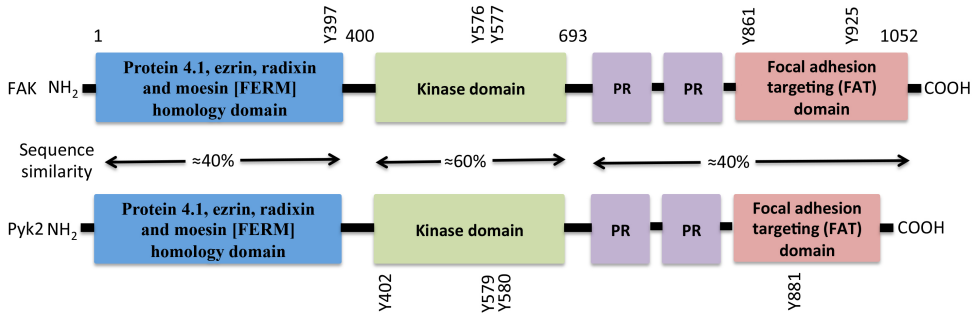

Figure 3: Homology structure of FAK and PYK2. PR: proline rich domain.

1.6 MicroRNAs

MicroRNAs (miRNAs) are small (~22 nucleotide long) non-coding RNAs (ncRNAs) which can modulate gene expression post-transcriptionally by binding to the 3'untranslated regions (UTR), coding sequences or 5′UTR of target messenger RNAs (mRNAs) which leads to inhibition of protein synthesis or mRNA degradation[66, 67]. There are about 2000 human miRNAs (miRBase v21), which may target 60% of the genome. On average one single miRNA can target ~200 mRNA transcripts. Interestingly, it has been suggested that even though a single miRNA may subdue the production of hundreds of proteins, this suppression frequently is rather minor (~2-fold) However, since miRNAs can regulate multiple proteins in a signaling pathway simultaneously, they may still have a major effect on specific biological processes.

Before the 1990s, miRNAs were thought to be insignificant in mammals and only essential in non-mammalian species. In 1993, it was discovered that lin-4 gene produced a 61-nucleotide precursor gene, which subsequently matures into an abundant 22-nucleotide transcript, instead of a protein product[68]. Successively it was found that lin-2 RNA product post-transcriptionally regulate LIN-14 translation and that lin-4 RNA has sequence complementarity to the 3'-UTR of the lin-14 gene[69]. It was not until 7 years later a second miRNA was found in *C elegans,* called let-7, which suppressed lin-14, lin-28, lin-41, lin-42, and daf-12 expression in developmental stage[70]. Subsequent detection of let-7 homologs in various species including humans inspired a substantial effort to clone small RNAs, demonstrating

that miRNAs are evolutionarily preserved across different species and frequently ubiquitously expressed.

1.6.1 Biogenesis and target recognition of microRNAs

Primary transcripts (pri-miRNAs) are generated from either introns of protein coding genes or from individual miRNA genes. In the nucleus, pri-miRNAs are further processed by Dicer and Drosha, two members of RNAse III family and DGCR8, dsRNA binding protein[71]. First, pri-miRNAs are processed by Drosha-DGCR8 complex into a ≈ 70-100 nucleotide long and hairpin shaped precursors called pre-miRNA. Subsequently pre-miRNAs are exported to the cytoplasm by Exportin-5/Ran-GTP complex and further processed by endonuclease Dicer in cooperation with transactivation response RNA binding protein (TRBP) which results in ≈ 20bp miRNA: miRNA duplex. The duplex is further processed by Argonaute (Ago) (possesses RNaseH-like endonuclease activity), which cleaves the 3' end of pri-miRNAs to create ac-pre-miRNA[72]. Subsequently one stand (called guide strand) of the miRNA: miRNA duplex is incorporated into miRNA-induced silencing complex (miRISC) and the remaining passenger miRNA strand or miRNA* is most commonly degraded but occasionally it can also be loaded into miRNA-RISC complex to function as mature miRNAs. It is still unclear as to how it is decided that which strand will go into the silencing complex but it is likely that the strand with least stable base pairing at the 5' end will have the higher propensity[66].

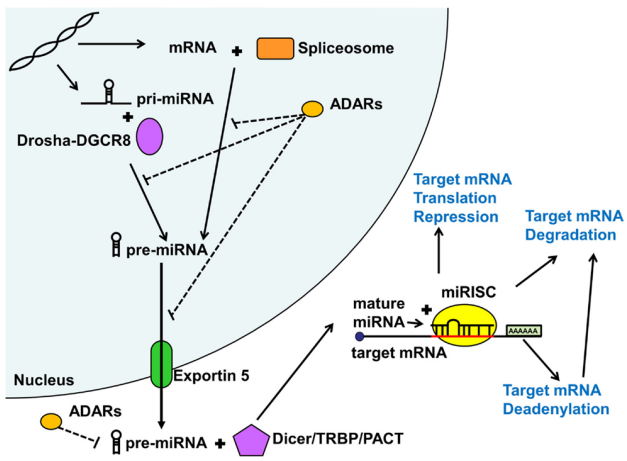

Figure 4: Biogenesis of microRNAs as described in 1.6.1. Image[73] is licensed under a Creative Commons Attribution License CC-BY 3.

Most frequently, miRNAs form an imperfect base pairing with 3'-UTR of the target mRNA and inhibit translation either by degrading mRNA by deadenylation mediated

by GW182 proteins or suppressing proteins synthesis[74, 75]. Achieving effective target recognition requires continuous base pairing of miRNA in nucleotide 2 to 7 (seed pairing), which is often supplemented by a pair in nucleotide 8 and a A in nucleotide 1[66].

Both Ago and GW182 proteins are primary components of silencing complex with most mammals possessing at least 4 different homologues of Ago (Ago1–Ago4). Ago interacts with GW182 through its N-terminal part (via GW repeats) and while c-terminal part of GW182 communicate with the poly (A) binding protein (PABP) to recruit deadenylases CCR4, NOT and CAF1[76, 77].

1.6.2 Vascular microRNAs

The first association between miRNAs and disease was revealed in 2002 when Calin et al showed the manifestation of B-cell leukemia with loss of miR-15 and miR-16[78]. The first cardiac miRNA-profiling study linking cardiac remodeling with dysregulation of various miRNAs in both mice and humans were published in 2006[79]. As a consequence of these findings, a lot of focus went into understanding how an alternation of specific sub-set of miRNA expression is associated with a particular disease. Even though various *in vitro* studies inferred essential well-defined roles for miRNAs in various aspects of cellular mechanisms, the major genetic evidence regarding the importance of miRNAs came through deletion of Dicer, a key endonuclease, which is responsible for processing most of the pre-microRNAs to mature microRNAs[80]. Deletion of Dicer in smooth muscle results in prenatal death in mice due to hemorrhage in the abdomen and skin[81]. The tamoxifen-inducible, conditional Dicer knockout mice have now been used to investigate the importance of miRNAs in various organs, including blood vessels[82-86]. Smooth muscle specific deletion of Dicer in adult mice causes lower blood pressure, reduced vascular contractility, myogenic response and triggers phenotypical switch in smooth muscle. Although adult mice die due to global loss of smooth muscle specific miRNAs around 12-14 weeks post tamoxifen treatment, it has been suggested that gastrointestinal but not vascular phenotype is accountable for the lethality[86].

There are several miRNAs found to be essential for normal function of the smooth muscle with the miR-143/145 cluster being the most crucial for smooth muscle contractile differentiation of smooth muscle[87-91]. Cordes et al demonstrated that SRF act synergistically with myocardin to regulate miR-143/145 expression and promote differentiation. MiR-143/145 also inhibits Elk-1 and KLF-4, which also interacts with SRF to promote a less differentiated and more proliferative smooth muscle phenotype[92]. Cheng et al. further reinforced role of the miR-143/145 cluster by demonstrating that overexpression of miR-145 elevated smooth muscle contractile

marker gene expression that was reduced when smooth muscle cells were treated with miR-145 inhibitor. They also showed that miR-145 is a potent modulator of vascular neointimal lesion formation following carotid artery balloon injury[93]. The importance of miR-143/145 in vasculature was further demonstrated by deletion of miR-143/145 that was associated with lower blood pressure but without cardiac abnormalities; severe decline in the number of contractile but increase in synthetic smooth muscle cells in the aorta and as well as in the femoral artery characterized by decrease in actin fiber, a thinner media and increased rough endoplasmic reticulum[94].

In a vascular injury model, inhibition of miR-221 decreased neointima thickness by ≈40% and the effects of miR-221 might be fortified by a synchronized up-regulation of miR-21[95, 96]. Mir-21 promotes smooth muscle proliferation while preventing apoptosis by targeting of the phosphatase and tensin homolog (PTEN) and up-regulating Bcl-2.

Using a stretch model in culture Song et al. suggested that miRNA-21 is upregulated with stretch and plays an important role in stretch-induced proliferation and apoptosis via activator protein 1 (AP-1) dependent pathway in human aortic smooth muscle cells[97]. Among other miRNAs that are changed due to stretch are mir-26a and miR-144/451. Mohamed et al. showed that in human airway smooth muscle cells, stretch selectively stimulates transcription of miR-26a located in the locus 3p21.3 of human chromosome 3 and induces hypertrophy via targeting glycogen synthase kinase-3β (GSK-3β)[98]. Turczyńska et al. showed that stretch in mouse portal vein can activate AMPK and activation of AMPK is correlated with downregulation of miR-144/451 cluster[99]. Furthermore, miR-146a has been reported to be mechanosensitive in primary human small airway epithelial cells (HSAEpCs) where it can regulate pressure-induced cytokine secretion and can target toll-like receptor proteins IRAK1 and TRAF6[100]. It has also reported that downregulation of miR-146a inhibited proliferative and migratory characteristics of vascular smooth muscle cells in vitro, while promoting Bax induced apoptosis[101].

2. Aims

The general aim of this thesis is to gain insight into the role of vascular smooth muscle cells in response to pressure/stretch or vascular injury. We aimed to elucidate:

1) Role of miRNAs in influencing stretch/pressure-induced signaling in the vascular wall (Paper I-III).

2) Involvement of PYK2, a non-receptor tyrosine kinase in stretch-induced calcium-handling mechanism and phenotype modulation in the vascular wall (Paper IV & V respectively).

3. Methods

3.1 Animal models

The Malmö/Lund animal ethics committee has approved all the experiments used in this thesis (M167-09 and M213-12). All investigations conform to Directive 2010/63/EU of the European Parliament[102].

3.1.1 Dicer knockout mice

As described in section 1.6.1, Dicer is a prerequisite for processing most pre-miRNAs into mature miRNAs. Thus deletion of Dicer has been widely used as an approach to investigate the global function of miRNAs. Since conditional smooth muscle deletion of Dicer results in embryonic lethality[103], we used an inducible and smooth muscle specific Dicer KO mouse, which was generated using cre-loxP recombination system[104]. A target gene (e.g., Dicer) can be deleted by inserting it between two recombinase recognition (loxP) sites while Cre can recognize and remove the target gene. Cre is a site-specific DNA recombinase, which identifies and facilitates recombination at the loxP recognition site for Cre, which is comprised of an 8-bp asymmetric spacer (determines the orientation of the loxP site) surrounded on both sides by 13-bp inverted repeats[105]. The floxed strain, generated by Merkenschlager and co-workers, harbors the loxP-flanked Dicer RNaseIII domain (exons 20 and 21), initially introduced into the germline by homologous recombination [106]. The second strain, generated by Offermanns and co-workers, provides the Cre recombinase $CreER^{T2}$ expressed from smooth muscle cell type-specific specific myosin heavy chain (SM-MHC) promoter region[107]. Cre recombinase is fused with mutated ligand binding domain (LBD) of the human estrogen receptor (ER^{T2}) and can be activated by *in vivo* by administrating an estrogen antagonist such as tamoxifen[86]. Dicer KO mice were then generated by crossing $Dicer^{flox/flox}$ and MHC-$CreER^{T2}$ (Figure 5).

The inserted MHC-$CreER^{T2}$ is on the Y-chromosome, and thus females are Cre negative. Male Dicer KO mice were administered with approximately 0.1 ml

tamoxifen (50 mg/kg/day) or vehicle (1:10 EtOH in sunflower oil) for 5 consecutive days at the age of 3–4 weeks. Mice administered with vehicle were used as controls. Any direct effect of tamoxifen on contraction was excluded by treating Cre negative mice with tamoxifen and evaluating 60mM KCl-induced contraction on mesenteric resistance arteries (MRA)[86]. All mice were on a mixed C57Bl/6 and SV 129 background.

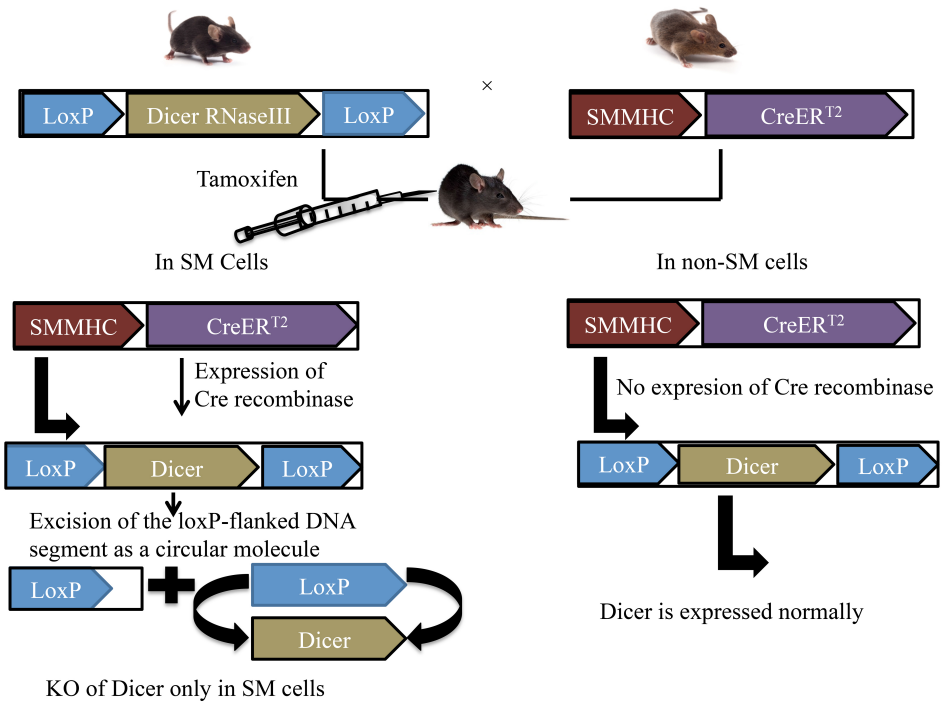

Figure 5: Generation of smooth muscle specific Dicer knockout mice. SMMHC: smooth muscle myosin heavy chain.

3.1.2 MicroRNA-143/145 knockout mice

The conserved miR-143 and miR-145 encoding genes reside in close proximity with each other on murine chromosome 18 (≈1.4 kb) and are transcribed from the same gene. Boettger et al. generated microRNA-143/145 knockout mice by substituting the miR-143/145 coding genomic region with a lacZ reporter, deleting the sequences coding for the mature miR-143 and miR-145 and a 1.3 kb fragment located between the 2 genes[94]. Mice were bred in-house.

3.1.3 Portal vein organ culture model

The portal vein transfers nutrient-rich blood from the spleen and gastrointestinal tract to the liver sinusoids. It has a dominant longitudinal smooth muscle layer and a thin inner circular smooth muscle layer (see Figure 6A). The smooth muscle of the portal vein shows phasic spontaneous activity caused by bursts of action potentials generating influx of calcium via voltage-gated channels. The longitudinal smooth muscle layer hypertrophies when a loose ligature is placed around the vein at the liver hilus, causing increased (2-3 fold) intraluminal pressure. The hypertrophy is characterized by increased expression of contractile smooth muscle marker proteins as well as increased contractile force following 7 days of obstruction[108-110]. The effect of pressure *in vivo* can be replicated *ex vivo* by stretching the portal vein longitudinally in organ culture. Stretch of the portal vein *ex vivo* results in larger cell size, increase of DNA and protein synthesis, activation of the ERK1/2 pathway and longitudinal remodeling[111]. It is worth noting that this *in vitro* model demonstrates the effects of physiological stretch of the vessel wall vs. the absence of stretch, and thus does not compare normal vs. hypertensive conditions, as does the *in vivo* portal hypertension model. After 3 days of culture in the stretched condition, portal vein strips maintain contractility to a similar level as that of fresh preparations whereas portal veins cultured without any load lose their contractile ability[111].

Serum is commonly used in cell culture to stimulate growth and proliferation and presence of serum is essential to maintain cell viability over prolonged time. Several studies have shown that organ culture in the presence of 10-20% of fetal calf serum (FCS) impairs contractility,[112-114] which could be due to the presence of vasoconstrictors causing increased intracellular calcium, since the calcium channel inhibitor verapamil partially preserves contractility in culture with 10% FCS[115]. Detrimental effects of FCS can be avoided by dialysis and by reducing its concentration[111]. In the studies described in this thesis, portal veins were cultured in DMEM-Hank's F12 (1:1) with 2% dialyzed FCS and 10^{-8} mol/L insulin, which stimulates growth at a low concentration of added protein. For short-term stretch (10 min), the weight was suspended with a thread during overnight incubation to allow vessels enough time to accommodate following mounting, and then released for 10 min before the vessel strip (for rat, half of the vessel was used) was frozen either in liquid nitrogen or in ice-cold 10% acetone-trichloroethanoic acid (TCA) – 10 mM dithiothreitol (DTT). Long-term effects of stretch/load were studied under continuous load for 24h-5d. The unloaded control strips were treated identically. The culture model is depicted in Figure 6B.

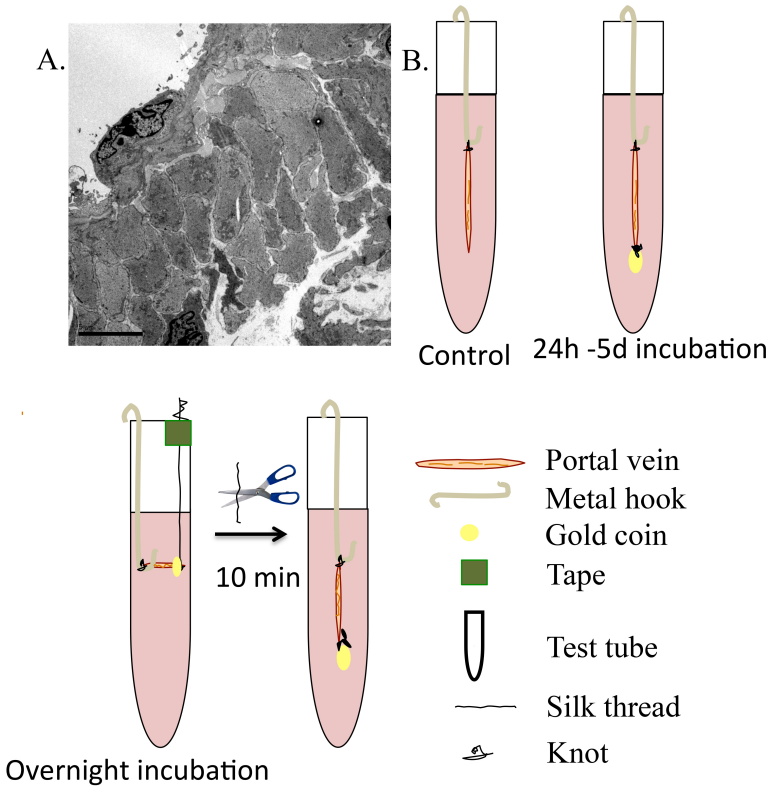

Figure 6: (A) Electron myograph of a mouse portal vein in transverse section. (B) Portal vein culture model. See text for details.

	Animal model	Vascular bed used
Paper I	Dicer KO (mouse)	Small mesenteric arteries, aortic vascular smooth muscle cells
Paper II	microRNA-143/145 KO (mouse)	Portal vein
Paper III	microRNA-143/145 KO (mouse)	Small mesenteric arteries
Paper IV	Rat	Portal vein
Paper V	Rat	Small mesentric arteries, carotid artery, smooth muscle cells from carotid artery

Table 2: Summary of animal models and vessels used from various vascular beds.

3.2 Chemicals

3.2.1 PYK2 inhibitor PF-4594755

A main limitation of tyrosine kinase inhibitors is their non-selective nature. Most commonly used commercially available tyrosine kinase inhibitors include genistein and tyrphostin A47. Whereas genistein inhibits the ATP-binding site[116], tyrphostin A47 interacts with the substrate-binding site[117]. Both these inhibitors have been reported to eliminate or mitigate constriction exerted by contractile agonists such as Angiotensin II (Ang II) or noradrenaline and also to affect calcium-handling mechanisms[118-121]. Even though these results indicate a crucial role of tyrosine kinases it is not possible to assess a role specifically of PYK2 in this context.

A more specific PYK2 inhibitor, PF-4594755, was developed by Pfizer Inc. by screening structure–activity relationships of a series of diamino pyrimidines. In biochemical assays the compound was found to be more than 100-fold selective for PYK2 over FAK and Src, and in NIH3T3 cells it inhibited PYK2 autophosphorylation at Tyr-402 with a calculated IC_{50} of 120 nM[122]. In the present experiments we used PF-4594755 (a kind gift from Pfizer Inc.) in concentrations of 0.5 or 1 μM.

3.3 Pressure myography

A pressure myograph system (Living Systems Instrumentation, St. Albans, Vermont, U.S.A) was used to investigate functional characteristics and effects of pressurization of small arteries. For our study (Paper I, II & V) we used $2^{nd}/3^{rd}$ order mesenteric arteries from mice (Paper I & II) or rat (V).

The main components of a pressure myograph include a chamber fitted with small cannulas or micropipettes for mounting vessels, a pressure servo system, a pressure monitor, and pressure transducers on the inflow and outflow side of the chamber (Figure 7A). The pressure servo system regulates intraluminal pressure via two peristaltic pumps. The pressure myograph chamber is fitted with a built in heating capability to ensure a constant temerature. A Nikon Diaphot 200 inverted microscope equipped with a charge-coupled device (CCD) camera is used to monitor the vessel in real time. The CCD camera is connected to a computer with software

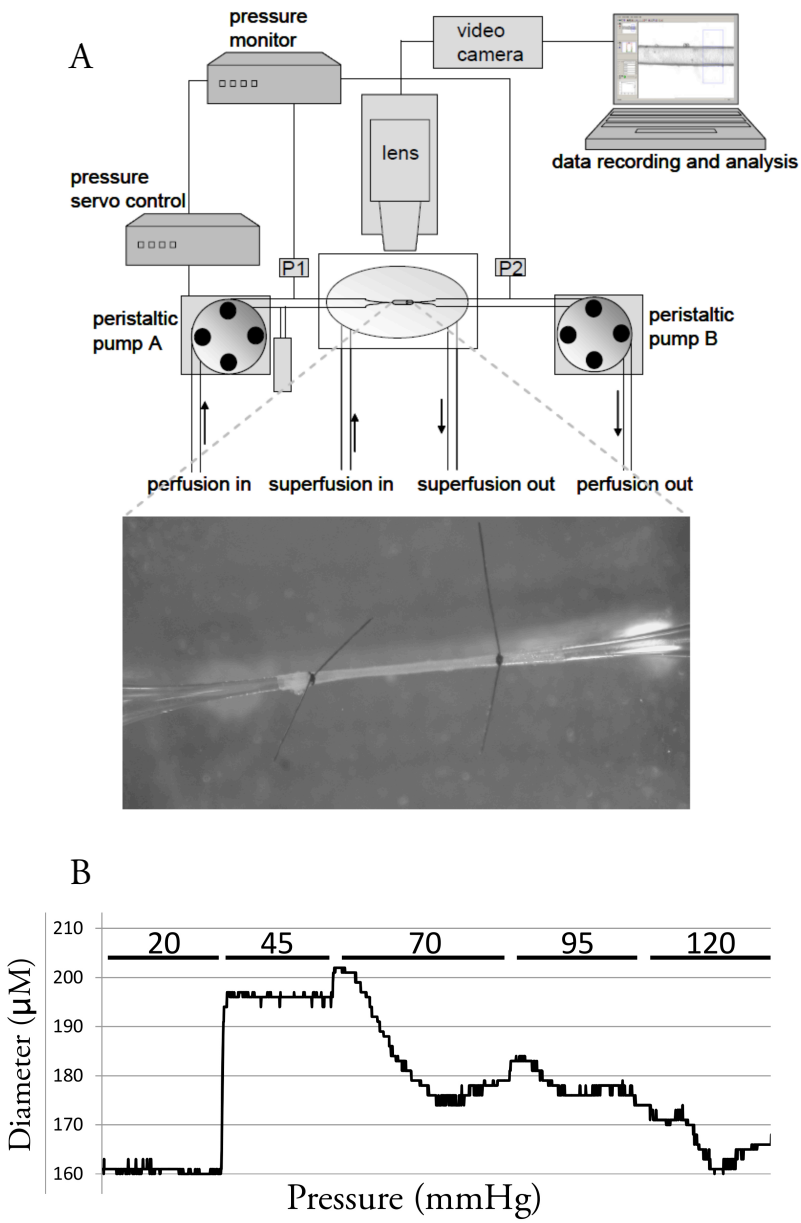

Figure 7: (A) Pressure myograph setup. (B) Example of a pressure-diameter recording. See text for details.

capable of detecting the edges of the vessel, thus measuring vessel diameter. The of pressure myograph system is depicted in Figure 7A. All pressure-induced myogenic response experiments were done in buffered saline solution (composed of 135.5 mM NaCl, 5.9 mM KCl, 2.5 mM CaCl2, 1.2 mM MgCl2, 11.6 mM glucose, and 11.6 mM HEPES, pH 7.4 @37°C). For overnight incubation of rat mesenteric arteries in the pressure myograph, DMEM-Hank's F12 (1:1) with 2% dialyzed FCS and 10 nM insulin was used, and the myograph chamber was placed inside a benchtop incubator. Stepwise increases in pressure by 25 mmHg increments resulted in myogenic constriction in the range of 70-120 mmHg, as shown by a typical recording in Figure 7B.

3.4 Extraction and quantification of messenger and microRNAs

RNAs were isolated using commercially available total RNA extract kit (miRNeasy Mini Kit, Qiagen, Germany). The first essential step of RNA analysis is the extraction of good quality RNA. Trizol based methods have long been used to extract high quality RNA from cell or tissue[123, 124]. Tissue samples are disrupted with TissueLyser LT and homogenized in QIAzol Lysis Reagent (Qiagen, Germany), which is a monophasic solution of phenol and guanidine thiocyanate. QIAzol prevents RNA degradation by blocking RNases and also helps in the lysis of tissue. Following addition of chloroform (20% vol. of QIAzol) the homogenate is shaken vigorously for 5 minutes to ensure proper mixture and then segregated into organic and aqueous phases with centrifugation (12,000 x g for 15 min at 4°C). RNA separates to the upper, aqueous phase and DNA and protein to the interphase and lower organic phase, respectively. The upper aqueous phase is transferred to a new tube and with addition of RNAase free ethanol, binding conditions for all RNA molecules from 18 nucleotides (nt) upwards was optimized. The ethanol-sample mixture was then pipetted to an RNeasy Mini spin column, which is specially designed to bind the RNAs while other contaminants are washed way. Subsequently, RNAs were eluted with RNAse free water. Concentration and quality of the extracted RNA was measured using spectrophotometer (Nanodrop, Thermo Scientific). For mRNA, a single-step quantitative real time polymerase chain reaction (qRT-PCR) was used to measure expression level, whereas mature miRNAs were first polyadenylated at the 3’ end by poly(A) polymerase and reverse transcribed into cDNA using oligo-dT primers. A degenerate anchor is attached to the 3' end of the oligo-dT primers and a

universal tag sequence on the 5', which allows amplification of mRNA or miRNA in the real-time PCR step using SYBR green. SYBR green binds to the double stranded DNA and emits fluorescence that can be measured in real time. Cycling conditions for real-time PCR includes an initial activation step (15min, 95°C): HotStarTaq DNA Polymerase is activated by this heating step. This is followed by three step cycling (around 35-40 cycles): 1) Denaturation (15s, 94°C): double stranded cDNA is denatured 2) Annealing (30s, 55°C): specific primers are added to the cDNA and 3) Extension (30s, 70°C for mRNA and 72°C for mRNA): polymerase add more primers along the length of the template. For mRNA, one addition step (30min, 50°C) was added before initial activation step to transcribe cDNA from mRNA since one-step uRT-PCR was used for mRNA detection. One of the pitfalls of SYBR Green is that it can bind to the "non-specific" primer dimers thus giving false positive results, which is avoided by careful analysis of melting curves[125]. A housekeeping gene with stable expression level was always used to analyze the relative expression with the delta delta $C_{(T)}$ method [126].

We have also used a customized qRT-PCR array (Qiagen, Germany) to measure expression level of smooth muscle specific mRNA and miRNAs, which works on the same principle as described before. Computational prediction tools such as Targetscan (http://www.targetscan.org), microCosm (http://www.mirbase.org), miRanda (http://www.microRNA.org) or PicTar (http://pictar.mdc-berlin.de) were used to discover potential mRNA targets of specific miRNA or vice versa. The databases use common approaches such as evolutionary conserved "seed region" (bases 2 to 8, see section 1.6.1 for detail), the thermodynamic stability of the miRNA:mRNA duplex and the existence of complex secondary structures adjoining the miRNA binding sites to predict targets[127]. PicTar not only allows perfect seed complementarity but also imperfect seed complementarity where one non-complementary mutation or insertion providing the free energy for binding of the miRNA: mRNA duplex is unchanged or does not include a G.U base pairing[128]. The "Context score" feature in TargetScan improves estimations for nonconserved sequences by taken into account features in the adjoining mRNA, including local A-U content and position (proximity to the 3'UTR is favored) [129]. A common tactic to improve specificity is to combine several target prediction tools and to look for overlapping targets. A downside of this approach is lower sensitivity compared to a single prediction tool.

The most common method to manipulate miRNA expression is to use miRNA mimics or miRNA inhibitors. MicroRNA mimics are synthetic duplex molecules chemically modified to increase stability and cellular uptake and to imitate the endogenous miRNA of interest. The "guide strand" of the synthetic miRNA mimic is similar to the miRNA of interest, whereas the "passenger strand" is altered and commonly linked to a molecule such as cholesterol for increased cellular uptake[130].

MicroRNA inhibitors can reduce the endogenous levels of a miRNA and are chemically modified antisense oligonucleotides containing the full or partial complementary reverse sequence of a target mature miRNA[131].

A comprehensive listing of methods used in miRNA research is beyond the scope of this thesis (reviewed elsewhere[128, 132, 133]) but most commonly used techniques in miRNA research are listed in table 3.

Detection	Target determination	Regulation
Microarray analysis	*Computational algorithms*	*Genetic manipulation of microRNAs*
Real time PCR	UTR analysis	*In vitro miRNA mimicry*
Deep sequencing	Transcriptome/proteome analysis	In vitro miRNA inhibition
Northern blotting	Pull down assays	In vivo miRNA regulation
In situ hybridization		

Table 3: Summary of techniques commonly used miRNA research. Methods used in this thesis is in italics. Modified from[133]

3.5 Extraction and quantification of proteins

Proteins from frozen vessels were extracted in 2% sodium dodecyl sulfate (SDS) buffer (Laemmli buffer) and protein concentration was determined with Biorad DC™ Protein Assay (modified Lowry method), which is a colorimetric assay based on the reaction of proteins with an alkaline copper tartrate solution and Folin reagent.

SDS is an anionic detergent that applies a negative charge to each protein in proportion to its mass and also removes any complex secondary/tertiary structure to make the protein molecule linear. Heating the sample for a brief period (70°C, 10 min) can further facilitate denaturation of proteins. Mercaptoethanol or DTT was used to retain the protein in denatured state. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate protein mixtures by their molecular size. Electrophoresis is performed by adding extracted proteins from tissue/cells onto a well in a porous matrix comprised of polyacrylamide. In an electric field, differently sized and negatively charged protein molecules in the sample move towards the positive charged anode through the matrix at different velocities. Since the charge-to-mass ratio is approximately equivalent in denatured protein molecules, the final separation of proteins is almost exclusively dependent on the disparities in relative molecular mass of the proteins.

At the end of the separation, the proteins were detected as bands separated according to size. Following separation, the proteins were electrically transferred onto nitrocellulose membranes with small pores of either 0,2 µM or 0,45 µM. The transferred proteins are bound to the surface of the membrane and are readily accessible to antibodies. Immersing the membrane in a solution containing blocking agent (1% casein or 5% milk) blocks all unspecific binding sites in the membrane. Following overnight incubation with a primary antibody, the membrane was washed with TBS containing 0.1% tween, and the antigen was identified by detection with a secondary horseradish peroxidase or fluorescently conjugated anti-IgG antibody. For enhanced chemiluminescence, a substrate was added to the membrane resulting in a luminescent reaction at the site of the antigen-antibody complex. The luminescence was detected by a CCD camera (LI-COR) for analysis.

3.6 Statistics

Numerical values are represented as standard error of mean (± S.E.M.) unless otherwise mentioned. For comparison between two groups, unpaired Student's t-test (parametric, assuming Gaussian distribution) was performed. For comparison between more than two groups one-way analysis of variance (ANOVA) was used with Bonferroni post-hoc testing for multiple comparisons. 2-way ANOVA was used when multiple factors were involved (for example pressure-diameter relationships). All analyses were performed using GraphPad Prism 5 (GraphPad Software Inc.). $p<0.05$ was considered statistically significant. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

4.Results and Discussion

4.1 MicroRNAs play an imperative role in the myogenic response and stretch-induced contractile differentiation

4.1.1 The myogenic response in resistance arteries is regulated by microRNAs

Dicer KO

As discussed in section 1.6.2, the endonuclease dicer is critical for processing of pre-miRNAs into mature miRNAs in the cytoplasm and disruption of this process results in embryonic lethality at E16.5 to E17.5 due to widespread internal hemorrhaging [103]. To overcome this, we used the tamoxifen-inducible smooth muscle specific conditional Dicer KO mice (discussed in methods). Using a stretched portal vein model, we have previously shown that spontaneous activity and stretch-induced contractile differentiation is lost in Dicer KO mice at 10 weeks post-tamoxifen treatment [84]. As a follow-up to this study, the main aim of Paper I was to investigate if acute pressure-induced myogenic tone in small arteries was dependent on the expression of miRNAs.

Following tamoxifen injections, inactivation of Dicer and miRNAs occur in a time-dependent manner. We first established the time course of deletion of smooth muscle enriched miRNAs. Among 48 miRNAs analyzed, 28 (≈43%) and 38 (≈58%) miRNAs were downregulated (2 fold or more) at the 2 week and 3 week time point, respectively. Four weeks after tamoxifen treatment, 58 (≈89%) of the miRNAs were significantly downregulated. The remaining 7 (≈11%) miRNAs could either be processed by a Dicer independent mechanism or, more likely, they are also expressed in other cell types present in the vascular preparation such as endothelial cells or fibroblasts. Since we wanted to avoid any general effect on contractile function and investigate the direct effects of miRNA deletion on pressure-induced signaling

mechanisms and myogenic responses, we specifically chose to use an early time point at five weeks post-tamoxifen treatment. Using a pressure myograph setup (see section 3.2 for details) we evaluated the pressure-induced myogenic response and found that this was entirely absent in Dicer KO MRA.

In a previous work, Albinsson et al. showed that Dicer KO, 6–8 weeks post-tamoxifen treatment, caused a decline in both systolic and diastolic blood pressures[86]. At ten weeks post tamoxifen (late time point) they observed a reduced media area and thickness but unchanged lumen diameter in aorta and they attributed this effect to loss of smooth muscle cells. At the early time point of five weeks, we measured media thickness, media area and lumen diameter to understand if loss of myogenic response could be due to arterial remodeling, but found no significant change in any of the measured parameters (Paper I). This discrepancy can be attributed to the time course of Dicer knockdown (early vs. late stage) or to different vascular bed (mesenteric vs. aorta) with different properties (resistance vs. elastic). Interestingly, at the early time point we have observed a significant decrease in distensibility indicating altered elastic properties of mesenteric arteries, which may compensate for part of the reduced myogenic tone. The reduced distensibility results in a similar active diameter in WT and Dicer KO at 70mmHg, which is likely to be close to the physiological pressure in small mesenteric arteries[134].

It was also shown that expression of some smooth muscle contractile marker proteins in aorta was reduced at both mRNA and protein level at ten weeks after tamoxifen treatment and this reduction resulted in both a reduced contractile response to depolarization by KCl and to the α_1-adrenergic receptor agonist phenylephrine[86]. To clarify the effect of Dicer deletion on expression of contractile marker proteins in MRAs at the earlier time point, we measured expression of several smooth muscle contractile markers at both messenger and protein level. Even though three out of four markers were reduced at the messenger level, only tropomysosin-1 was significantly reduced at the protein level. This indicates a minor effect of miRNA deletion on the assessed contractile marker proteins at this early time point in MRAs and this is thus unlikely to have a significant effect in the loss of the myogenic response.

MicroRNA-143/145 KO

The miR-143/145 cluster is one of the most highly expressed in the smooth muscle and it has been previously reported that deletion of miRNA-143/145 causes decreased blood pressure[94]. Since myogenic tone could play an important role for blood pressure regulation in miR-143/145 KO mice, the aim of the second study was to evaluate pressure-induced responses in small mesenteric arteries deficient of the miRNA-143/145 cluster. Boettger et al. demonstrated that when challenged with angiotensin II, miR-143/145 KO mice show abridged increase in systolic blood pressure

compared to wild type mice[94]. Furthermore, saphenous arteries from these mice showed reduced contraction in response to phenylephrine, AngII, and calcium. Myogenic tone was impaired in both homozygous and heterozygous mice but unlike Dicer KO, distensibility was unaltered, indicating unchanged elastic properties in miR-143/145 MRAs. The loss of myogenic tone in heterozygous mice is interesting since contractile function in response to depolarization is maintained in these mice, suggesting that myogenic responses are exceptionally sensitive to the expression of this miRNA cluster.

Using KO mouse models several previous reports have shown that expression of miR-143/145 cluster is essential for maintaining normal contractile phenotype[94, 135]. The phenotypic switch is associated with enlarged and increased number of rough endoplasmic reticulum, a known phenomenon associated with the synthetic phenotype. Even though Elia *et al.* reported decreased expression of smooth muscle alpha actin (*Acta2*) and smooth myosin heavy chain (*Myh11*) transcripts, this is contradicted by other studies where expression of smooth muscle contractile protein was unchanged[136]. We investigated phenotypic switch as reason for the loss of myogenic responses in miR-143/145 KO MRAs. Using custom-made mRNA arrays for smooth muscle markers, we analyzed 42 genes associated with either the synthetic or contractile phenotype of smooth muscle cells (Table 4). Interestingly, established smooth muscle marker genes such as calponin (*Cnn1*), smooth muscle myosin heavy chain (*Myh11*) and SM22α (*Tagln*) were unchanged at both mRNA and protein (data not shown), which is in agreement with the report of Xin et al. Osteopontin (*Spp1*) is known to be associated with the synthetic smooth muscle phenotype shift and is also unchanged in MRAs.

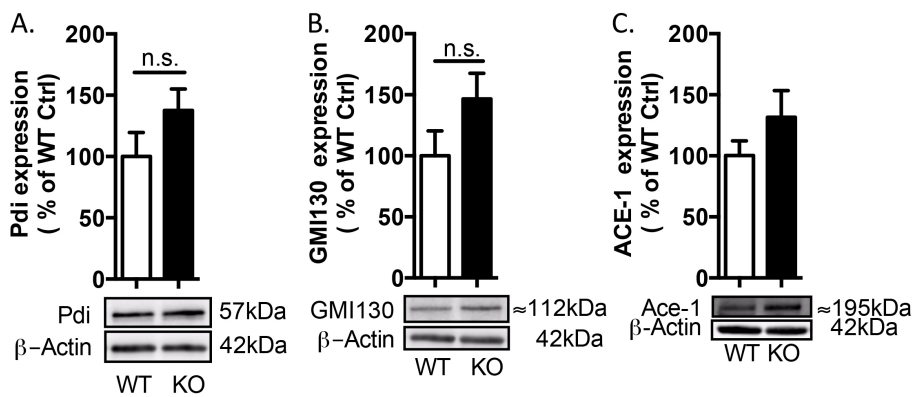

Figure 8: Expression of Pdi 9 (A), GM130 (B) and Ace-1 (C) is measured using western blot.

We also measured protein expression of protein disulfide isomerase (Pdi), a marker of Endoplasmic Reticulum (ER) and GM130, a Golgi family of proteins and marker of

Golgi expression since increased expression ER and Golgi proteins are associated with smooth muscle phenotype shift[94, 135]. Expression of either PDI or GM130 was not significantly altered in miR-143/145 MRAs. Boettger et al attributed loss of contractile phenotype in miR-143/145 KO aorta to increased expression of angiotensin converting enzyme-1 (ACE-1). ACE-1 converts angiotensin I to angiotensin II (Ang II) by excluding two C-terminal residues. Ang II is a peptide hormone that exerts various effects on vascular smooth muscle including contraction. While low amounts of Ang II can stimulate contractile differentiation of smooth muscle cells, Ang II signaling is rapidly desensitized and excessive stimulation can therefore result in a state of "angiotensin resistance" [137].

Gene name	Gene symbol	Fold Regulation (vs. WT ctrl)
Serum response factor	Srf	2,0857
Myocardin	Myocd	1,4031
3 MKL (megakaryoblastic leukemia)/myocardin-like 1	Mkl1	3,3768
GATA binding protein 6	Gata6	1,8794
GATA binding protein 4	Gata4	1,347
Calponin	Cnn1	1,0281
Myosin, heavy chain 11, smooth muscle	Myh11	-1,1474
Calcium channel, voltage-dependent, L type, alpha 1C subunit	Cacna1c	1,3193
Prominin 1	Prom1	1,4788
Chloride channel accessory 1	Clca1	15,3113
Decorin	Dcn	1,4078
ADAM metallopeptidase with thrombospondin type 1 motif, 7	Adamts7	-2,324
Transient receptor potential cation channel, subfamily C, member 6	Trpc6	1,5363
Syncoilin	Sync	4,4459
Neuropilin 2	Nrp2	-1,1298
Integrin, alpha 8	Itga8	-1,4339
Synaptopodin 2	Synpo2	-1,5404
Dystrophin	Dmd	-2,1523
Potassium large conductance calcium-activated channel, subfamily M, beta member 1	Kcnmb1	-1,7659
Leiomodin 1 (smooth muscle)	Lmod1	-1,9857
Phosphatase and actin regulator 4	Phactr4	-1,9178
Kruppel-like factor 4	Klf4	-1,1923
Kruppel-like factor 5	Klf5	-1,9884
Smoothelin	Smtn	-3,2342
Secreted phosphoprotein 1/Osteopontin	Spp1	-1,1086
Angiotensin I converting enzyme	Ace	-1,5446
Rho-associated, coiled-coil containing protein kinase 1	Rock1	-1,3385
Myocyte enhancer factor 2C	Mef2c	-3,7399
Protein phosphatase 1, regulatory subunit 12A	Ppp1r12a	-1,8113

Myosin, light chain 9, regulatory	Myl9	-1,2127
Rhodopsin	Rho	-1,7252
Calcium/calmodulin-dependent protein kinase II gamma	Camk2g	1,1519
Calcium/calmodulin-dependent protein kinase II delta	Camk2d	-1,0211
Protein tyrosine kinase 2 beta	Ptk2b	-1,0669
Transgelin	Tagln	-1,0761
Caveolin 1,	Cav1	1,4235
SLIT-ROBO Rho GTPase activating protein 1	Srgap1	-1,4482
Cofilin 2 (muscle)	Cfl2	-1,2595
Slingshot protein phosphatase 2	Ssh2	2,8257
Tenascin C	Tnc	-2,9182
Sarcoglycan, gamma	Sgcg	-1,5279
Thrombospondin 4	Thbs4	-1,2161

Table 4: List of vascular smooth muscle related gene expression in miR-143/145 KO MRAs.

However, we found that in miR-143/145 KO MRAs (Paper III), expression of ACE-1 is unchanged at the protein level. Taken together, these results indicate that unlike other vascular beds (aorta, saphenous artery), markers of smooth muscle phenotype in small resistance arteries is not altered due to deletion of miR-143/145 cluster and thus is a highly unlikely a cause for loss of myogenic response.

4.1.2 Expression of microRNA-143/145 is necessary for maintaining stretch-induced contractile differentiation of portal vein

Stretch is an essential factor for maintaining contractile phenotype in portal vein[111, 138]. A previous report showed that global loss of miRNAs in smooth muscle results in loss of stretch sensitivity in portal vein due to the loss of L-type calcium channels[84]. Influx of Ca^{2+} is essential for activation of RhoA and SRF and subsequent expression of smooth muscle contractile markers, which is dependent on translocation of myocardin-related transcription factor (MRTF) to the nucleus. To understand the role of miRNA-143/145 in the context of influencing contractile differentiation we used a portal vein culture model (Paper II), as described in section 3.1.3. Following 24h organ culture, stretch increases expression of contractile smooth muscle marker (*Myh11*, *Tpm1*, *Des*, *Cnn1*) in wild type (WT). However, in miR-143/145 KO portal veins, this response was reduced. Expression of smooth muscle markers (SM22α, α-Actin and Calponin) was also decreased at the protein level as well as the expression of MLCK, suggesting an impairment in actin-myosin cross bridge formation, which is necessary for contraction of smooth muscle. Force generated by phosphatase inhibitor

Calyculin A, which is primarily Ca^{2+}-independent, was also decreased (35%), roughly equivalent to the decrease of media thickness (32%). The amplitude but not frequency of spontaneous activity as well as KCl-induced force was decreased in miR-143/145 KO portal vein. Since spontaneous activity depends on Ca^{2+} signaling, we focused our investigation on calcium handling mechanism.

4.2 Role of miRNAs in smooth muscle calcium handling

4.2.1 Five week deletion of smooth muscle dicer affects the activity but not the expression of calcium channels in MRAs

Previously, Albinsson et al. reported that ten weeks of dicer deletion has a profound effect on smooth muscle contractile machinery, partly due to inhibition of actin polymerization[86]. Since our goal was to understand the direct effects of smooth muscle miRNA deletion on pressure-induced myogenic responses, we used five weeks post-tamoxifen as an early time point (as described in section 4.1.1) (Paper I). We tested the functionality of the contractile machinery using the phosphatase inhibitor Calyculin A in nominally calcium-free conditions. Calyculin A unspecifically inhibits myosin light chain phosphatase and elicits a contraction without an increase of cytosolic calcium concentration [139]. We did not detect a significant change in Calyculin-A induced contraction suggesting that loss of the myogenic response in Dicer KO MRAs at five weeks is not due to alteration of the smooth muscle contractile machinery. We then tested the effect of depolarization using 60mM KCl as depolarizing agent and found that KCl-induced contraction was significantly reduced in Dicer KO MRAs. Since depolarization of smooth muscle is associated with increase of intracellular calcium [140], we measured KCl induced calcium influx in cannulated MRAs using the green fluorescent calcium indicator fluo-4, which can detect calcium concentration in the range of 100nM to 1μM [141]. Fluo-4 intensity was decreased in depolarized KO MRAs suggesting impaired calcium influx, which is further supported by blunted pressure-induced myosin light chain phosphorylation in pressurized MRAs, since influx of calcium is essential for phosphorylation of myosin light chains. Interestingly, by using BayK8644 (300nM), an opener of L-type calcium channels, we managed to completely rescue myogenic tone in KO MRAs. This suggests that there are enough L-type calcium channels on the membrane of the dicer KO mice for BayK8644 to alter the opening probability.

Voltage dependent calcium channels (VDCCs) are complex multisubunit structures consisting of a plasma membrane bound α_1 subunit, a cytoplasmic $Ca_v\beta$ subunit and a predominantly extracellular $\alpha_2\delta$ subunit. There is also a γ subunit but its expression is limited mainly to skeletal muscle [142]. The α_1 subunit consists of four homologous domains (I-IV); each of the single domains constituted of six transmembrane segments (S1–6) and an intracellular amino- and carboxyl-termini. This subunit is mainly responsible for the functional properties known to be essential for pressure induced contraction including Ca^{2+} permeability and voltage sensitivity hence often a target for pharmacological calcium channel modulators [143, 144]. We have previously observed significant loss of $Ca_v1.2$ (voltage-dependent, L type, alpha 1C subunit) in portal vein [84] and bladder [85] at 10 weeks after tamoxifen treatment. To establish if reduced calcium influx during pressure-induced myogenic response is due to loss of voltage gated calcium channels, we measured expression of the pore forming subunit (Cacna1c/$Ca_v1.2$) at the messenger and protein level and found them to be unaltered. This is thus not the cause of the reduced calcium influx in MRAs. We subsequently investigated the involvement of the $\alpha_2\delta$ auxiliary subunit since it has been reported to promote plasma membrane trafficking and modify voltage dependence of activation properties of the pore forming α_1 subunit[145, 146]. In Dicer KO MRAs, we did not detect any significant change in expression of $\alpha_2\delta$-1 compared to WT MRAs, which ruled out any role of the $\alpha_2\delta$-1 for the dysfunctional calcium influx. Between four $Ca_v\beta$ family members, only $Ca_v\beta_2$ and $Ca_v\beta_3$ protein is detected in smooth muscle even though mRNA is present for all of them [146]. $Ca_v\beta_2$ deletion is embryonically lethal due to cardiovascular abnormalities [147] but $Ca_v\beta_3$ null mice are viable and have unaltered contractility and normal blood pressure, thus unlikely to have a direct effect on abolished myogenic response in Dicer KO arteries[148]. Taken together these results (Paper I) indicate that expression of the calcium channel subunits is not influenced by smooth muscle miRNA deletion at the early five-week time point and that decreased activity of the calcium channel might be responsible for loss of myogenic tone in Dicer KO MRAs

4.1.2 Inactivation of the PI3K/Akt pathway may be responsible for the reduced L-type calcium channel activity in Dicer KO resistance arteries

Phosphoinositide 3-kinase (PI3K), a lipid and protein kinase has recently emerged as an important mediator of blood pressure regulation (reviewed in [149]). Vecchione et al. first reported that PI3Kγ null mice not only have diminished contractile responses to angiotensin II and intracellular Ca^{2+} mobilization, but they are also protected from

angiotensin II-induced vascular damage [150]. The same group subsequently demonstrated that genetic deletion of PI3Kγ as well as pharmacological inhibition resulted in a blunted pressure-induced myogenic response in mesenteric arteries [151]. PI3Kγ activation by G-protein-coupled receptors (GPCR) or RTK as well as non-nRTK results in phosphorylation of phospatidylinositol (4, 5) bisphosphate (PIP2) at the D-3 position, which generates phosphatidylinositol (3,4,5) triphosphate (PIP3). PIP3 acts as secondary messenger and recruits pleckstrin homology (PH) domain containing serine/threonine kinase protein kinase B (PKB)/Akt influencing their translocation to the plasma membrane where it can be phosphorylated by PDK1 (Phosphoinositide-dependent kinase 1) at threonine 308 and cause a conformational change to activate Akt [152 153, 154].

It has been previously reported by several groups that Akt plays a prominent role in regulation of Ca^{2+} channels in cardiomyocytes and neuronal cells [155-157]. To test the hypothesis that loss of calcium channel activity might be responsible for the abated myogenic response, we investigated the role of PI3K/Akt in Dicer KO MRAs. First we established that inhibition of the PI3K pathway by LY-294002 (10μM) results in the abolishment of myogenic tone in WT MRAs, confirming an essential need of PI3K activation for a typical myogenic response (Paper I). We then demonstrated that pressure-induced activation of Akt in cannulated MRAs is impaired in Dicer KO. Very recently it has been reported that an increase of intraluminal pressure in cannulated cerebral artery causes phosphorylation of Akt and oxidative inactivation of phosphatase and tensin homolog (PTEN) [158]. The phosphatase PTEN is a tumor suppressor that functions as a negative regulator of PI3K/Akt pathway, which dephosphorylates PIP3 at position D-3 on the inositol ring [159, 160]. Using the target prediction algorithm *targetscan*, we found several miRNAs that can target PTEN and several of them have been experimentally validated [161-168] and highly expressed in smooth muscle. Most miRNAs that target PTEN (Figure 9) are downregulated by five weeks of tamoxifen treatment, suggesting more than one miRNAs might cause an up-regulation of PTEN in Dicer KO vessels. In addition to the established PTEN-targeting miRNAs we have confirmed miR-26a as a miRNA that target PTEN using miRNA mimics in aortic smooth muscle cells. MiR-26a is interesting because it has been previously reported that it can be up- regulated by mechanical stretch in airway smooth muscle [98].

Quignard et al. showed that Ang II -induced activation of L-type Ca^{2+} channels and subsequent influx of Ca^{2+} involves PI3Kγ in portal vein myocytes [169]. It has been previously reported that angiotensin II can phosphorylate Akt at Ser-473 and stimulate its kinase activity via the AT_1 receptor. The PI3K inhibitor LY- 294002 can block this effect [170]. The AT_1 receptor, which can be activated in the absence of ligands and directly by stretch, is essential for a typical myogenic response in mesenteric and renal resistance arteries [171]. In our model, Ang II was able to induce

phosphorylation of Akt in both WT and Dicer KO arteries despite an increase in PTEN expression. This may be due to the fact that Ang II can inhibit PTEN activity and thereby bypass the increase in PTEN expression in Dicer KO[172]. The mechanism for the effects of PI3K/Akt on L-type calcium channel activity could involve $Ca_v\beta_2$, as it has been demonstrated that Akt dependent phosphorylation of $Ca_v\beta_2$ is responsible for increasing $Ca_v1.2$ density in the plasma membrane inhibiting degradation of $Ca_v1.2$ protein by preventing PEST sequence (signals for rapid protein degradation) to be recognized [173].

Interestingly, a short pre-incubation with Ang II (300 nM) can rescue myogenic tone in Dicer KO mice, an effect that is inhibited in presence of LY- 294002 (10µM) and candesartan (1µM). It is tempting to speculate that pre-treatment with 300nM Ang II can phosphorylate a large pool of Akt despite PTEN-induced inhibition and restore $Ca_v1.2$ kinetics or density in the plasma membrane to allow sufficient calcium influx, which rescues pressure induced myogenic response in Dicer KO MRA.

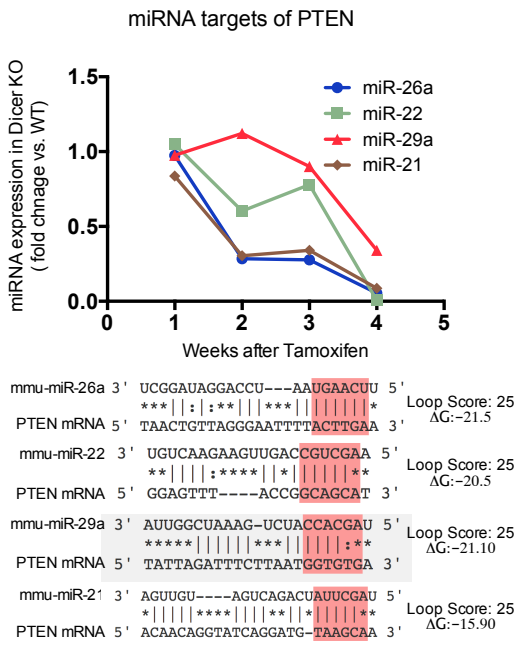

Figure 9: Expression of miRNAs after 1,2,3,4 weeks after tamoxifen treatment is measured using qRT-PCR. Binding sites of miR-26a (position 1263-1285, 1/7 target positions are shown), miR-22 (position 2779-2796, 1/7 target positions shown), miR-29a position 1698-1720,1/6 target positions are shown) and miR-21 (position 3709-3735, 1/3 target position is shown) against 3'UTR of PTEN is shown. Vertical lines indicate base pairing in the seed region. Additional nucleotides are furthermore responsible for the binding strength. The free energies and Loop Score were acquired using the FindTar3 database [174].

4.1.3 Reduced calcium influx and myosin light chain phosphorylation in miR-143/145 knock out mesenteric arteries

Similar to Dicer KO MRAs, KCl-induced depolarization was significantly reduced in homozygous MRAs from miR-143/145 KO mice, which is in agreement with previous reports from Boettger et al. (saphenous arteries)[94]. However, depolarization in heterozygous MRAs was unchanged. Calyculin A-induced contraction in calcium free condition was not significantly decreased in homozygous KO mice suggesting that the smooth muscle contractile machinery is intact, which is in line with the previous data regarding maintained contractile phenotype of the KO MRAs. The myogenic response was rescued in heterozygous (miR-143/145$^{-/+}$) mice using L-type calcium channel opener BayK8644 (300 nM) and non-selective K^+ channel blocker Tetraethylammonium chloride (TEA, 1mM) but not in homozygous (miR-143/145$^{-/-}$) mice. This is consistent with decreased Ca^{2+} influx upon KCl-induced (60mM) depolarization, even though expression of pore forming subunit of L-type calcium channel ($Ca_v1.2$) is upregulated. Collectively, these data suggest that 1) even though $Ca_v1.2$ expression is increased, its activity is decreased thus preventing influx of Ca^{2+} and 2) since BayK8644 or TEA did not rescue myogenic response in homozygous miR-143/145 KO mice (Paper III), this indicates a possible disruption also in downstream mechanisms regulating contraction.

Interestingly Horita et al., has reported that knockdown of SRF is associated with decrease of miR-143 and increase of miR-21, which is associated with downregulation of PTEN[175]. In miR-143/145 KO MRAs miR-21 expression is increased (Figure 10A) which is in agreement with Horita et al. However, expression of PTEN was unaltered (Figure 10B), thus ruling out any effect of PTEN in the regulation in $Ca_v1.2$ activity in these animals.

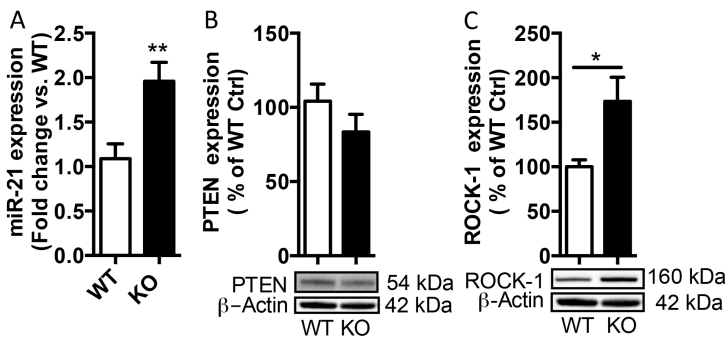

Figure 10: (A) Expression of miR-21 was measured using qRT-PCR (B) PTEN and (C) ROCK-1 expression was measured using western blot.

We also investigated signaling mechanisms downstream of calcium signaling responsible for contraction of smooth muscle. Expression of MLCK was severely reduced, likely contributing to the loss of pressure-induced myogenic response. Expression of myosin phosphatase target subunit 1 (MYPT1) was decreased (≈ 60%) as well, which might be counterintuitive since decreased MYPT1 would enhance contraction by increasing myosin phosphorylation. Furthermore, ROCK1 is significantly increased (≈74%) in the miR-143/145 KO (Figure 10C), which would tend to inhibit any remaining expression of MYPT1. In the miR-143/145 KO, myosin light chain phosphorylation in response to U46619 (300nM), a thromboxane A_2 (TP) receptor agonist, was significantly impaired. However, MYPT1 phosphorylation (MYPT853) in response to U46619 was maintained, suggesting that calcium sensitization pathways are functional in miR-143/145 KO MRAs. However, this does not seem to compensate for the loss of MLCK in miR-143/145 KO arteries.

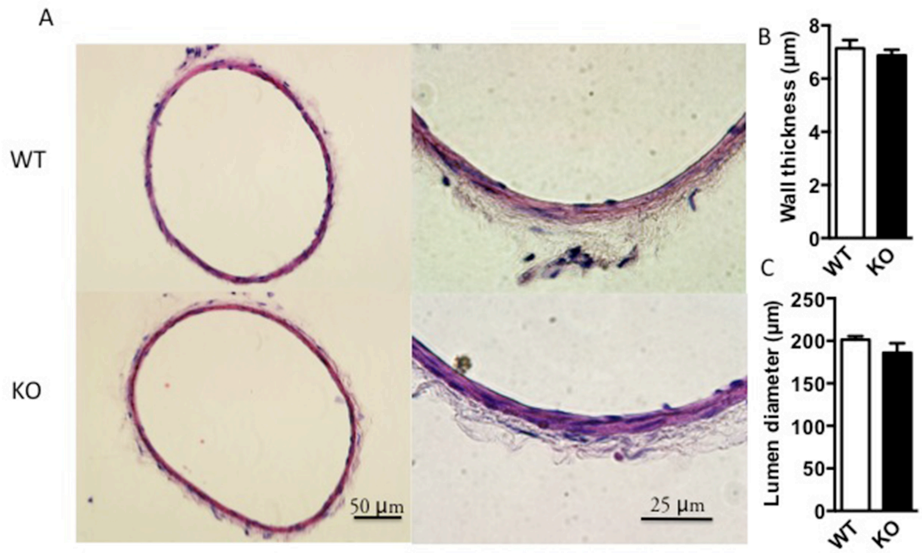

Figure 11: (A) Hematoxylin and eosin staining of WT and miR-143/145 MRAs (B) Wall thickness and (C) Lumen diameter is unaltered in miR-143/145 mice. See text for details.

These results re-emphasize the importance of calcium influx as an upstream modulator in pressure-induced myogenic response. It is tempting to speculate that decreased MYPT activity is a compensatory mechanism for the decreased MLCK activity but this is likely ineffective since myogenic tone is entirely abolished in miR-143/145 KO MRAs. This is likely due to both a severe loss in calcium channel influx and a reduced expression of MLCK. However, since BayK8644 did not rescue myogenic tone in homozygous KO arteries, there are two possibilities: 1) BayK8644

did not manage to restore pressure induced calcium influx, suggesting that miR-143/145 deletion affects another mechanisms that influences calcium channel activity, perhaps involving allosteric inhibition or channel desensitization 2) miR-143/145 deletion causes an impairment of downstream mechanisms, which is essential for myogenic tone (such as MLCK activity, actin polymerization). Thus, even if calcium influx were restored in the presence of BayK8644, it would be unable to restore myogenic tone. Unlike aorta, wall thickness is unchanged (Figure 11) in miR-143/145 KO MRAs suggesting that loss of smooth muscle mass is not a likely reason for lost myogenic tone, which is supported by the fact that the Calyculin A-induced response was unaffected in KO MRAs.

4.1.4. Decreased expression of pore forming subunit of L-type calcium channel in miR-143/145 portal vein.

It has been previously reported that loss of global smooth muscle miRNAs caused decrease of $Ca_v1.2$, the pore-forming subunit of L-type calcium channel in bladder and portal vein [85, 99]. Since inhibition of miR-145 with miRNA inhibitor decreased transcript levels of L-type calcium channel (*Cacna1c*) in WT aortic cells to about the same levels as dicer KO (10 weeks post tamoxifen), Turczyńska et al. hypothesized that miR-145 was partly responsible for the decreased expression of $Ca_v1.2$. In accordance with this theory, expression of *Cacna1c* (mRNA) as well as $Ca_v1.2$ (protein) was significantly decreased in miR-143/145 KO portal vein (Paper II). Ronkainen et al. have previously reported that expression of $Ca_v1.2$ is regulated by downstream regulatory element (DRE) binding transcription factor DREAM, which is activated by calmodulin kinase II (CaMKII)[176]. Furthermore, Cordes et al. have previously shown that CaMKIIδ is target of miR-145[92]. Similar to Dicer KO portal veins, expression of both CaMKIIδ and DREAM was increased in miR-143/145 KO portal vein (Paper II). Since DREAM negatively regulates $Ca_v1.2$ expression, it may explain the loss of calcium channels. As discussed before, since Ca^{2+} influx via L-type channels is essential for spontaneous activity and maintaining stretch-induced contractile differentiation, decreased expression of calcium channels can explain the reduction of these responses in miR-143/145 portal vein. In addition loss of smooth muscle mass can contribute to lack of contractile responses in the KO mouse model. ACE-1 upregulation was previously attributed as the reason for loss of contractile phenotype in miR-143/145 aorta[94] but expression of ACE-1 was unchanged in spontaneously active miR-143/145 KO portal veins, suggesting its role to be less imperative in the context of maintaining contractile phenotype in portal veins compare to that of aorta[94].

4.3 Protein tyrosine kinase 2 as a mediator of calcium handling mechanisms in the vascular wall

4.3.1 PYK2 as mediator of stretch-induced calcium signaling in the portal vein

Intracellular Ca^{2+} plays two distinctive roles in smooth muscle: on the one hand it induces contraction in response to appropriate stimuli, and on the other hand it acts as a second messenger to induce transcriptional activation of genes involved in contraction, differentiation, growth and proliferation. Thus a closely knitted spatial-temporal regulation of Ca^{2+} handling mechanisms is essential in phenotype regulation of smooth muscle cells[177]. Pressure-induced responses of blood vessels involve increased L-type calcium channel activity [178, 179] and activation of Src[180], Moreover, Src is able to activate L-type calcium channels[181] and Src activation requires auto-phosphorylation of PYK2 at Y402 (see section 1.5.1). It is therefore tempting to speculate that PYK2 plays an important role in stretch-induced calcium handling mechanisms in smooth muscle and that it may mediate calcium-dependent trophic effects via its association with Src and its downstream signaling pathways.

Ren et al. showed that stretch-induced expression of smooth muscle markers in portal vein is inhibited by blockade of L-type calcium channels by verapamil (1 μM)[182]. In contrast 2-APB (30 μM), a non-selective blocker of store-operated Ca^{2+} entry, inhibited overall protein synthesis in stretched portal vein but did not affect expression of smooth muscle markers. This brings into question the causes of the apparent specificity of downstream signaling in response to Ca^{2+} entry. We investigated the role of PYK2 in this process (Paper IV). Short-term (10 min) stretch was found to activate PYK2, which was inhibited by the small molecule inhibitor PF-4594755 (1 μM). The compound also inhibited stretch-induced phosphorylation of Akt and FAK but had only a partial effect on ERK1/2. Notably, however, stretch-induced (10 min) ERK1/2 activation is insensitive to both verapamil and 2-APB[182]. In long-term (5 days) organ culture of portal vein, PF-4594755 (1 μM) profoundly inhibited overall protein synthesis but did not abolish stretch-sensitive synthesis of the smooth muscle marker SM22α, which is similar to the effects of 2-APB as discussed earlier.

To further investigate the dependence of PYK2 activation on calcium entry mechanisms, portal veins were cultured in parallel over 3 days in the presence of PF-4594755 (0.5 μM), nifedipine (1 μM), 2-APB (30 μM), and the combination of PF-

4594755 with nifedipine and 2-APB, respectively. PYK2 remained activated in stretched portal vein during the 3-day culture and this was inhibited by PF-4594755. Unlike short-term effects, long-term cultures with PF-4594755 inhibited basal as well as stretch-activated PYK2 phosphorylation. This effect was mimicked by 2-APB but not by nifedipine, which did not affect basal PYK2 phosphorylation but eliminated its stretch sensitivity. The combination of PF-4594755 with nifedipine inhibited PYK2 phosphorylation to the same low level in both unstretched and stretched portal veins, while the combination of PF-4594755 with 2-APB did not modify the effects of either inhibitor alone. PF-4594755 also inhibited Akt and ERK1/2 phosphorylation. Thus, unlike short-term effects, long-term stretch-induced phosphorylation of ERK1/2 is dependent on Ca^{2+} influx [17]. Taken together, the results obtained using calcium entry blockers suggest that PYK2 plays a role in overall protein synthesis and that this requires calcium influx via non-voltage dependent (e.g., store-operated) calcium channels. However, PYK2 is also activated by stretch via a mechanism involving L-type channel activity.

PYK2 phosphorylation is involved in growth signaling pathways, and in particular AngII is a well-known activator of PYK2[183]. Moreover, the AngII receptor is activated by stretch[171]. In the presence of the AngII antagonist Losartan (1 μM) the stretch sensitivity of both PYK2 and Akt phosphorylation was abolished, suggesting that PYK2 activation by stretch may involve Ang II receptors, possibly in a ligand-independent manner[54]. Losartan does not have any effect on contractile marker expression at the transcript level, similar to PF-4594755.

Interestingly, the levels of PYK2 and Akt phosphorylation were increased with culture time under basal condition. This might reflect increased intracellular calcium concentration, possibly due to increased non-voltage dependent calcium channel expression with increasing culture time and phenotype modulation[184]. It may also explain the effect of PF-4594755 on basal PYK2 phosphorylation in long-term culture. Portal vein cultured for 3 days under stretched condition in the presence of PF-4594755 show increase in spontaneous activity compared to control vessels cultured without the inhibitor, whereas responses to cirazoline-induced α_1-adrenergic stimulation and to 60-mM KCl induced depolarization were unchanged. These findings perhaps present us with indirect evidence of preserved phenotype in the presence of PF-4594755. We investigated this further in arterial preparations using an *ex vivo* balloon injury model.

4.3.2 Effect of PYK2 inhibition on vascular phenotype modulation and apoptosis

The intravascular pressure is an essential stimulus for maintaining the contractile phenotype. As shown in paper V, pressurization of small mesenteric arteries not only causes increase of smooth muscle markers like myocardin, calponin and SM22α but also causes phosphorylation of PYK2, which is inhibited by PF-4594755. In contrast to physiological stretch by the normal blood pressure, excessive stretch and tissue injury may elicit shift to a more proliferative phenotype. To investigate the role of PYK2 in these responses, isolated rat carotid arteries in organ culture were exposed to balloon injury by a catheter inflated to 2 atm for 2 min and then cultured for a further 48 h. PYK2 phosphorylation increased in the balloon-injured arteries, and this was prevented by PF-4594755 (0.5 μM) in the culture medium (Paper V). Organ culture *per se* decreased smooth muscle contractile marker expression at the messenger level (*Myh11*, *Acta2*, *Tagln*) and increased the extracellular matrix protein osteopontin (*Spp1*) when compared to freshly frozen carotid samples. These effects were enhanced in response to balloon injury. PF-4594755 partially rescued the contractile phenotype of uninjured arteries during culture, but particularly prevented the effects of balloon injury. Thus the PYK2 inhibitor retards smooth muscle phenotype shift in culture and in response to tissue injury. These results suggest that PYK2 has distinct functions in contractile as opposed to synthetic smooth muscle. One possible explanation for this involves the role of Rho signaling in the different phenotypes.

Rho is activated by heterotrimeric G-proteins via $G_{12/13}$ coupling to the RhoGTPase nucleotide exchange factors (RhoGEFs) p115, PDZ and LARG, activating Rho via pathways involving non-receptor tyrosine kinases, including PYK2[27]. Rho/Rho Kinase (ROCK) exerts major effects on actin polymerization, stimulating cell migration and also proliferation, possibly via interaction with the MAPK pathway (reviewed by[185]). The effects of Rho/ROCK in the quiescent contractile cells of the normal vascular wall are however most evident as an increase in contractility mediated via ROCK-dependent inhibition of MYPT, with ensuing increase of calcium sensitivity[27]. In addition, Rho/ROCK-stimulated actin polymerization enhances the expression of smooth muscle marker proteins as described in Section 1.3.2. Accordingly, it was recently shown that knocking out $G\alpha_{12/13}$ in mice enhances the synthetic phenotype in smooth muscle cells and greatly increases neointima formation following carotid artery ligation, while knocking out $G\alpha_{q/11}$ has the opposite effects[31]. This shows that the two G-protein stimulated cascades need to be in balance for an appropriate response to vessel injury.

With respect to the role of PYK2 in the injury response, our results suggest that its pharmacological inhibition predominantly tends to preserve the contractile

phenotype and to reduce neointima formation, as also recently demonstrated in vivo using a PYK2 knock-down approach[62]. This is consistent with the inhibition of DNA synthesis and migration of vascular smooth muscle cells by PF-4594755 observed in our study. It does however not suggest that possible Rho/ROCK inhibition by the PYK2 inhibitor has a negative effect on smooth muscle contractile differentiation, which the inhibitor may instead partially rescue by reducing the elevation of MAPK and Akt signaling in tissue injury.

In intact small mesenteric arteries, on the other hand, PF-4594755 caused a small but significant inhibition of U46619-induced contraction. This compound activates thromboxane A_2 receptors which couple to $G_{12/13}$, causing calcium sensitization by MYPT inhibition[186]. In contrast, there was no inhibitory effect on contractions elicited by α_1-receptor stimulation by cirazoline, which activates $G_{q/11}$, recently shown to couple to Rho/ROCK via p63RhoGEF[187]. These results suggest that PYK2 activates Rho/ROCK primarily via $G_{12/13}$, which is in accordance with previous reports[188, 189], and in addition the results show that this mechanism, while detectable in contractile tissue, seems to be of limited significance in mediating phenotype shift after vascular injury. It should be added, however, that the injury response, involving increase in both Rho and MAPK signaling, might be finely tuned temporally, an aspect requiring further study. This also applies to the role of PYK2 in promoting apoptosis following vessel injury, as suggested by the effect of PF-4594755 in preventing increased BAX/Bcl2 following balloon injury in vitro.

Summary & future directions

The profound influence of myogenic responsiveness as a local regulator of microvascular hemodynamics fosters numerous key questions while considering the pathophysiological conditions comprising vascular dysfunction (e.g. vascular complications associated with diabetes, hypertension, vasospasm, ischemic stroke etc.). Small resistance arteries respond to a sudden increase in intraluminal pressure by inducing a myogenic response. However, a sustained increase in intraluminal pressure will result in persistent myogenic constriction in the resistance vasculature, which attempts to keep the capillary pressure constant during this period to prevent organ damage. Vasoconstriction for extended period of time can cause inward eutrophic remodeling (increased wall thickness and reduced lumen diameter) and/or a reduced arterial distensibility in order to counteract the increased wall stress.

Targeting pathways involved in myogenic response mechanisms has been proposed as a therapeutic option. Arguably, 'resetting' vascular resistance can be advantageous in ischemic as well as hyperdynamic situations to normalize blood flow. Since heterogeneous signaling mechanisms exist among vascular beds, it might be possible to achieve a target-specific effect. A theoretical example of such an approach involves targeting BK_{Ca} channels, which act as a negative feedback regulator of the myogenic response. BK_{Ca} channels are more important in regulating myogenic tone in cerebral arteries[52] than in skeletal muscle arterioles[190].

In this thesis we have demonstrated that non-coding miRNAs are key players in regulating mechanosensitive vascular tone (Paper I-III) and phenotype (Paper II), which might open up new opportunities to pharmacologically target pressure-induced myogenic response pathways. There are exciting new studies which not only established miRNAs as a feasible target for therapeutic purposes for various diseases including cancer[191] and cardiovascular diseases[192] but also as a marker for disease diagnosis[193]. There are two main advantages of using miRNAs in therapeutics: 1) the short sequence of miRNAs makes it relatively easy to mimic or inhibit its function and 2) due to its conserved nature it is relatively easy to translate from pre-clinical safety studies to clinical trials. There are two main approaches presently utilized to modulate miRNAs. The first one involves antimiRs, antisense oligonucleotides or miRNA sponges, which are chemically modified to improve their biostability,

binding affinity and pharmacokinetic properties. The second approach involves synthetic RNA duplexes (again, chemically modified for the same reason as antimiRs). Each of the approaches has their limitations and advantages but most common concerns include off-target effect, limited uptake, bioavailability and stability. One approach that has been used is to overcome these issues is liposome-based delivery methods. One example of this is called MRX34, a miR-34 mimic that is currently being tested in phase I clinical trials in patients with unresectable primary liver cancer[194]. The other example of miRNA based therapy, which is in phase IIa clinical trial is called miravirsen (antimiR-122), and is shown to reduce serum Hepatitis C viral (HCV) load levels in patients chronically infected with HCV with no measurable indication of resistance[195].

As we continue to understand more on how microRNAs influence arterial remodeling and myogenic tone, hopefully we will move more towards using that knowledge to develop therapeutic tools superior to the ones that are currently available, the most common of which are the ACE inhibitors[196], which have significant side effects[197].

	*Dicer KO portal vein [84]	Dicer KO resistance artery [82]	miR-143/145 KO portal vein	miR-143/145 KO resistance artery
Myogenic response/spontaneous activity	Lost	Lost	Decreased	Decreased
Remodeling	?	No	Yes	No
Expression of contractile proteins	Decreased	Unchanged	Decreased	Unchanged
Expression of $Ca_v1.2$	Decreased	Unchanged	Decreased	Increased
Ca^{2+} independent contraction	?	Unchanged	Decreased	Unchanged
KCl induced Depolarizatiom	Decreased	Decreased	Decreased	Decreased
Main mechanism	Reduced expression of $Ca_v1.2$, increased expression of DREAM	Inactivation of PI3K/Akt pathway due to increased expression of its repressor, PTEN.	Reduced expression of Cav1.2, increased expression of DREAM	Reduced calcium influx. Decreased MLCK expression.

Table 5: A summary comparison of different properties of portal vein/mesenteric artery from two different KO mice. * Not part of this thesis.

Both members of the FAK family of nRTKs (FAK and PYK2) are critical modulators of cellular signaling pathways and not only exert tremendous influence on cell survival, proliferation and migration but also synchronize the growth response to cytoskeletal dynamics. They have emerged as targets for treatment of cancer, inflammation and osteoporosis[198]. Due to the high degree of homology between PYK2 and FAK, until recently it was challenging to generate pharmacological inhibitors that selectively target PYK2. In this thesis, using a small molecule inhibitor of PYK2, we investigated its role in stretch-induced signaling in the vascular wall.

The PYK2 inhibitor PF-4594755 is more than 100-fold selective over FAK[122]. One interesting finding, with possible clinical implication, was that PF-4594755 inhibited phenotype modulation and apoptosis in balloon-injured carotid arteries. Shift of smooth muscle phenotype from contractile to a non-contractile synthetic state has been reported in diseases including systemic hypertension[199, 200], atherosclerosis[201], Marfan syndrome[202], asthma[203], obstructive bladder disease[204], abdominal aortic aneurysm[205] and numerous gastrointestinal and reproductive disorders[206]. Thus it is of great interest to gain a deeper understanding of molecular mechanisms involved in phenotype modulation to develop therapeutic targets, since current treatment strategies that target these pathways are limited. Cholesterol-lowering statins prevent progression of atherosclerosis and promote smooth muscle differentiation *in vivo*[207] but have side effects that include myopathy[208], elevated level of transaminases[209] and increased risk of diabetes[208]. Rapamycin has been shown to promote smooth muscle differentiation[210] and to inhibit its proliferation and matrix synthesis, but it also has side effects that include lung toxicity[211] and insulin resistance[212]. Local delivery of rapamycin analogs using drug-eluting stents has been reported to inhibit restenosis in the coronary arteries for some patients but patients with diabetes are prone to higher risk[213]. For aortic aneurysm, surgery is the only available option and it might go undetected until rupture. All these data indicate a need for alternative approaches to target smooth muscle phenotype shift. Our data suggest that inhibiting PYK2 might be part of such an approach.

Conclusions

The following conclusions can be drawn based on data obtained in this thesis:

- Pressure-induced myogenic response and calcium signaling in small mesenteric arteries is extremely sensitive to loss of miRNAs.
- Loss of smooth muscle specific miRNAs following five weeks of dicer KO directly affects pressure-induced signaling mechanisms, including activation of the PI3K/Akt pathway.
- Unlike larger vessel preparations, deletion of the miR-143/145 cluster did not have any influence on contractile gene expression in smooth muscle of small resistance arteries even though the pressure-induced myogenic response was completely abolished in both homozygous and heterozygous animals.
- PYK2 signaling is activated by stretch and mediates growth responses to voltage-independent calcium influx in spontaneously active vascular smooth muscle.
- A small-molecule PYK2 inhibitor retards smooth muscle phenotypic shift in organ culture and vascular injury.

Svensk sammanfattning

Blodkärlen har den viktiga uppgiften att reglera blodflödet till varje del av kroppen, vilket sker genom att de justerar sin diameter som svar på olika stimuli. Den glatta muskulaturen i kärlväggen möjliggör snabba förändringar i kärlens diameter genom att antingen dra sig samman eller slappna av från ett normaltillstånd av partiell sammandragning, så kallad tonus. Detta sker framför allt i mindre artärer som kallas resistensartärer på grund av sin förmåga att reglera kärlträdets resistens. Vid långvariga förändringar av kärltonus, till exempel vid högt blodtryck (hypertoni) kan det uppstå kroniska förändringar i kärlväggen som benämns kärlremodellering, som en följd av strukturella förändringar i vävnaden. Till exempel kan det ske en permanent minskning av kärlets diameter även då muskulaturen är helt avslappad, en ökad bindvävsproduktion och en förändring av kärlens elastiska egenskaper. Dessa strukturella förändringar kan leda till förvärrad hypertoni, organskada till följd av dåligt blodflöde och ökad risk för ateroskleros. Dessa effekter kan även leda till skadliga förändringar i hjärtat då det konstant får arbeta mot ett högre tryck. Hur förändringarna i kärlväggen regleras på en molekylär nivå är ännu inte helt klarlagt. Det är sannolikt att den glatta muskulaturen spelar en viktig roll i processen och det är möjligt att den förändrade mekaniska belastningen av de glatta muskelcellerna kan initiera remodelleringen.

För att studera de molekylära mekanismerna bakom effekterna av mekanisk belastning i glatt muskulatur har vi använt oss av två organmodeller där intakta kärl från möss och råttor studeras utanför kroppen (*ex vivo*). I den ena modellen har vi använt oss av portavenen som är ett kärl som spontant kontraherar rytmiskt vid belastning. Då portavenen huvudsakligen har längsgående glatt muskulatur så kan detta kärl sträckas genom att en vikt appliceras i ena änden av kärlet. Den sträckta portavenen kan sedan hålla levande i så kallad organodling i upp till sju dagar. I den andra metoden används små resistensartärer som trycksätts i en så kallad tryckmyograf. I denna apparat kan kärlets diameter samtidigt studeras och i avhandlingens första studie användes denna metod för att studera de akuta effekterna av ett ökat tryck. Detta involverar en kontraktion som kallas myogen tonus och som är ett direkt svar på en tryckökning i kärlen. Kontraktionen initieras av ett inflöde av kalciumjoner i de glatta muskelcellerna som därefter leder till en interaktion mellan

de kontraktila proteinerna aktin och myosin. Denna interaktion leder till en sammandragning av cellerna och en minska kärldiameter.

Både kalciuminflödet och kontraktionen påverkas av uttrycket av de proteiner som är involverade i processen och en nyupptäckt mekanism för reglering av proteinuttryck är så kallade mikroRNA. Det finns hundratals olika mikroRNA i cellen och vart och ett kan reglera syntesen av ett flertal olika proteiner. I denna avhandling har vi studerat mekanismerna bakom både akuta och kroniska effekter av förändrat mekaniskt stimuli med fokus på betydelsen av kalciumsignallering och mikroRNA. Det ökade kalciuminflödet som sker vid mekanisk belastning reglerar inte bara kontraktionen utan kan även påverka remodellering av kärlväggen. Vi har därför studerat hur ett kalciumkänsligt enzym som heter PYK2 påverkas av en förändring i kärlväggens sträckningsgrad.

För att undersöka mikroRNAs effekter har vi använt oss av musmodeller som saknar antingen samtliga eller vissa specifika mikroRNA molekyler. Resultaten av studierna kring mikroRNAs betydelse visar att effekterna av mekanisk belastning är kritiskt beroende av dessa molekyler. Avsaknad av samtliga mikroRNA leder till en snabb förlust av den myogena kontraktionen trots att inga andra markanta förändringar skedde i kärlväggen. Likaså kunde vi visa att ett minskat uttryck av två specifika mikroRNA ledde till fullständig förlust av den myogena kontraktionen samt en minskad känslighet för långvariga förändringar i mekanisk sträckning. En del av dessa förändringar kan bero på en minskad aktivitet av kalciumkanaler. För att studera effekterna av sträckberoende kalciumsignallering i de glatta muskelcellerna fokuserade vi på det kalciumkänsliga enzymet PYK2. PYK2 kopplar till flera intracellulära signalvägar för tillväxt och differentiering och är av intresse både för cancerforskningen och för forskningen om osteoporos, eftersom den påverkar celltillväxten såväl som kärlnybildning och benbildande cellers funktion. Eftersom PYK2 regleras av integriner (proteiner i cellmembranet som kan förmedla mekaniska signaler) och kalcium spelar det möjligen en generell roll för anpassningen av vävnader som ben och blodkärl till mekanisk belastning. Vi använde en ny specifik PYK2-hämmare för att undersöka effekter på kärlmuskulaturen i portavenen och små resistensartärer. Vi fann PYK2 aktiveras av sträckning samt att hämning av PYK2 reducerar celltillväxt och proliferation i kärlväggen och kan reversera vissa av de skadliga förändringar som sker vid kärlskada.

Sammanfattningsvis så har vi identifierat nya mekanismer för sträckkänslig signallering i kärlväggen som involverar mikroRNA och PYK2. Resultaten i denna avhandling leder till en ökad förståelse för varför skadliga förändringar kan ske i kärlväggen vid en ändrad mekanisk belastning och öppnar nya möjligheter för behandling av hjärt-kärlsjukdomar.

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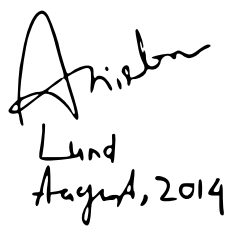

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