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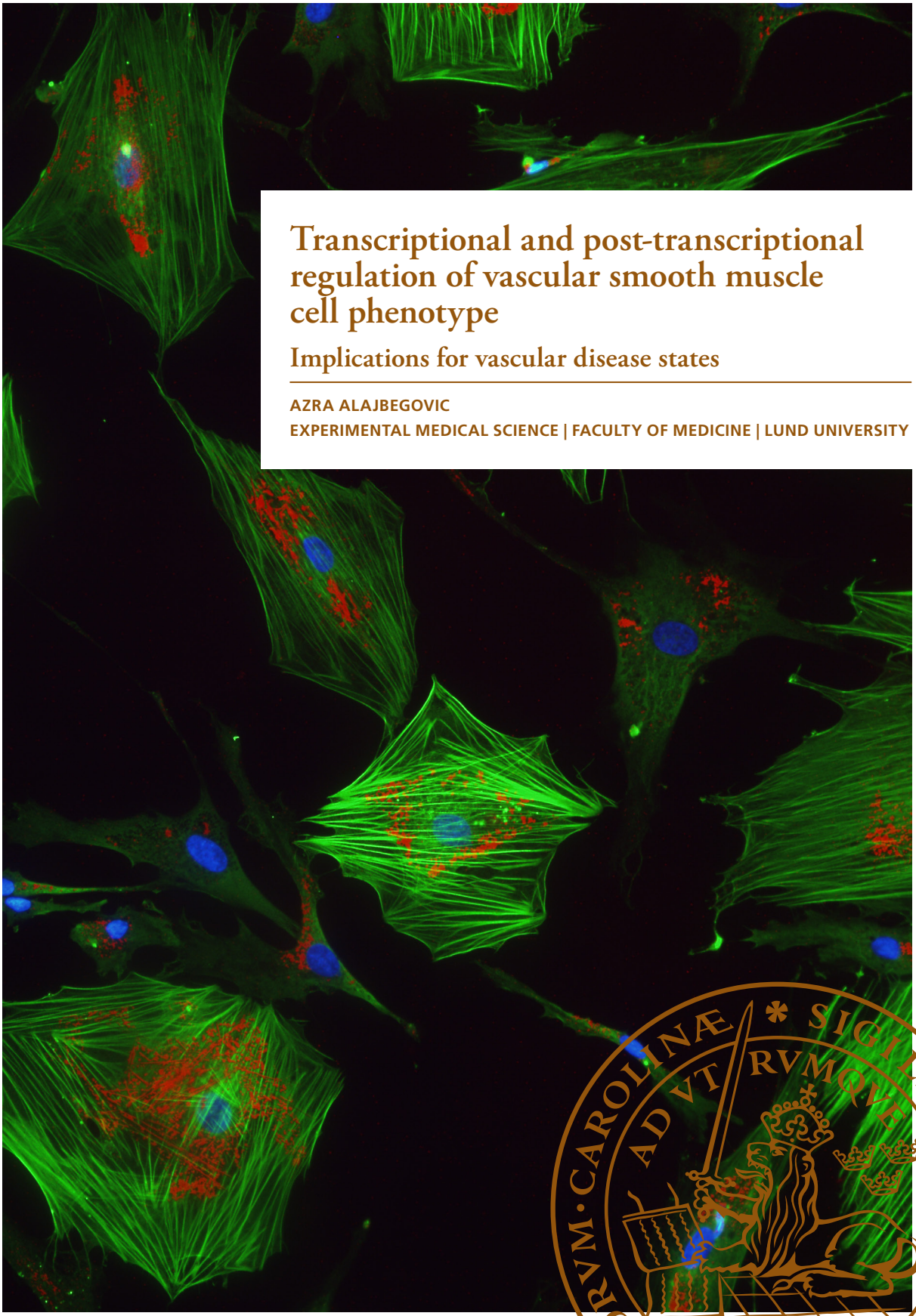

Transcriptional and post-transcriptional regulation of vascular smooth muscle cell phenotype

Implications for vascular disease states

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Abstract

As the world population is pushing toward 8 billion, cardiovascular diseases (CVD) remain the leading cause of death worldwide, representing 30% of all global deaths. A large body of work has recognized that smooth muscle cells (SMCs) surrounding the blood vessels play a prominent role in the development and progression of cardiovascular diseases.

SMCs are highly specialized cells with the main function to maintain vascular tension and thereby regulate blood pressure and blood flow. SMCs retain remarkable plasticity. In response to changes in external cues, SMCs can modulate their phenotype from a highly mature contractile phenotype to a synthetic, proliferative phenotype. Although beneficial during key physiological processes such as wound healing, phenotypic modulation can contribute to the development and progression of several vascular disease states. Despite extensive studies on the transcriptional programs that define smooth muscle phenotype, the endogenous regulators that control smooth muscle specificity are still far from understood. The aim of this thesis was to gain further insight into the transcriptional and post-transcriptional regulation of gene expression that occurs during disease development and how these changes affect the function of the vascular wall.

The work in the following papers has identified previously unknown mechanisms by which small non-coding RNAs (miRNAs), actin polymerization and transcriptional regulators MRTFA and GATA6 can contribute to the changes in vascular smooth muscle observed in vascular disease states. In summary, we show that actin polymerization and MRTFA regulate a profile of miRNAs that are downregulated in patients with mildly dilated aorta. Moreover, we demonstrate a novel role for MRTFA in lipid accumulation and foam cell formation. We further demonstrate the importance of miRNA-143 and miRNA-145 for vascular function and for adaptation to hypertension. Lastly, we show that GATA6 regulates migration of SMCs. A deeper understanding into the underlying molecular mechanisms is crucial in order to develop new efficient therapeutic approaches against cardiovascular disease states.

Key words vascular disease, smooth muscle cell phenotype, microRNA, actin polymerization, hypertension, foam cell, aneurysm, cell migration

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Fluorescently labeled human vascular smooth muscle cells. Nuclei (blue), actin (green) and cholesterol (red).

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“The more I learn, the more I realize how much I don't know”
- Albert Einstein

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II. Holmberg J, Bhattachariya A, **Alajbegovic A**, Rippe C, Ekman M, Dahan D, Hien TT, Boettger T, Braun T, Swärd K, Hellstrand P, Albinsson S. Loss of Vascular Myogenic Tone in miR-143/145 Knockout Mice is Associated With Hypertension-Induced Vascular Lesions in Small Mesenteric Arteries. *Arterioscler Thromb Vasc Biol*. 2018 Feb;38(2) :414-424.

III. **Alajbegovic A,** Holmberg J, Albinsson S. MRTFA promotes conversion of human coronary artery smooth muscle cells into lipid-loaded foam cells. Manuscript

IV. **Alajbegovic A**, Kawka K, Holmberg J, Albinsson S. Transcription factor GATA6 promotes migration of human coronary artery smooth muscle cells. Manuscript

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Albinsson S, Della Corte A, **Alajbegovic A**, Krawczyk KK, Bancone C, Galderisi U, Cipollaro M, De Feo M, Forte A. Patients with bicuspid and tricuspid aortic valve exhibit distinct regional microrna signatures in mildly dilated ascending aorta. *Heart Vessels.* 2017 Jun;32(6):750-767.

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Populärvetenskaplig sammanfattning

Hjärt- och kärlsjukdomar är den ledande dödsorsaken i Sverige, och även i andra delar av världen. I Sverige dör 4 av 10 till följd av hjärt- och kärlsjukdomar, vilka drabbar organ i cirkulationssystemet såsom hjärta och blodkärl. Riskfaktorer som påverkar när och vem som drabbas inkluderar utöver ärftliga anlag även rökning, förhöjda blodfetter, högt blodtryck samt fysisk inaktivitet.

Idag finns det inget botemedel för hjärt- och kärlsjukdomar utan enbart förebyggande åtgärder för att minska risken att drabbas av dessa sjukdomar såsom motion och hälsosam kost. Utöver detta finns medicinering som blodtryckssänkande samt kolesterolsänkande läkemedel. Om detta inte är tillräckligt finns ett tredje alternativ som är operation. Operationen innebär bland annat att man vidgar ett kärl som har blivit tilltäppt för att återställa blodflödet. Trots imponerande framsteg ökar fortfarande antalet dödsfall till följd av hjärt- och kärlsjukdomar. Att hitta nya, billiga och effektiva behandlingsalternativ är därför oerhört viktigt.

Forskningsstudier har visat att hjärt- och kärlsjukdomar orsakas av strukturella förändringar i kärlväggen. Den glatta muskulaturen som omringar kärlväggen reglerar kärlets diameter genom sin kontraktila förmåga och spelar en central roll vid förändringar av kärlväggens struktur. De glatta muskelcellerna utsätts ständigt för mekaniska krafter genererade av blodflödet och blodtrycket. Cellerna påverkas även av skador, vilket stimulerar celltillväxt och produktion av bindväv. I samband med kärlskada kan glatta muskelcellernas karaktäristiska egenskaper (fenotyp) förändras, genom att de tappar sin kontraktila förmåga och börja dela och röra på sig. Denna förändring kan bidra till kärlförträngning vilket kan ge allvarliga konsekvenser såsom hjärtinfarkt och stroke.

I denna avhandling har vi identifierat nya, tidigare okända mekanismer, vilka kan bidra till de strukturella och funktionella förändringar i kärlväggen som har observerats vid kärlsjukdomstillstånd såsom åderförkalkning, högt blodtryck samt kärlförträngning. En bättre förståelse för de mekanismer som reglerar glatta muskelcellers fenotyp skulle kunna identifiera potentiella terapeutiska mål mot kärlsjukdomar.

Selected Abbreviations

3' UTR – 3' untranslated region

ACTA2 - smooth muscle alpha actin

Ang II - angiotensin II

BAV - bicuspid aortic valve

chol – cholesterol

CNN1 - calponin 1

DON - donors

F-actin - filamentous actin

G-actin - globular actin

HCASMC - human coronary artery smooth muscle cell

HKG - housekeeping gene

Jasp – jasplakinolide

KO – knockout

LatB – latrunculin B

LC_{20} - myosin 20 kDa light chain

LDL - low-density lipoprotein

mAoSMC - mouse aortic smooth muscle cell

miRNA – microRNA

MLCK - myosin light chain kinase

MLCP - myosin light chain phosphatase

MRTFs – myocardin-related transcription factors

MYH11 - smooth muscle-specific myosin heavy chain

MYOCD - myocardin

RhoA – Ras homolog gene family, member A

SMC – smooth muscle cell

SRF – serum response factor

SYNPO2 - synaptopodin-2

TAV - tricuspid aortic valve

WT - wild type

Prologue

As the world population is pushing toward 8 billion, cardiovascular diseases (CVD) remain the leading cause of death worldwide, representing ~ 30% of all global deaths [1]. While hereditary factors play a role in disease development, behavioural risk factors such as smoking, physical inactivity and unhealthy diet are independent risk factors linked to cardiovascular events. CVD comprise significant health and economical burdens, estimated to cost the EU economy €210 billion a year [2]. Despite impressive advances in medical therapy with lipid-lowering drugs and well-established surgical interventions including balloon angioplasty, the number of deaths from cardiovascular diseases are on the rise. It is therefore of outmost importance to improve disease control with early detection to decrease the burden of disease.

A large body of work has recognized that smooth muscle cells (SMCs) surrounding the blood vessels play a prominent role in the development and progression of cardiovascular diseases. SMCs are highly specialized cells with the main function to maintain vascular tension and thereby regulate blood pressure and blood flow. In contrast to many other cell types, SMCs retain remarkable plasticity. In response to changes in external cues, SMCs can modulate their phenotype from a highly mature contractile phenotype to a more synthetic, proliferative phenotype. Although beneficial during key physiological processes such as wound healing following an injury, phenotypic modulation can contribute to the development and progression of several vascular disease states including atherosclerosis, hypertension and restenosis post-angioplasty. More recently, it has been demonstrated that SMCs can reprogram to other cell types, suggesting a much bigger role for SMCs then previously assumed.

Despite extensive studies on the transcriptional program that defines smooth muscle phenotype, the endogenous regulators that control smooth muscle specificity are still far from understood. Expanding the knowledge of the underlying molecular mechanisms, including transcriptional and post-transcriptional, that govern SMC phenotype is therefore of great importance.

Introduction

The cardiovascular system

The cardiovascular system was first described in the modern concept by an English physician, William Harvey in 1628 [3]. The circulatory system comprises the heart and a complex network of blood vessels that permit blood to circulate and transport oxygen, nutrients and waste products throughout the body. Hence, the cardiovascular system is vital for survival by maintaining body homeostasis.

Blood vessel structure

Blood vessels are divided into three major types; the arteries, which transport oxygenated blood away from the heart, capillaries, which enable the exchange of nutrients and waste between the blood and tissue, and the veins, which carry deoxygenated blood back to the heart. Because of their different roles, their structures are also different. Arteries have in general thicker walls and a smaller lumen than veins. Some veins also contain valves to keep the blood flowing in one direction. Although they differ in structure and function, blood vessels share certain features. The vascular wall of arteries and veins is composed of three histologically distinct layers or tunics; *tunica intima*, *tunica media* and *tunica adventitia* (Figure 1). Intima is the innermost layer, consisting of a single, continuous, layer of endothelial cells. It functions as a semipermeable barrier for fluids and other components in the blood. The endothelium rests on a basal membrane, a thin layer of extracellular matrix enriched in laminins, heparane sulfate proteglycans, hyaluronan, fibronectin and type IV collagen [4, 5]. The media is the middle layer of the vessel wall, which mainly consists of smooth muscle cells supported by connective tissue consisting of type I and III collagen, elastin and proteoglycans [6]. The contractile capacity of smooth muscle cells regulates lumen diameter and subsequently blood pressure and blood flow. Tunica adventitia is the outermost layer composed of fibroblasts, nerves and small blood vessels (*vasa vasorum)* innervated in a network of extracellular matrix composed of fibrillar collagen [6, 7]. The abundance of each component varies depending on vascular bed. While collagens provide tensile strength, elastin provides elasticity needed to accommodate changes in pressure and the pulsatile nature of blood flow. Proteoglycans and hyaluronan retain viscoelasticity making it resistant to

compression forces [6]. In larger muscular arteries, the three concentric layers are separated by an internal and external elastic lamina.

Arteries are further grouped into three main types; elastic (conductive) arteries, muscular (distributing) arteries and arterioles. The elastic arteries are located closest to the heart and include the aorta and pulmonary artery. The walls of these arteries are enriched in elastin, which allow maintenance of constant blood pressure between heart contractions. Muscular arteries follow the elastic arteries and include the femoral artery and mesenteric arteries. The medial layer of muscular arteries contains less elastic fibers and more smooth muscle cells. These arteries distribute blood to different parts of the body by branching into arterioles. Arterioles are small arteries that deliver blood to capillaries. Because of their small lumen these arteries generate "peripheral resistance" which allow them to regulate blood flow according to the need of target tissues or organs [8]. By controlling the peripheral resistance, small arteries also regulate mean arterial blood pressure (MABP).

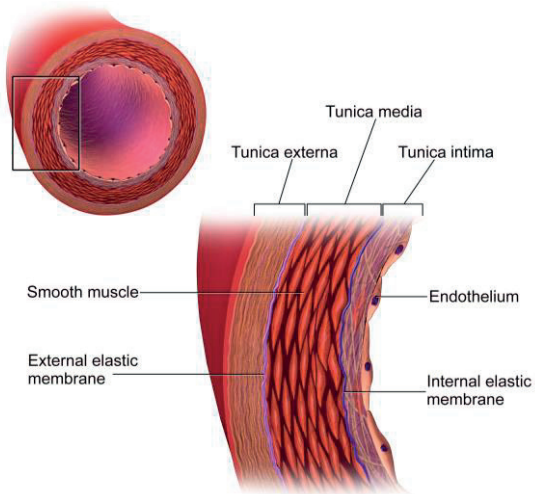

Figure 1. Structure of an artery composed of three distint layers; tunica intima, tunica media and tunica adventita (externa) [9].

Blood vessel function

The regulation of vascular tone is essential for proper regulation of blood flow and pressure. Vascular smooth muscle contractility is controlled by several intrinsic and extrinsic factors. Intrinsic factors include substances that are released locally e.g. endothelial factors such as nitric oxide and myogenic mechanisms originating

from smooth muscle cells. The intrinsic mechanisms allow the blood flow to be accommodated according to a tissue's metabolic demand. The extrinsic factors include humoral factors and neuronal regulation by sympathetic nerves [10]. In addition to these factors, blood vessels *in vivo* are constantly exposed to mechanical stimuli. Small resistance arteries, in particular arterioles, can regulate blood flow in response to mechanical load resulting from stretch elicited by the intraluminal blood pressure and shear stress exerted on the endothelium by the blood flow. The former is termed *the Bayliss effect*. William Bayliss was a British physiologist, who discovered that stretch of the vascular wall resulting from increased intraluminal pressure activates smooth muscle cells, eliciting vessel contraction defined as *myogenic tone* [11]. According to law of Laplace $T = P \cdot r$, loss of myogenic tone increases vessel radius (r) leading to increased wall tension (T) (Figure 2). Wall tension is an opposing force to the distension exerted by the intraluminal pressure (P). The wall tension can be normalized to wall thickness (w) and expressed as wall stress (σ): $\sigma = T/w$. Hence, since $\sigma = P \cdot r/w$, wall stress can be increased by dilation of the vessel, even if blood pressure is constant. The blood vessel has two main ways to reduce wall stress. One is by decreasing the vessel radius e.g. through myogenic contraction. The other mechanism is by different types of vascular remodeling affecting the wall:lumen ratio.

The mechanisms underlying stretch-induced myogenic response are elusive. Several ion channels have been implicated in mechano-activated vasoconstriction including transient receptor potential channel 6 (TRPC6) and voltage-gated potassium channels (K_v). However, studies have demonstrated that these may not be the primary mechanosensors responsible for myogenic vasoconstriction. In particular, emerging evidence suggest angiotensin II type 1 receptor (AT_1R) as a novel mechanosensor that can be directly activated by mechanical stimuli including intraluminal pressure. Knockout of AT_1R in mouse mesenteric arteries completely abolishes myogenic tone. Thus, mechanical activation of AT_1R is a key contributor to myogenic autoregulation and subsequently blood pressure regulation [12].

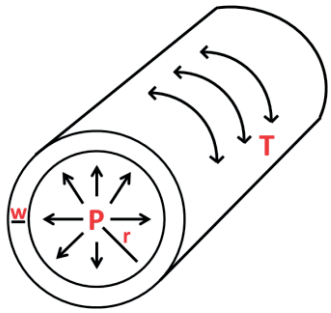

Figure 2. Laplace's law describes the relationship between wall tension (T), intraluminal pressure (P) and vessel radius (r). Wall tension increases as a result from elevated intraluminal pressure, increased vessel radius (r) or decreased wall thickness (w).

Vascular smooth muscle cell

Smooth muscle contraction

Smooth muscle contractility is dependent on cross-bridge cycling between contractile proteins actin, thin filaments, and myosin, thick filaments. Contraction is initiated by mechanical stimuli or by various agonists binding to specific receptors on the cell membrane (Figure 3).

Calcium-dependent contraction

Contraction is mediated by elevation in intracellular calcium (Ca^{2+}) concentrations resulting in activation of myosin motor activity. The increase in free Ca^{2+} arises from calcium release from intracellular stores, mainly the sarcoplasmic reticulum (SR), and influx from extracellular space through Ca^{2+}- channels, initiated by stretch or agonist binding to receptors. The primary target of the initial increase in cytosolic Ca^{2+} is calmodulin, a calcium-binding protein. Binding of calcium to calmodulin induces conformational change of the calmodulin molecule and subsequently activation of downstream targets including myosin light chain kinase (MLCK). MLCK is an enzyme that phosphorylates regulatory myosin light chains on myosin in the presence of ATP. Energy released from ATP hydrolysis then drives actin and myosin to slide past each other resulting in muscle contraction. Conversely, a decrease in intracellular Ca^{2+} results in smooth muscle relaxation [13, 14].

RhoA/Rho kinase signaling

The small GTPase RhoA and its downstream targets, play a key role in sustaining contraction through calcium sensitization by regulating myosin light chain

phosphatase (MLCP) activity. MLCP acts by dephosphorylating the myosin light chain of myosin, promoting smooth muscle relaxation. RhoA activity is regulated by Rho-guanine nucleotide exchange factors (Rho-GEFs) which enables the transition of Rho from an inactive (GDP-bound) state to an active (GTP-bound) state. The active RhoA-GTPase activates numerous downstream targets including Rho kinase (ROCK), a serine/threonine kinase, which has been shown to interact with MLCP. ROCK inhibits MLCP activity by phosphorylation, causing an elevation in the amount of phosphorylated myosin light chain, which subsequently leads to an increase in contractility [13, 14] In addition to Ca^{2+}-sensitization, RhoA is involved in rearrangement of the actin cytoskeleton and in regulation of gene expression important for force generation.

Actin polymerization

Actin belongs to a family of highly conserved proteins existing in six isoforms, grouped into three classes: α, β and γ. Smooth muscle expresses four different isoforms, non-muscle β and γ actin, and smooth muscle α and γ actin. The former isoforms are thought to have purely cytoskeletal functions, whereas the latter isoforms are important components of the contractile machinery. In vascular smooth muscle, α-actin is the predominantly expressed actin-isoform [15, 16]. Actin cytoskeletal dynamics, together with myosin, is crucial for intracellular force generation required for smooth muscle contractility. Actin filaments in vascular smooth muscle cells undergo ongoing rearrangements through their disassembly (depolymerization) and assembly (polymerization). The rate of actin polymerization is increased when vascular smooth muscle cells are exposed to mechanical strain or contractile agonist stimulation. Actin exists in two different forms inside the cells, either as monomeric globular actin (G-actin) or polymeric filamentous actin (F-actin) [17] .The process of actin polymerization is activated by several signaling pathways, primarily the Rho/Rho-kinase pathway. As a response to activation of Rho-kinase, downstream target LIM kinase is phosphorylated causing inactivation of actin depolymerizing factor, cofilin [18-20]. As a consequence, G-actin polymerizes into filamentous F-actin inside the smooth muscle cells. The pool of G- and F-actin can be pharmacologically manipulated providing changes in F:G- actin ratio. Jasplakinolide (jasp) is a compound that stabilizes filamentous F-actin whereas latrunculin B (latB) inhibits assembly of monomeric G-actin, a process which is independent of myosin light chain phosphorylation or intracellular calcium concentrations [21, 22].

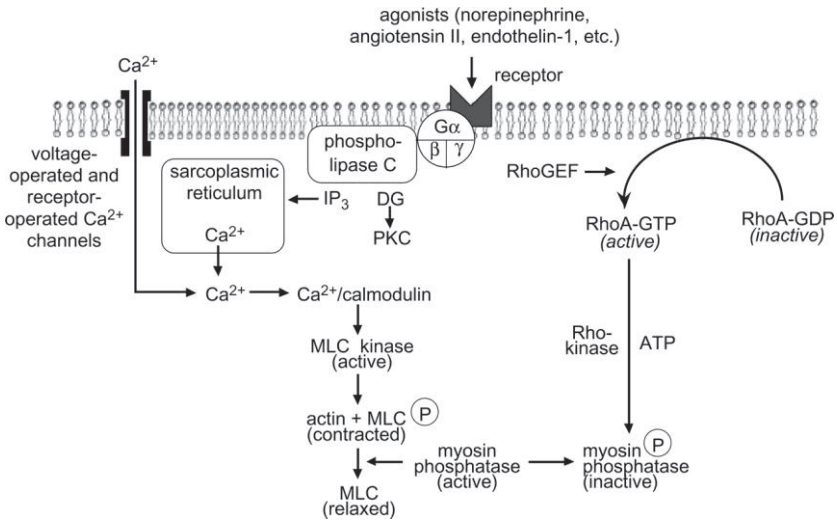

Figure 3. Schematic representation of smooth muscle regulation. Contractility of smooth muscle is initiated by an increase in intracellular calcium (Ca^{2+}) concentrations following mechanical (stretch) stmiuli or binding of various agonists (humoral, neurotransmittors) to specific receptors on the cell membrane. Mechanical stimuli elicts contraction through depolarization of the cell membrane likely via the opening of voltage-dependent calcium channels (L-type calcium channels). In addition, a number of chemical stimuli including angiotensin II and endothelin-1 can elict contraction by binding to membrane-bound G-coupled receptors (GPCRs). Contractility is further regulated by the RhoA/ROCK signaling pathway via myosin light chain phosphatase (MLCP) [13].

Smooth muscle plasticity

During vascular development, the main function of SMCs is to proliferate and synthesize extracellular matrix (ECM) proteins, components of the vascular wall. In adult vessels, however, SMCs are normally present in a differentiated contractile state, characterized by a low rate of proliferation and migration, expression of unique repertoire of proteins, ion channels, receptors and signaling molecules required for the cell's contractile function. Unlike cardiac and skeletal muscles which are considered terminally differentiated, SMCs within adult tissue retain remarkable plasticity. In response to changes in local environmental cues, which involve mechanical forces, injury, cell-cell and cell-matrix interactions, SMCs can switch from a more quiescent contractile phenotype to a proliferative, migratory phenotype, commonly termed "synthetic" smooth muscle cells (Figure 4). This process was first described as dedifferentiation but is now also often referred to as phenotypic modulation or switching. The course of phenotypic modulation requires substantial changes in gene expression patterns over a period of time. Differentiated contractile SMCs are associated with high expression of smooth muscle markers, proteins necessary for the contractile function of SMCs. These smooth muscle markers include cytoskeletal proteins (desmin), actin-binding proteins (calponin, SM22α, synaptopodin 2) and contractile proteins

(tropomyosin, α-actin, myosin heavy chain). Tropomyosin 4 and Myosin heavy chain embryonic are typically expressed in immature SMCs and considered markers of the synthetic phenotype. Despite growing evidence demonstrating the occurrence of SMC plasticity, we do not have a full understanding about the details of molecular mechanisms regulating SMC phenotypic modulation [23, 24].

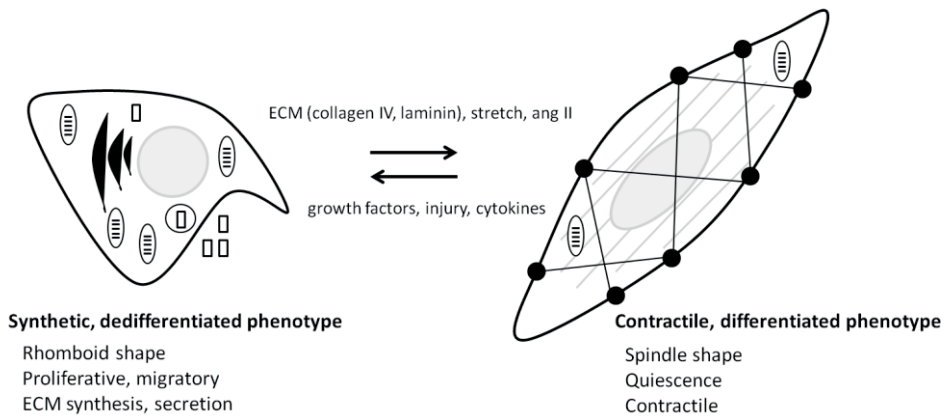

Figure 4. Smooth muscle phenotypic plasticity. Different characteristics of contractile vs. synthetic phenotype.

Transcriptional regulation

The transcriptional program that defines SMCs includes serum response factor (SRF), a ubiquitously expressed transcription factor that regulates a variety of genes involved in growth and muscle differentiation. The specificity of SRF to regulate smooth muscle-specific genes depends on its interaction with co-factors including the myocardin family of transcriptional coactivators; myocardin (MYOCD) and myocardin-related transcription factors A and B (MRTFA/MKL1 and MRTFB/MKL2). SRF can also bind other co-factors which compete for SRF binding including the ternary complex factors (TCFs), effectors of MAPK signaling (Figure 5) [25]. TCFs regulate genes involved in growth and proliferation, hence resulting in a more synthetic proliferative phenotype. SRF belongs to the MADS family of transcriptional factors that share a conserved MADS-box domain involved in DNA-binding. SRF binds to specific A/T-rich DNA sequences ($CC(A/T)_6GG$), known as CArG boxes, crucial for SRF-dependent transcription [26, 27]. Whereas, MRTFA/B are expressed in a variety of embryonic and adult tissues [28], myocardin expression is confined to cardiac and smooth muscle [29]. MRTFs possess a unique RPEL domain that promotes binding to monomeric G-actin in the cytoplasm, rendering MRTFs inactive [17]. Stimuli that activate the Rho-signaling pathway and promotes actin polymerization

causes MRTF to be released from G-actin and transported into the nucleus where it activates SRF [30, 31]. SRF/MRTF and SRF/myocardin activation promotes VSMC differentiation and cytoskeletal organization by regulating the expression of smooth muscle marker genes including SM α-actin, calponin and myosin heavy chain (SM-MHC). In contrast, myocardin is insensitive to changes to actin dynamics and is constitutively active in the nucleus. However, despite the fact that both myocardin and MRTF control the transcription of contractile genes in smooth muscle, these factors may play a reciprocal role in pathological vascular remodeling [32].

In addition to myocardin family of coactivators, gene transcription in vascular smooth muscle is regulated by several other transcription factors including GATA6. The GATA family of zinc-finger transcription factors plays an essential role in developmental processes and cell-specific gene transcription. The members of the GATA family act through interactions with DNA regulatory elements containing the motif 5'-(A/T)GATA(A/G)-3'. In vertebrates six GATA genes have been identified and categorized into two subfamilies; GATA-1/2/3 and GATA-4/5/6. GATA6 is the predominantly expressed GATA factor in smooth muscle [33, 34]. Because of their ability in restricting other cell-lineages it was proposed that GATA6 may regulate VSMC differentiation. Several studies demonstrate that GATA6 plays a role in maintaining the differentiated contractile phenotype of VSMC by regulating the expression of smooth muscle specific genes [35, 36]. In addition, GATA6 has been shown to promote cell cycle arrest [37] and reduce neointima formation *in vivo* following vascular injury [38, 39]. However, studies have also shown that GATA6 can induce expression of genes associated with the synthetic function of SMCs [40, 41]. This effect of GATA6 may be caused by repressive or stimulatory effect on myocardin activity depending on target gene [42]. Despite the compelling amount of data demonstrating a role for GATA6 in regulation of VSMC phenotype, the details regarding the mechanisms underlying GATA6 remain unclear.

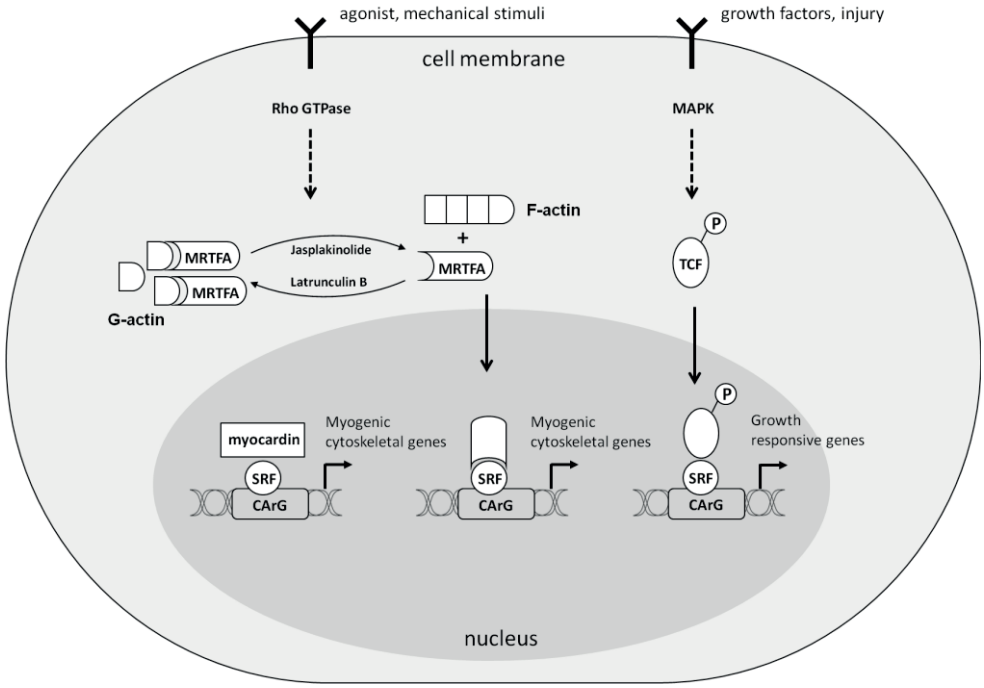

Figure 5. Regulation of smooth muscle phenotype in response to various stimuli. Activation of the myocardin family of coactivators induce the expression of genes necessary for the contractile function of smooth muscle. Growth factors and injury elicit mitogen-activated protein kinase (MAPK) signaling cascade, which activates TCFs. TCFs repress smooth muscle genes by triggering the displacement of myocardin/MRTFs from SRF. TCF-SRF binds to CARG boxes activating transcription of growth responsive genes.

Post-transcriptional regulation

microRNAs (miRNAs) represent a novel class of endogenous, small non-coding RNAs (20-25 nucleotides) involved in gene regulation at the posttranscriptional level. They were first described in 1993 [43, 44] and came to be known as miRNAs [45-47]. Since then they have been extensively studied in a range of cell types and biological processes. The genomic location for miRNA sequences can be intronic, intergenic and more rarely exonic [48]. miRNAs can share the promoter of their host gene or be regulated by independent promoters [49]. miRNAs are transcribed in the nucleus as a double stranded hairpin transcript, known as primary miRNA (pri-miRNA), and then further processed by the Drosha/DGCR8 complex into precursor miRNA (pre-miRNA) [50, 51]. The pre-miRNA is exported to the cytoplasm [52-54] and further cleaved by the endonuclease Dicer generating the mature double-stranded miRNA [55, 56]. The mature miRNA is then incorporated into the RNA-induced silencing complex (RISC), which include members of the Argonaute (Ago) protein family. All four mammalian Ago proteins participate in miRNA-mediated repression. Ago2 is the

only one with endonucleolytic activity, cleaving mRNA. Only one strand of the miRNA duplex is loaded into the RISC complex. The miRNA strand containing the thermodynamically less stable 5' end is usually chosen as the mature "guide" strand. The less abundant strand is termed the "passenger" strand (miRNA*) [57]. Studies have shown that the passenger strand can, in some cases, also be active in silencing [58, 59]. miRNAs binds to their target messenger RNA (mRNA) through Watson-Crick base-pairing between the regulatory sequence “seed region” on the miRNA (comprising nucleotides 2-8) and a complementary sequence on the mRNA, typically at the 3’untranslated region (3’UTR) [60]. The miRNA-RISC complex induces translational repression of target mRNA. In many cases this is associated with degradation of the mRNA transcript (Figure 6) [61-65].

Approximately 30% of the human gene set is predicted to be regulated by miRNAs, emphasizing the importance of miRNA regulation [66]. A large number of miRNAs have now been identified, but only a small fraction of these miRNAs have been assigned biological functions. Studies have demonstrated involvement of miRNAs in vascular development and phenotypic control of VSMCs. Experiments involving smooth muscle Dicer knockout mice resulted in loss of smooth muscle miRNAs, and impaired contractile force of vessels associated with loss of contractile proteins, suggesting miRNAs as key mediators of vascular smooth muscle development and function [67-69]. Among the miRNAs described so far, miR-143 and miR-145 have received much of the attention. miR-143 and miR-145 are highly expressed in VSMCs as a bicistronic cluster under the control of SRF and members of the myocardin family of coactivators [70]. Both miR-143 and miR-145 have been shown to be required for VSMC differentiation by several mechanisms including, but not limited to, Kruppel-like factor 4 (KLF4), KLF5 and Elk-1, all of which are suppressors of contractile smooth muscle gene expression [71-73]. In addition, several studies have demonstrated miR-145 to play a role in reducing neointima formation *in vivo* in rat carotid arteries upon balloon injury [73, 74]. Moreover, miR-143 and miR-145 have been implicated in vascular disease states such as atherosclerosis [75] and aneurysm [76, 77].

In addition to miR-143/145, a number of other miRNAs have been shown to play a role in smooth muscle. For instance, increased expression of miR-31 downregulates the expression of its target, cellular repressor of E1A-stimulated genes (CREG), a transcription factor highly expressed in differentiated cells [78]. Furthermore, miR-21 has been shown to be upregulated in atherosclerosis [79], aortic aneurysm [80] and neointimal lesions [74]. miR-21 promotes SMC proliferation by regulating the expression of its target phosphatase and tensin homolog (PTEN), a known tumor suppressor [74]. In parallel, up-regulation of miR-146a, miR-221and miR-222 has shown to promote VSMC proliferation and dedifferentiation by targeting KLF4 [81] and cell cycle inhibitors p27 (Kip1) [82], p57 (Kip2) [83], respectively.

Importantly, miRNAs can be manipulated using oligonucleotide inhibitors (anti-miRs) or miRNA mimics, representing a new frontier in modern medicine to modulate biological pathways [84]. Although much remains to be learned regarding the delivery, pharmacokinetics and miRNA biology, several miRNA-targeted therapeutics have already reached clinical studies including miR-34 [85], miR-122 [86] and miR-29b for treating cancer, hepatitis and fibrous scar formation respectively [87]. However, there is currently no miRNA-directed therapeutics against vascular disease.

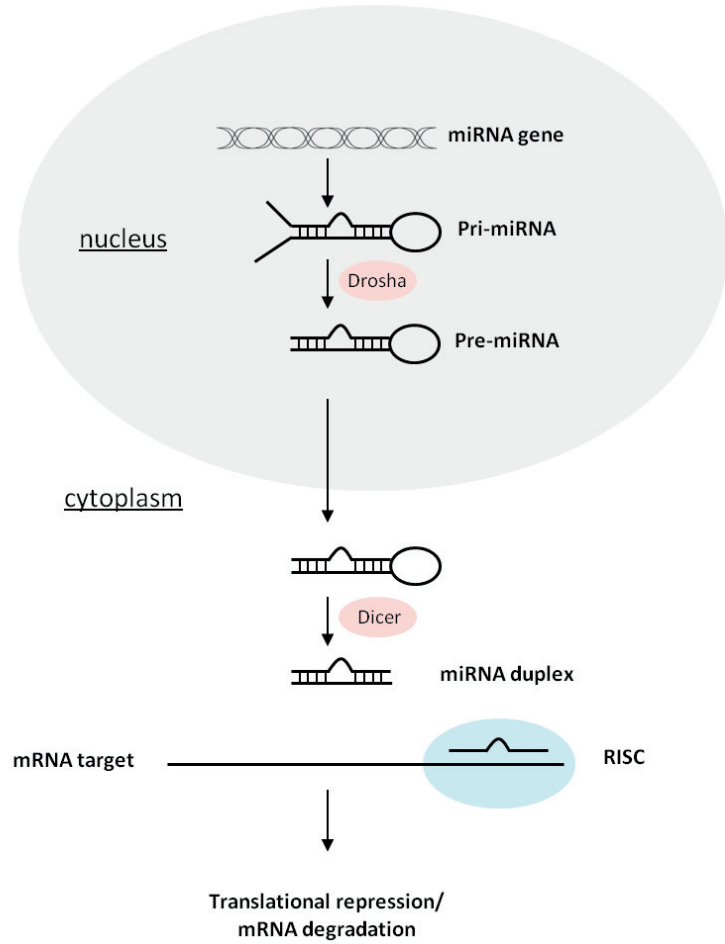

Figure 6. Schematic illustration of miRNA biogenisis.

Smooth muscle plasticity in vascular disease

Although beneficial during certain circumstances such as wound healing following a vascular injury, phenotypic modulation of smooth muscle plays a prominent role in the pathophysiology of many vascular disease states. The most common and well-studied vascular diseases include hypertension, atherosclerosis and restenosis accompanied by VSMC migration and excessive proliferation. Reversing the phenotypic modulation is therefore of great interest to control the development and progression of these diseases.

Atherosclerosis

Atherosclerosis is the leading cause of death in the Western world [88]. Despite significant advances made in pharmacological therapies (e.g. statins) and surgical interventions (bypass, angioplasty), atherosclerosis remains the most prevalent cause for cardiovascular disease.

Atherosclerosis is a chronic inflammatory disease of large and medium-sized arteries resulting in gradual narrowing of vessel lumen leading to obstruction of normal blood flow. It is characterized by the formation of atherosclerotic plaques composed of inflammatory cells including macrophages, build-up of modified lipoproteins, phenotypically modified SMCs and lipid-laden cells, termed foam cells. A vulnerable plaque is a high-risk plaque that can rupture and form a thrombus, leading to major clinical manifestations such as stroke and myocardial infarction [89].

In the development and progression of atherosclerosis, SMCs dedifferentiate and start to proliferate and migrate towards the intima. Dedifferentiated SMCs synthesize ECM proteins which form a fibrous cap that protects the plaque from rupturing. Hence, the general dogma has been that SMCs act athero-protective whereas macrophages, which engulf modified lipoproteins and become foam cells, are atherogenic and detrimental for plaque stability [89]. However, the use of novel lineage-tracing techniques has challenged this historical view. Several emerging reports have shown that SMCs can transdifferentiate (reprogram) into other cell types including macrophages [90]. Approximately 30% of cells identified as macrophages in atherosclerotic lesions are SMC-derived [91]. Notably, studies have demonstrated that up to 70% of all foam cells in atherosclerosis originate from SMCs and not from macrophages as previously assumed [92]. The molecular mechanism and clinical significance behind SMC transdifferentiation and foam cell formation remains largely undefined.

Hypertension

High blood pressure, or hypertension, is defined as systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg (140/90). In US, however, new blood pressure guidelines state that normal blood pressure is under 130/80. Hypertension affects an estimated 1.13 billion people worldwide and is the leading risk factor for cardiovascular disease, including atherosclerosis. In many cases existing therapies are not sufficient in controlling blood pressure. Hypertension remains therefore a major health problem [93]. A key hallmark of hypertension is increased peripheral resistance due to functional or structural changes to the arteries [94, 95].

Alterations in the structure of blood vessels is termed vascular remodeling and involve changes in VSMCs of both large and small arteries. These changes include proliferation and hypertrophy of VSMCs, reorganization of cells around the vessel lumen and changes in ECM composition. Vascular remodeling is a heterogeneous process and may involve a decrease or increase in vessel lumen diameter, termed, *inward* or *outward* remodeling, respectively. This can occur with no change in media cross-sectional area and is then called *eutrophic* remodeling. Conversely, vessels with increased or decreased cross-sectional area are considered *hypo-* and *hypertrophic* respectively. When the cross-sectional area is unchanged but the vessel lumen is reduced, the wall-to-lumen ratio is enhanced [96]. In essential hypertension, inward eutrophic remodeling of small resistance arteries has been observed, with reduced lumen and increased wall:lumen ratio [95, 97]. Hypertrophic remodelling with increased vascular growth is often observed in secondary hypertension [97]. Initially, these structural changes are adaptive, aiming to normalize wall stress, but in pathological conditions they become maladaptive, restricting blood flow, crucial in maintaining proper hydraulic pressure in downstream capillaries. The exact role of remodeling as a cause or effect of hypertension is still not fully understood and warrants further studies.

Finding ways to prevent the progression of hypertension by targeting vascular remodeling will be essential for global health. The cellular changes underlying vascular remodeling in hypertension have been attributed to signaling pathways activated by vasoactive agents, growth factors, mechanical factors (stretch) and physical forces (shear stress, pressure). These processes activate signaling pathways that modulate VSMC contractility, cell migration/proliferation/hypertrophy, apoptosis and inflammation [97, 98]. Mechanical stress leads to the activation of integrins, G-protein coupled receptors (GPCRs) and tyrosine kinase receptors [95]. Angiotensin II (ang II) is one of the factors known to play an important role in hypertensive vascular remodeling. Ang II is released/ synthesized in response to pressure and binds to its receptor, AT_1R. Activation of AT_1R leads to vasoconstriction and cellular growth by activating a

cascade of signaling pathways including phospholipase C (PLC), tyrosine kinases and mitogen-activated protein kinases (MAPKs), RhoA/ROCK that regulate the function of VSMCs [99]. The significance of ang II in the pathogenesis of hypertension has been demonstrated by studies targeting AT_1R and angiotensin-converting enzyme (ACE), which results in lower blood pressure and reversal in arterial remodeling.

Aneurysm

Aneurysms are local distensions of a blood vessel caused by a weakening in the arterial wall. They are defined as an increase in vessel diameter by more than 50% of the normal arterial size. Ruptured aneurysm causes serious clinical complications, which are fatal in many cases. Aneurysms can occur in any blood vessels but the most common is aortic aneurysms. Global Burden of Disease 2013 study listed aortic aneurysm among the top 10 leading causes for cardiovascular disease-related deaths [100]. Aneurysm is characterized by a distinct mechanism of disease progression including VSMC apoptosis, degradation of ECM proteins and elastin fragmentation. Phenotypic modulation of VSMCs has also been reported in aneurysm accompanied with increased cell proliferation, increased cell apoptosis, reduced expression of contractile proteins and increased expression of metalloproteinases (MMP2 and MMP9), which results in widespread ECM degradation [101, 102]. Mutations in smooth muscle-specific proteins *ACTA2* [103] and *MYH11* have been shown to cause aortic aneurysm [104]. Notably, mutations in *ACTA2* is the most prevalent cause of thoracic aortic aneurysm, with more than 40 identified mutations. Mutations in *ACTA2* leads to a perturbed actin cytoskeleton and reduced contractility, which leads to reduced ability to withstand mechanical stress and subsequently increasing the risk for aneurysm [105].

Moreover, several miRNAs have been shown to be deregulated in aneurysm including the miR-29 family, which targets mRNA encoding ECM proteins [106, 107]. Importantly, inhibiting miR-29 *in vivo* using antisense oligonucleotides has been shown to abrogate aortic dilation in mice [108, 109]. Smooth muscle-enriched miRNAs, miR-143 and miR-145, have been shown to be reduced in human aortic aneurysm [76] as well as in intracranial aneurysms [110]. Overexpression of miR-145 reduces ang-II induced aortic aneurysm, possibly via downregulation of MMP-9 expression [111]. In a recently published study miR-134 was identified as a novel regulator of VSMC phenotype and vascular remodeling through targeting STAT5b and ITGB1. Induced expression of miR-134 *in vivo* suppressed aortic dilation and media degeneration in thoracic aortic dissection [112]. Reversing smooth muscle phenotypic modulation can however result in the loss of mechanisms that protect against aneurysm development. Several groups have demonstrated increased expression of miR-21 in aortic and

cranial aneurysm [80, 110]. Notably, inhibition of miR-21 leads to an increase in aortic dilation whereas overexpression of miR-21 showed protective effects on aneurysm expansion. This effect of miR-21 is likely due to its effect on increasing cell proliferation and decreasing apoptosis in the aortic wall through its target PTEN [80].

Restenosis

Phenotypically modified SMCs is not only a hallmark of atherosclerosis and hypertension but also a result of balloon angioplasty and in-stenting. These are invasive procedures to widen obstructed vessels due to pathological conditions including atherosclerotic lesions. Despite widespread use of angioplasty technique, restenosis (re-narrowing of blood vessels) remains a major clinical problem, limiting the long-term success. Although drug-eluting stents have dramatically reduced the occurrence of restenosis, it still remains a major clinical challenge. Angioplasty includes dilating the vessel by inflating a balloon inside the artery. This process exposes the vessel to elevated stretch leading to vessel damage. SMCs respond to repair the damage by phenotypic modulation and migration towards the lumen resulting in neointima formation and restenosis [113]. Understanding the factors that contribute to this pathophysiology is therefore fundamental to develop strategies to improve the outcome following invasive interventions.

Aims

The overall objective of the thesis was to gain further insight into the molecular mechanisms that regulate vascular smooth muscle cell phenotype. Elucidating the transcriptional and post-transcriptional regulation of vascular smooth muscle phenotype is of outmost importance in developing efficient therapeutic approaches against cardiovascular disease states.

The specific aims were:

Paper I: Identify miRNAs regulated by actin polymerization and MRTFA in smooth muscle

Paper II: Investigate the importance of miR-143/145 for vascular mechanosensing and adaption to hypertension

Paper III: Study the role of MRTFA in smooth muscle lipid accumulation and foam cell formation

Paper IV: Investigate the importance of GATA6 for regulation of smooth muscle gene expression and function

Material and Methods

This section is a brief overview of methods applied in this thesis. A more detailed description of the methods and techniques used can be found in the method-section in the respective papers.

Cell culture

Primary vascular smooth muscle cells from human or mouse were used throughout this thesis. Primary cells can be defined as cells isolated directly from a biological tissue, in contrast to cell lines, which have been continuously passaged over a long period of time and are immortalized. Although primary cells have a limited lifespan they provide a number of advantages. Most importantly, primary cells reflect the true diversity of living tissue, offering more biologically relevant data compared to cell lines.

Primary vascular smooth muscle cells can be obtained from tissue using enzymatic digestion. This is a process that involves tissue dissection and isolation of cells. The cells are then placed in a cell culture flask under sterile condition and cultured in cell medium supplemented with antibiotics and serum, necessary for cell growth and function. The cells are kept in an incubator at 37˚C in 5% CO_2.

To study the functional importance of a specific gene we have utilized replication deficient virus constructs as gene delivery tools. They offer great advantage as they infect animal cells with high efficiency. Throughout the thesis we have used various adenovirus constructs to overexpress specific genes.

This method was used in paper I-IV.

Animal models

While cell culture systems allow for a controlled and safe environment to perform experiments, they do not reflect the complex three-dimensional environment of a living animal. Hence, animal models can be very useful tools.

The mouse is the most frequently used animal model. To investigate the functional importance of a specific gene *in vivo*, a knock-out (KO) mouse model is used. The gene of interest can either be deleted from all tissues in the body (global KO) or from a particular tissue (conditional KO). In this thesis we used a global miR-143/145 KO mouse model generated by Boettger et al. [71]. In the KO mice, the coding region for miR-143/145 is replaced with a lacZ (beta-galactosidase) reporter. Beta-galactosidase activity can be assessed by staining with x-gal to monitor transcription of these miRNAs. All animal work was performed according to national and international guidelines and approved by the Animal Ethics Committee. This method was used in paper II.

Patient material

Not all results obtained in animals are relevant for humans as some molecular mechanisms may differ significantly between species. Hence, at some point all findings must be confirmed in human tissue. In collaboration with a research group in Italy we obtained human aortic biopsies from patients with mild dilation of the aorta as well as from healthy donors. These human biopsies were used to study early molecular mechanisms leading to aneurysm formation. In addition, in collaboration with a research group in Spain, we obtained intact human renal arteries and cells cultured from the same arteries to study SMC phenotypic modulation. Informed consent was obtained from all patients prior to their inclusion. This method was used in paper I.

Assessing mRNA and miRNA expression

Quantitative polymerase chain reaction (qPCR) is a highly sensitive, quick and easy to perform laboratory technique that allows for relative quantification of mRNA and miRNA gene expression. The first step includes cell lysis and RNA extraction followed by PCR. For PCR the following components are required: DNA polymerase, nucleotides (building blocks to create DNA), a gene-specific primer and sample DNA. For mRNA and miRNA expression analysis, RNA was used as the starting template. To obtain DNA, the RNA was reverse transcribed into complementary DNA (cDNA) using the enzyme reverse transcriptase (RT).

The PCR consists of three repeating steps performed in a thermocycler. Each step is devoted to a specific function: denaturing, annealing and elongation (Figure 7). The first step includes heating the sample to 95˚C which separates the two DNA strands. Next, the sample is cooled which allows the primers (forward and reverse)

to bind to a specific sequence on the DNA strands. The third step involves heating the sample to 72˚C, the optimal temperature for the DNA polymerase. The DNA polymerase initiates the synthesis of new DNA strands using the primers as a starting point and adding the nucleotides present in the sample mixture. Each cycle doubles the copy number of the gene of interest. The whole process is repeated 40 times, following an exponential pattern until it reaches a plateau, which is when most reagents have been used up. This enormous amplification capacity enables analysis of gene expression in extremely low sample concentrations.

To record amplification in real-time several fluorescence-based assays have been developed. SYBR Green is a highly sensitive dye which emits fluorescence only when bound to double-stranded DNA (dsDNA). The fluorescence intensity is proportional to the amount of dsDNA in the sample. SYBR Green is not sequence specific and detects all newly generated dsDNA. Hence, a melt curve analysis is performed to ensure specificity of the product, only one peak should be observed.

The PCR products are quantified by considering the cycler number, at which the fluorescent signal is detected above background noise. This is referred to as the cycle threshold (C_T). The more target DNA present initially, the lower the C_T value will be. Relative expression can be determined by normalizing the expression data to a control gene (housekeeping gene), whose expression does not change under different experimental conditions.

This method was used in paper I-IV.

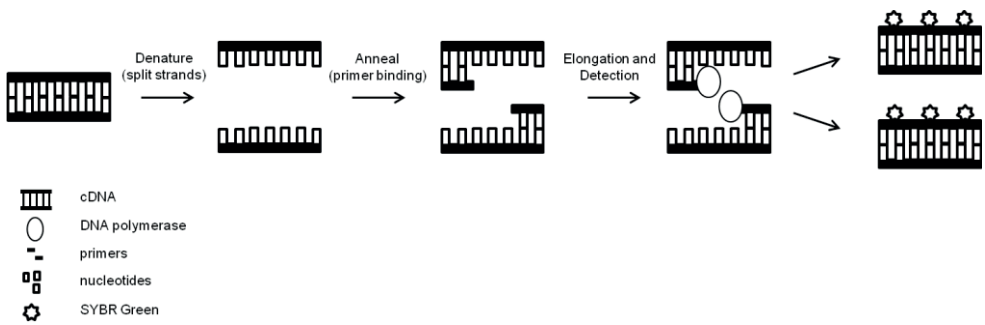

Figure 7. Schematic of the main steps in a PCR including denaturing, annealing and elongation.

Determining protein expression

Protein expression does not necessarily reflect the expression of its gene transcript. Thus, it is highly recommended to complement the mRNA analysis with

measurement of protein expression. Western blot is a widely used analytical technique to measure protein expression. The first step includes cell lysis and protein purification and determination. The lysis buffer contains sodium dodecyl sulfate (SDS), a compound that denatures proteins and gives them a negative charge. Equal amount of protein (5-30µg) is loaded to each lane on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).The proteins migrate through the gel towards a positively charged electrode. Proteins are separated on the gel based on their molecular weight. After separation, the proteins are transferred onto a nitrocellulose membrane by applying a constant current. This is based on the same principle as during gel electrophoresis. After transfer, the membrane is "blocked" in casein solution. Casein prevents non-specific protein binding by applied antibodies. Next, the membrane is incubated in a solution with a primary antibody specific to the target protein. After careful washing to remove unbound antibody, the membrane is incubated with a secondary antibody. The secondary antibody recognizes and binds to the primary antibody. To enable detection, the secondary antibody is conjugated to an enzyme, horse radish peroxidase (HRP). The enzyme is activated by introducing a substrate for HRP, which generates a chemiluminescent signal. Within a certain range, the signal is proportional to the amount of bound secondary antibody, and therefore functions as an indirect measure of the amount of protein present. Relative protein expression is determined by normalization to a control gene, known to stay constant between control and experimental sample.

Limitations to this technique include, but are not limited to, off-target binding by the primary antibody. Each antibody should thus be validated against a positive protein control, to ensure specificity. In addition, during the detection it is important to make sure that the bands of interest lie within the linear range for detection, as saturation may occur in highly abundant targets.

This method was used in paper I-III.

Immunofluorescence

Immunofluorescence (IF) is a technique used on biological samples (tissue sections or cells) to detect specific target-antigens using antibodies. This method is primarily used to detect the localization of a specific target but can also be used as a "semi-quantitative" method to assess the expression level of a protein. There are two main IF methods with different principles, primary (direct) and secondary (indirect). Direct IF uses one antibody, responsible for both target-binding and detection. Indirect IF uses two antibodies - a primary antibody specific for a target

protein and a secondary labeled antibody that facilitates detection. The detection is possible due to a fluorophore conjugated ("attached") to the bound antibody, which allows visualization of the target under a fluorescent microscope (Figure 8).

Indirect IF was used in this thesis as it provides higher sensitivity, important for low abundant antigens, and greater flexibility since secondary antibodies are inexpensive and come in a wide range of colors.

This method was used in paper II-III.

Figure 8. Illustration of immunofluorescence technique. For indirect immunofluorescence, a primary antibody and a secondary antibody are used. The primary antibody binds to a target protein while the secondary antibody is labeled with a fluorophore allowing detection.

Assessing myogenic tone and contractility

Resistance arteries respond to increased intravascular pressure by reducing their diameter through contraction. This myogenic contraction permits resistance arteries to maintain constant blood flow to downstream organs, contribute to regulation of blood pressure and maintain capillary hydrostatic pressure constant. Pressure myography is a technical apparatus that records changes in vessel diameter in response to changes in intraluminal pressure or flow. Contraction is only observed in the presence of extracellular calcium. In the absence of calcium, only a passive distension is observed. The difference in vessel diameter in the presence and absence of extracellular calcium determines the degree of active myogenic tone. Experiments can be conducted using resistance arteries from various vascular beds. Second or third order mesenteric arteries from mice were used in our experimental setups. To determine the myogenic response, 4-5 mm arterial segments are mounted in a bath chamber between two glass cannulae. The

cannulae are connected to two pressure transducers which monitor the intravascular pressure on the inflow and outflow side. These are connected to an adjustable servo system that regulates intravascular pressure via a peristaltic pump. Throughout the experiment the temperature is kept constant at 37˚C (Figure 9).

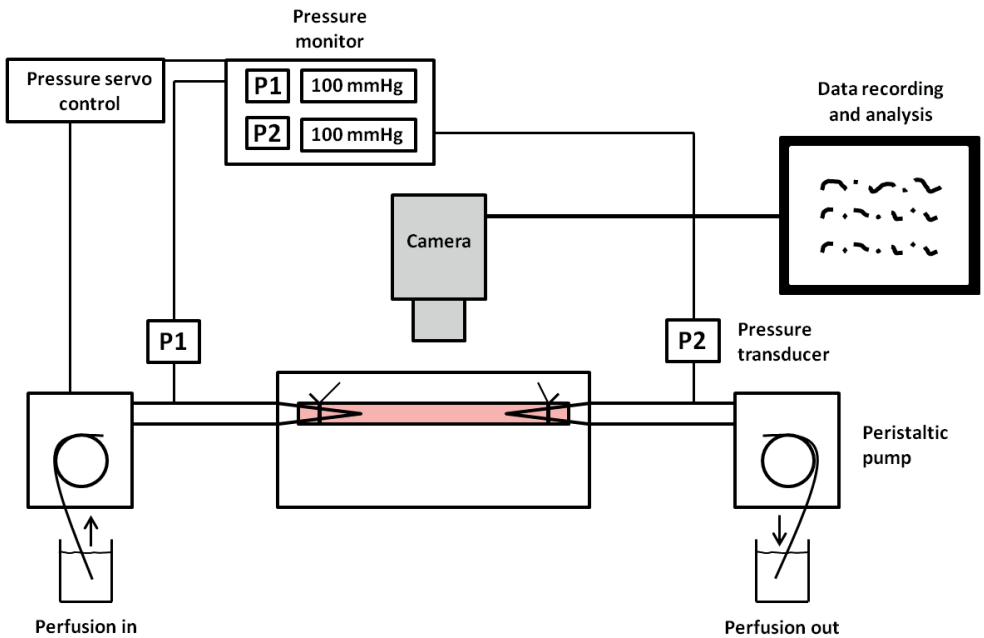

Figure 9. Illustration of pressure myography set-up for determination of vessel diameter at various intraluminal pressures.

Wire myography is a technique used to measure force generated from small arteries *ex vivo* under isometric conditions (where the internal circumference is held constant). Intact mesenteric artery segments, 1.5-2 mm in length, are mounted between two wires attached to a force transducer that records wall tension developed by the vessel and a micrometer which controls the vessel circumference (Figure 10). The preparations are kept in a chamber containing physiological salt solution at 37˚C. The vessels are held at an internal circumference L_0, for maximal force development. Following mounting and equilibration, each preparation is activated by high KCl to determine active force development and to ensure tissue viability. This is followed by determining the contractile responses to various contraction stimuli in the form of a dose-response curve. The wire myography can also be used to evaluate endothelial function and to analyze passive biomechanical

properties of vessels by determining the passive internal circumference-tension relationship.

Myography was used in paper II.

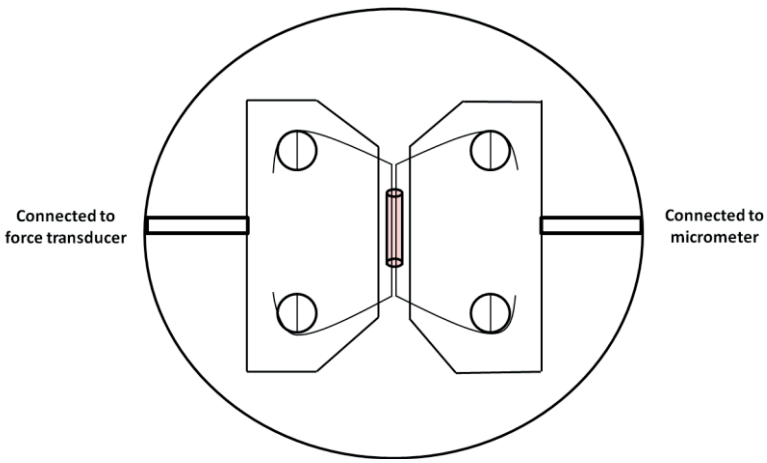

Figure 10. A schematic of wire myography apparatus for measuring force produced by an artery.

Results and Discussion

The following section is a summary of key findings in Paper I-IV. For details, readers are referred to the original papers.

In order to develop new efficient therapeutic approaches against cardiovascular disease states it is crucial that we understand the transcriptional and post-transcriptional regulation of gene expression that occurs during disease development and how these changes affect the function of the vascular wall. The work in the following papers has identified previously unknown mechanisms by which the small non-coding RNAs, actin polymerization and transcriptional regulators MRTFA and GATA6 can contribute to the changes in vascular smooth muscle observed in vascular disease states.

Paper I

Regulation of microRNA expression in vascular smooth muscle by MRTFA and actin polymerization

Actin is a ubiquitously expressed protein in smooth muscle that in addition to mediating mechanical stability and contractility is involved in transcriptional gene regulation via myocardin related transcription factor A (MRTFA). Increasing evidence points toward a crucial role for actin polymerization and MRTFA activity in disease development. Although regulation of protein-coding genes by actin/MRTFA has been demonstrated previously, some of the effects behind actin/MRTFA-mediated transcription may be regulated by small non-coding microRNAs (miRNAs). Hence, the aim of this study was to identify a profile of miRNAs that were transcriptionally regulated by actin polymerization and MRTFA expression.

Analysis was performed in both mouse and human vascular smooth muscle cells using a miRNA PCR array designed with primers for miRNAs associated with cardiovascular diseases. We identified five miRNAs (miR-1, miR-22, miR-143, miR-145 and miR-378a) that were regulated by dynamic changes in actin and MRTFA expression. Several of these miRNAs have already been shown to play an important role in smooth muscle but their transcriptional regulation was not well understood.

The phenotypic state of SMCs plays a key role in multiple vascular disease states. By comparing the miRNA expression in intact arteries with proliferating VSMCs derived from the same tissue we could demonstrate that all actin/MRTF-regulated miRNAs, except miR-22, were associated with the differentiated contractile phenotype of smooth muscle.

An important aspect for miRNA-directed therapies is to determine the abundance of a miRNA in specific tissues to limit unwanted off-target effects. A prime example is miR-122 inhibitor, Miravirsen, which has completed phase II clinical trials for treatment of hepatitis C. miR-122 expression is considerably dominant in the liver, which results in limited off-target effects of Miravirsen [114]. This notion prompted us to investigate the tissue specificity of the identified actin/MRTFA-regulated miRNAs. Except for miR-22, all miRNAs were enriched in muscle tissue. miR-143 and miR-145 were enriched in smooth muscle whereas miR-1 and miR-133 were highly expressed in both smooth and striated muscle.

To understand disease development and progression it is crucial to determine the early molecular events. The dynamic properties of actin cytoskeleton and subsequently MRTFA activity plays an important role in multiple vascular disease states including aneurysm [103]. Hence, the question arose as to whether actin-MRTFA-regulated miRNAs were altered in the early events of aneurysm formation. We found a dramatic decrease in polymerized actin and significant downregulation of actin-regulated miRNAs (miR-1, miR-143 and miR-145) in patients with mild aortic dilations compared to healthy controls, suggesting that these factors may play a role in resisting mechanical forces in the vascular wall (Figure 11).

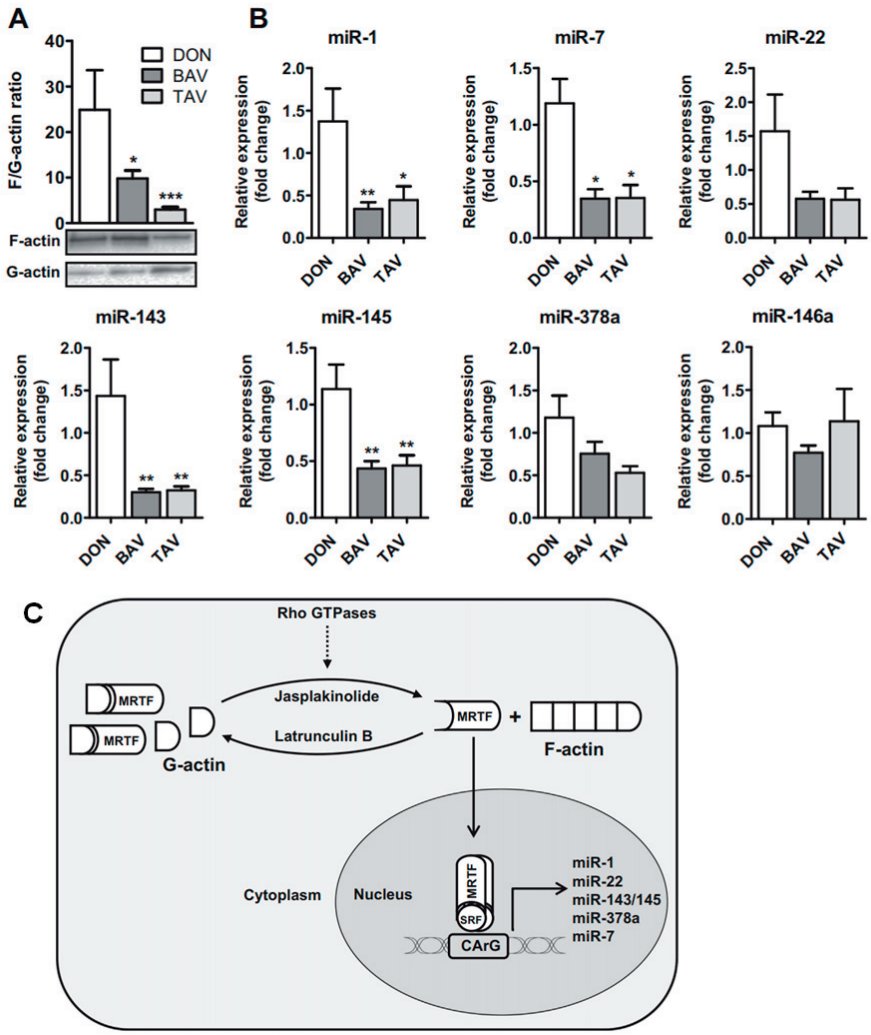

Figure 11. Actin/MRTFA-regulated miRNAs in patients with mild aortic dialtions.(A) Actin polymerization was assesed in aortic biopsies from healthy donors (DON) and patients with mild aortic dilations with bicuspid (BAV) or tricuspid (TAV) aortic valves by evaluating the F/G-actin ratio (n=3-9). (B) Expression of actin/MRTFA-regulated miRNAs was assesed in indicated patient groups (n=6-12). (C) Schematic model illustrating the role of actin polymerization and MRTFA activity on miRNA expression in smooth muscle. Data are presented as mean ± SEM. * p <0.05, ** p <0.01, ***p < 0.001 [115].

Paper II

Loss of vascular myogenic tone in miR-143/145 knockout mice is associated with hypertension-induced vascular lesions in small mesenteric arteries

Despite extensive knowledge regarding the events leading to contraction of arteries the mechanisms behind the mechano-sensing properties of vascular smooth muscle cells leading to myogenic constriction remain less certain. We have previously shown that Dicer-dependent miRNAs are critical for the development of myogenic tone. To this end, we investigated the importance of the two most highly expressed miRNAs in smooth muscle, miR-143 and miR-145, for pressure-induced myogenic constriction. This was done using miR-143/145 KO mice.

Using wire and pressure myography we could demonstrate that deletion of miR143/145 impairs force generation of the contractile system due to several factors including reduced calcium influx as a result of reduced L-type calcium channel expression and alternations in the contractile machinery including myosin light chain (LC_{20}) and myosin light chain kinase (MLCK). Moreover, we show for the first time that knockout of miR-143/145 completely abolishes pressure-induced myogenic tone in small mesenteric arteries. The mechanism behind myogenic responsiveness remains elusive. In previous studies we have shown that loss of myogenic tone in inducible Dicer KO mice is rescued by pretreatment with angiotensin II (ang II), an effect that is likely dependent on activation of the PI3-kinase/Akt signaling pathway [116]. However, miR-143/145 KO arteries were unresponsive to pretreatment with ang II. Emerging evidence has demonstrated a key role for angiotensin II type 1 receptor (AT_1R) for mechano-induced myogenic tone. Deletion of AT_1R in mesenteric arteries results in complete loss of myogenic tone [12]. In particular, AT_1R has been shown to be downregulated in miR-143/145 KO mice [71]. Thus, downregulation of AT_1R together with aberrant protein expression and reduced calcium signaling likely explain the loss of myogenic tone in small arteries of miR-143/145 KO mice.

Dysfunctional autoregulation of myogenic tone increases vessel diameter and subsequently elevates intraluminal pressure and blood flow in downstream vascular beds, increasing the risk for end organ damage. Hence, the question arose as to how small resistance arteries can cope with loss of myogenic responsiveness in a hypertensive setting. To the best of our knowledge, this had not been tested previously. We therefore investigated the importance of myogenic tone for vascular integrity in myogenically active vessels in mice subjected to chronic infusion of ang II subcutaneously to induce hypertension. Four weeks following treatment, analysis of blood pressure revealed similar increase in blood pressure in WT and KO mice. Because KO mice lack myogenic tone it is unlikely that the elevation in blood pressure was due to increased peripheral resistance. Instead,

retention of salt and water in the kidneys was likely responsible for the elevated blood pressure levels. In a hypertensive setting *in vivo*, loss of miR-143/145 resulted in the development of vascular lesions in small mesenteric arteries, possibly caused by the lack of myogenic tone. Arteries from KO mice developed multiple lesions at bifurcations and distal ends with inflammation, neointima hyperplasia, adventitial remodeling, SMC dedifferentiation, elastic lamina fragmentation and loss of polymerized actin in the media (Figure 12). The mechanisms initiating these events are unclear but we hypothesized, based on calculations on wall stress, the loss of myogenic tone and location of the lesions, that the extensive mechanical strain exerted on the vessel wall at bifurcations leads to events classical of the vascular response-to-injury mechanism. Additional studies are warranted to find out how abolished myogenic tone affects downstream vascular beds.

The expression of Galectin-3 (MAC-2) was dramatically increased in miR-143/145 KO lesions. Galectin-3 is a member of the lectin family of proteins and binds to several substrates including signaling molecules, receptors and ECM proteins to influence a wide range of biological processes including inflammation, cell cycle, apoptosis and fibrosis. In particular, studies have implicated Galectin-3 in the development of pulmonary hypertension of various experimental models. A recent study using the rat as a model system identified the smooth muscle cells as the predominant cell type expressing Galectin-3 in hypertensive pulmonary artery. They demonstrated that Galectin-3 confers a migratory, proliferative and fibrotic cell phenotype, contributing to pulmonary vascular remodeling. This was evident by employing pharmacological inhibitors and genetic deletion of Galectin-3 which could prevent and reduce pulmonary hypertension by preventing remodeling and fibrosis [117]. Thus, an attractive future study would be to investigate the therapeutic potential of Galectin-3 in hypertension-induced vascular lesions.

Altered expression of miR-143/145 has been documented in several human clinical settings including pulmonary hypertension, atherosclerosis and aneurysm. Notably, in pulmonary hypertension miR-145 expression is increased and treatment with inhibitors against miR-145 protects against disease development [118, 119]. However, in aneurysm, miR-143/145 is decreased. Hence, studies demonstrate a reciprocal role for miR-145 in vascular disease states. Our findings suggest that therapeutic inhibition of miR-145 for the treatment of pulmonary hypertension may result in adverse effects in arteries regulating myogenic tone and subsequently target organs.

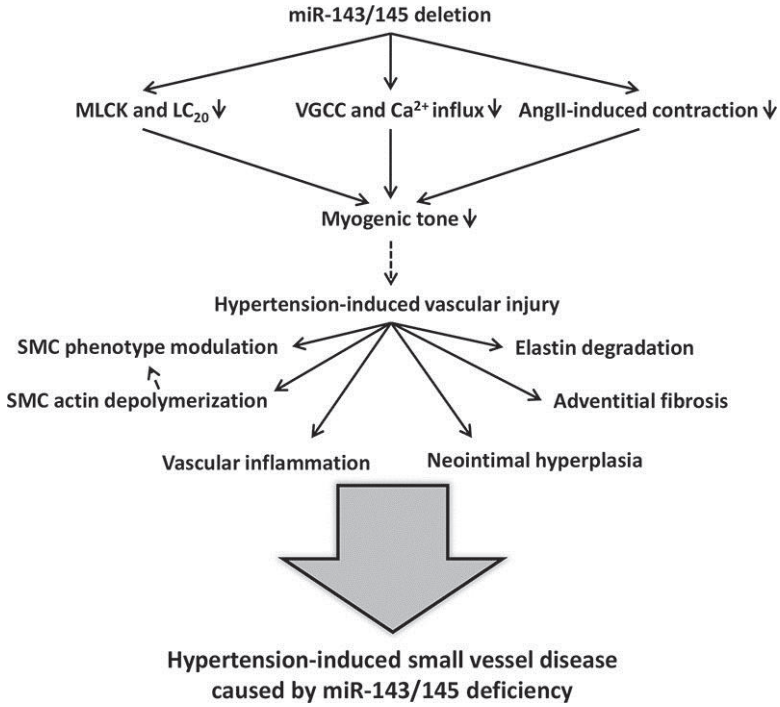

Figure 12. Deletion of miR-143/145 results in complete loss of myogenic tone in small mesenteric arteries. In a hypertensive setting this leads to damaging levels of mechanical stress in downstream vascular beds resulting in multiple vascular lesions [120].

Paper III

MRTFA promotes conversion of human coronary artery smooth muscle cells into lipid-loaded foam cells

A hallmark of atherosclerosis is the presence of lipid-loaded foam cells. A recently published study showed that approximately 70% of all total foam cells in atherosclerotic lesions originate from smooth muscle cells and not macrophages as assumed previously. The mechanisms underlying this process are poorly understood. While it is well established that the myocardin family of transcriptional co-activators play an important role in smooth muscle differentiation, studies have also demonstrated that induction of MRTFA expression is key in disease progression including atherosclerosis. Hence, in this study we investigated the effect of actin-sensitive transcriptional co-activator MRTFA for SMC foam cell formation.

To explore the role of MRTFA on foam cell formation we treated human coronary artery smooth muscle cells with a wide variety of lipids including cholesterol,

native LDL and oxidized LDL (oxLDL). We show for the first time that MRTFA potentiates lipid accumulation in VSMCs and thus promotes foam cell formation (Figure 13). We could further corroborate these findings using pharmacological compounds, ISX and CCG-1423, which promote or reduce MRTFA transcriptional activity respectively.

The general view is that unmodified LDL is taken up by the LDR receptor (LDLR) whereas oxLDL is taken up the by scavenger receptors. To inquire whether MRTFA regulates receptors for lipid uptake we analyzed the gene expression following MRTFA overexpression. Interestingly, MRTFA significantly increased levels of LDLR, whereas the expression of scavenger receptors was downregulated or unaltered suggesting that other mechanisms may be involved in MRTFA-mediated lipid accumulation. Emerging evidence has demonstrated a receptor-independent route known as macropinocytosis by which cells take up lipids. Considering that MRTFA potentiated lipid accumulation independent of lipid source we tested whether inhibition of macropinocytosis could prevent lipid accumulation following MRTFA overexpression. Administration of widely accepted inhibitors of macropinocytosis; amiloride, its derivate EIPA and PI3K-inhibitor (LY294002), we observed significant decrease in intracellular lipids. However, most inhibitors of endocytic pathways suffer from poor specificity. Hence, to confirm the involvement of macropinocytosis in MRTFA-mediated lipid accumulation, these findings will need to be complemented with markers of macropinocytosis such as latex beads and dextran.

This discovery uncovers new aspects of myocardin family of transcriptional activators in SMC function and their role in disease progression. Mechanical forces have been shown to induce MRTFA transcriptional activity through the Rho family of GTPases [121, 122]. The same family of GTPases have been shown to play a role in membrane ruffling and formation of macropinosomes [123]. It will be interesting to investigate whether changes in wall tension and stretch are coupled to lipid accumulation. We are currently investigating if cells cultured on substrates with various rigidities alter lipid accumulation via MRTFA nuclear activity. Moreover, in contrast to myocardin, MRTFA is expressed in a wide array of cells including macrophages. It will be important to establish whether MRTFA-mediated effects on foam cell formation are SMC-specific or if it is a general mechanism.

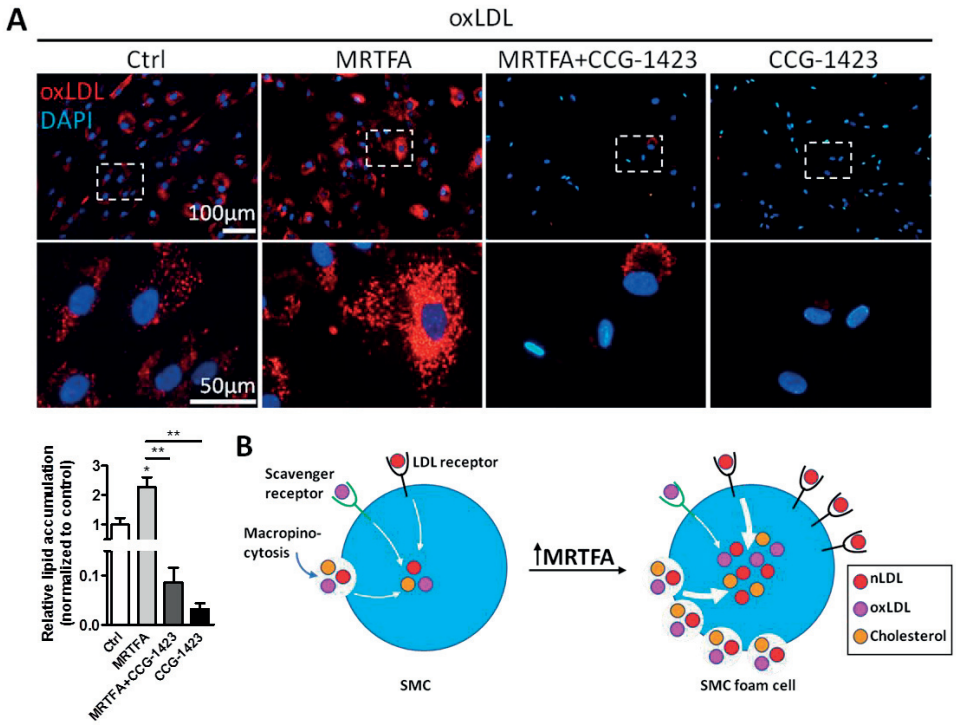

Figure 13. Increased lipid accumulation in vascular smooth muscle cells upon MRTFA overexpression. (A) Representative images of human coronary artery smooth muscle cells treated with/without MRTFA-encoding virus and/or MRTFA-inhibitor CCG1423 incubated with fluorescently-labeled oxidized LDL (4µg/ml) for 96 hours. Lipid accumulation was quantified and normalized to control.(B) Suggested model for MRTFA-mediated accumulation of intracellular lipids. MRTFA overexpression increased expression of LDLR and macropinocytosis. Data are presented as mean ± SEM (n=3). * p <0.05, **p < 0.01.

Paper IV

GATA6 promotes migration of human coronary artery smooth muscle cells

In contrast to the archetypical and more studied myocardin family of transcriptional co-activators, GATA6 is a less studied regulator of smooth muscle phenotype. In this paper, we investigated the importance of GATA6 in regulating gene expression and smooth muscle function *in vitro*. For this purpose we used smooth muscle cells obtained from coronary arteries of human origin.

To determine the effect of GATA6 on smooth muscle differentiation we analyzed the expression of SMC marker genes (*MYH11, ACTA2, CNN1, SYNPO2*) following GATA6 overexpression. In line with previous studies we observed a positive effect of GATA6 on the expression of these contractile markers. In parallel, reduced expression of GATA6 downregulated *ACTA2* expression,

however, no change in the expression of *CNN1* or *SYNPO2* was observed. This difference is not entirely surprising because *ACTA2* [36] and *MYH11* [35] are, to date, the only two smooth muscle markers demonstrated to harbor functional GATA6-binding sites in their promoter. However, gene transcripts do not necessarily reflect protein expression. Hence, it will be important to analyze the expression of SMC markers at the protein level.

Phenotypically modified SMCs are well known to be associated with changes in proliferative and migratory rates. While it is well established that GATA6 facilitates anti-proliferative effects on VSMCs, until a couple of months ago, the effect of GATA6 on VSMC migration had not been documented. This prompted us to explore the role of GATA6 on VSMC migration. Surprisingly, we found that overexpression of GATA6 promotes smooth muscle migration and that downregulation of GATA6 inhibits migration (Figure 14). We know from earlier studies that the role of GATA6 in smooth muscle is more complex than that of myocardin family of coactivators. It has been shown that GATA6 can regulate expression of genes associated with the synthetic SMC. This effect may be due to the activating or inhibitory effect of GATA6 on myocardin activity depending on target gene [42]. Importantly, while SMC dedifferentiation is often associated with reduced expression of SMC markers and increased rates of proliferation and migration it's important to keep in mind that these are separate biological events regulated by distinct signaling pathways [124].

To gain insight into the molecular mechanisms underlying GATA6-regulated cell migration a complete microarray screen of GATA6-sensitive gene transcription was performed. This screen identified several members of the TGF-β superfamily to be deregulated following GATA6 overexpression. TGF-β superfamily is well-known to play an important role in several vascular disease states and to have a wide array of effects on SMC function. This encouraged us to investigate whether TGF-β superfamily plays a role in GATA6-mediated cell migration. By employing chemical inhibitors of the TGF-β and BMP signaling pathways we obtained preliminary results suggesting a role for TGF-β superfamily in cell migration. While TGF-β is thought to protect against atherosclerosis, accumulating evidence indicate a positive role for TGF-β signaling in the development of hypertension and restenosis. In parallel, increased VSMC migration is considered to play an important role in stabilizing atherosclerotic plaques whereas in hypertension and restenosis, VMSC migration is involved in disease progression via neointima formation.

For future work it will be important to elucidate the involvement of TGF-β superfamily in GATA6-induced cell migration and to determine if GATA6 or TGF-β superfamily of proteins could be used to promote atherosclerotic plaque stability by stimulating SMCs to strengthen the fibrotic cap. The *in vitro* studies

were planned to be complemented by *in vivo* studies on the first smooth muscle specific and inducible GATA6 KO mouse. Unfortunately, we were not able to confirm a knockdown of GATA6 in this mouse model and the present study is therefore limited to *in vitro* results.

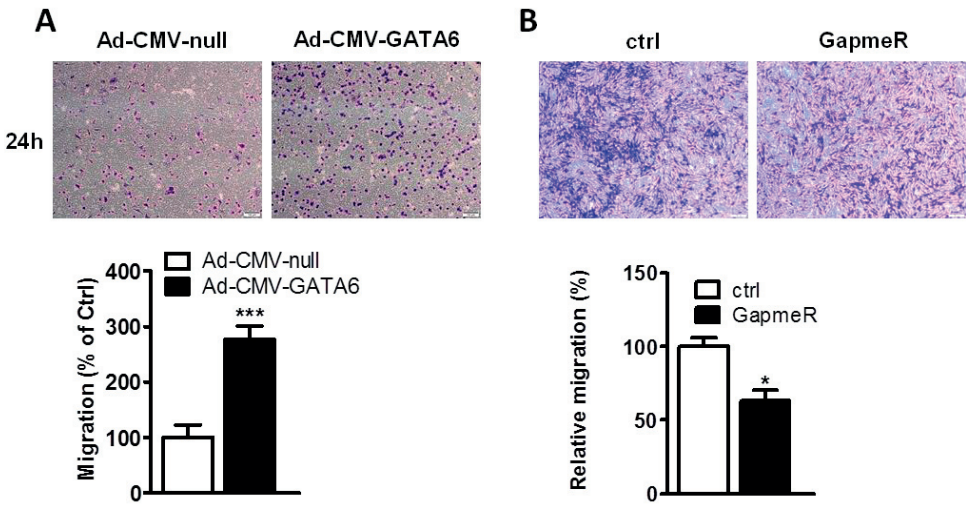

Figure 14. GATA6 promotes migration of human vascular smooth muscle cells. Migration of human coronary artery smooth muscle cells with (A) increased GATA6 expression (Ad-CMV-GATA6) or (B) decreased GATA6 expression (GapmeR) was assessed using a transwell migration assay. Photos were taken and analyzed at indicated time points. Data are presented as mean ± SEM (n=3-6). * $p < 0.05$, *** $p < 0.001$.

Conclusions

Based on the results obtained in papers I-IV we can conclude the following

- Actin polymerization and MRTFA regulate a profile of miRNAs (miR-1, miR-22, miR-143, miR-145 and miR-378a) that are (1) associated with the contractile differentiated SMC phenotype and (2) enriched in muscle containing tissue. Using patient biopsies from mildly dilated aorta we could demonstrate reduced actin polymerization that was associated with reduced expression of actin/MRTFA-regulated miRNAs.
- Knockout of miR-143/145 completely abolishes myogenic tone of small mesenteric arteries. This results in vascular lesions under hypertensive conditions as a result of elevated wall stress with adventitial remodeling, neointimal hyperplasia, loss of polymerized actin in the media and inflammation.
- Actin-sensitive transcriptional co-activator MRTFA enhances lipid accumulation in human coronary artery smooth muscle cells, resulting in foam cell formation.
- Transcription factor GATA6 promotes migration of human coronary artery smooth muscle cells. Moreover, a microarray screen identified hundreds of GATA6-sensitive genes including several members of the TGF-β superfamily.

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