Notch Signaling Guides Vascular Smooth Muscle Cell Function

DISSERTATION

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Abstract

Proper blood vessel formation and function is crucial for embryogenesis, wound healing, pregnancy and diseases. The interaction of endothelial cells and vascular smooth muscle cells/pericytes is critical for assembling and maintaining blood vessels. Previously, our lab and other groups showed that endothelial cells play an important role in governing vascular smooth muscle cell functions. To further investigate the signaling mechanisms that govern the interaction of vascular cells, we screened for genes and microRNAs whose expression was significantly altered by the endothelial cell-smooth muscle cell interaction.

One of those altered genes was SYNDECAN-2, which encodes a heparin sulfate proteoglycan (HSPG) surface receptor. SYNDECAN-2 is known to be important for developmental processes including angiogenesis in vitro and in vivo, however the role of SYNDECAN-2 in vascular smooth muscle cells has not been identified. Our results show that endothelial cells induce mRNA expression of SYNDECAN-2 in smooth muscle cells by activating Notch receptor signaling. Both NOTCH2 and NOTCH3 contribute to the increased expression of SYNDECAN-2 and are themselves sufficient to promote its expression independent of endothelial cells. Syndecan family members serve as coreceptors for signaling molecules and interestingly, our data show that SYNDECAN-2 regulates Notch signaling and physically interacts with NOTCH3. Notch activity was attenuated in smooth muscle cells made deficient in SYNDECAN-2, and this specifically
prevented the expression of differentiation marker smooth muscle α-actin (ACTA2). These results show a novel mechanism in which Notch receptors control their own activity by inducing the expression of SYNDICAN-2 that then acts to propagate Notch signaling by direct receptor interaction.

miR-145 is considered as a smooth muscle specific microRNA, and has been implicated in vascular smooth muscle cell differentiation, but its mechanisms of action and downstream targets have not been fully defined. Our lab previously showed that endothelial cells promote smooth muscle differentiation, and here we show that the miR-143/145 gene cluster is induced in smooth muscle cells by coculture with endothelial cells. Endothelial cell-induced expression of miR-143/145 is augmented by Notch signaling and accordingly expression is reduced in Notch receptor-deficient cells. To further define the function of miR-145 in smooth muscle cells, we screened putative target genes to identify over represented signaling pathways. Our results revealed that the TGFβ pathway has a significantly high number of miR-145 target genes, and we show that TGFβ receptor II (TGFBR2) is a direct target of miR-145. Extracellular matrix (ECM) genes, which are regulated by TGFBR2, were attenuated by miR-145 overexpression, and miR-145 mutant mice exhibit an increase in ECM synthesis. Furthermore, activation of TGFβ signaling via angiotensin II infusion revealed a pronounced fibrotic response in the absence of miR-145. These data demonstrate a specific role for miR-145 in the regulation of matrix gene expression in smooth muscle cells, and suggest that miR-145 acts to suppress TGFβ-dependent ECM accumulation and fibrosis, while promoting TGFβ-induced smooth muscle cell differentiation. Our findings
offer evidence to explain how TGFβ signaling exhibits distinct downstream actions via its regulation by a specific microRNA.
Dedication

This document is dedicated to my family.
Acknowledgments

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CHAPTER 1

Introduction

Endothelial / Vascular Smooth Muscle Cells Interaction

Blood vessel formation is a tightly regulated process that serves a critical role in both health and disease [1,2]. There are several processes of vessel formation: vasculogenesis, angiogenesis and arteriogenesis. Vasculogenesis refers to the de novo vessel formation via the differentiation of angioblasts and occurs primarily in the formation of the dorsal aorta, endocardium and blood island vessels in the embryo [3-5]. Angiogenesis and arteriogenesis refer to the subsequent stabilization of the newly-formed vessels through the recruitment of smooth muscle cells and pericytes, which also contribute to the expansion the arterial networks [3-5].

The formation of vascular networks involves a variety of cell types and must be precisely and delicately regulated (Figure 1). Initially, endothelial progenitors differentiate into endothelial cells, which coalesce to form a nascent vascular bed. The naked endothelial cells secrete growth factors, such as Ang-1 (Angiopoietin 1), PDGF (Platelet-derived growth factor) and TGFβ1 (Transforming growth factor beta 1), to recruit smooth muscle cells and pericytes. Once the nascent vessel is covered and stabilized by smooth muscle cells and extracellular matrix (ECM), the vessel is durable and matured, and able to support sustained blood flow [1].
Abnormal angiogenesis is involved in a wide range of diseases. Excessive angiogenesis is a critical contributing factor for diseases such as cancer, obesity and diabetic retinopathy [1,2]. Conversely, insufficient angiogenesis contributes to diseases like stroke, restenosis and myocardial infarction [1,2]. Therapeutic strategies designed to manipulate angiogenesis in these diseases have been applied clinically. One example is anti-vascular endothelial growth factor (VEGF) treatment in cancer and diabetic retinopathy [2]. In cancer, tumor angiogenesis from the pre-existing vessels supports the rapid growth of the tumor mass, and VEGF is a critical player in this process. Anti-VEGF treatment has been demonstrated to restrict the abnormal vessel growth and eventually limit the growth of the tumor. In diabetic retinopathy, excessive microvessels in the retina cause intraocular hemorrhaging and inhibiting angiogenesis through anti-VEGF treatment culls vascular branching. In contrast, a strong angiogenesis inducing factor, hypoxia is being investigated in terms of its potential for treating ischemic diseases [2].

Recently, it has become apparent that interactions between endothelial cells and mural cells, including vascular smooth muscle cells, pericytes and fibroblasts, are required for proper blood vessel formation [6,7]. Endothelial cells recruit mural cells to the nascent vessel tube and guide the differentiation of vascular smooth muscle cells. The differentiated vascular smooth muscle cells maintain the integrity of blood vessel and regulate vascular tone. Failure of this heterotypic cell interaction can lead to severe and sometimes even lethal cardiovascular defects, such as tumor angiogenesis, hereditary hemorrhagic telangiectasia (HHT), vascular malformations, and diabetic retinopathy [7]. Several different signaling pathways have been implicated in regulating the development
and maintenance of vasculature via endothelial-mural cell interaction, including TGF-β signaling, Angiopoietin/endothelium-specific tyrosine kinase-2 (Tie2) signaling, PDGF-B/PDGFR-β signaling, Sphingosine-1-phosphate (S1P)/endothelial differentiation gene (Edg) signaling and Notch signaling [7]. For example, PDGF-BB secreted from endothelial cells is responsible for the recruitment of PDGFR-β-expressing mural cells to the developing blood vessel [8-11]. In return, the recruited mural cells produce survival and antiproliferative factors to stabilize the nascent vessel [12,13]. The disruption of PDGFB/ PDGFR-β signaling is implicated in many cardiovascular diseases, such as atherosclerosis, pulmonary arterial hypertension and diabetic retinopathy [14-16].

The interactions between endothelial cells and mural cells are not only critical for vessel maturation but also for maintaining vascular tone. Vascular tone is controlled by the contraction and relaxation of vascular smooth muscle cells. Endothelial cells secrete vasoactive soluble factors, such as Nitric oxide (NO) and prostacyclin, to regulate the contraction state of vascular smooth muscle cells [17,18]. A decrease in NO levels caused by endothelial cell dysfunction plays an important role in hypertension [19]. Therefore, proper interaction between endothelial cells and smooth muscle cells is critical for the development and function of vessels.

Previously, Lilly lab utilized a 3D culturing system and showed that the endothelial cell-formed blood tubes were robustly enhanced with coculturing with mural cells [20]. Notch signaling has been found to be critical for this interaction of endothelial cells and mural cells. More detailed mechanisms behind are waiting to be clarified.
Vascular Smooth Muscle Cell

Smooth muscle is involuntary and non-striated muscle tissue. Located in the blood vessel wall, urinary bladder, uterus, reproductive tracts, gastrointestinal tract, respiratory tract and iris of the eye, the major function of smooth muscle is contraction. The greater elasticity of smooth muscle is important to maintain contractility and stretch in certain organs. In large blood vessels, vascular smooth muscle cells surrounding the endothelial layer receive cues from endothelial cells and consequently adjust contractility to maintain vessel tone, blood pressure and blood flow distribution [21].

Vascular smooth muscle cells are derived from diverse origins, predominantly from local mesoderm populations. The vascular smooth muscle cells in branchial arch-derived vessels are originated from neural crest and vascular smooth muscle cells in coronary arteries are from the proepicardial organ. It has also been suggested that circulating bone marrow stem cells also invest in vascular smooth muscle cells under certain pathological conditions [22]. Besides the diverse origins, vascular smooth muscle cells also show diverse phenotypes. One distinct character of vascular smooth muscle cells is their plasticity [21]. In early embryonic development, vascular smooth muscle cells are highly proliferative and migratory. In late embryonic development, vascular smooth muscle cells are highly synthetic, producing more ECM, and begin the process of induction of contractile marker proteins. The adult vascular smooth muscle cell has a relatively low proliferation, migration and synthesis rate, but is highly contractile. When
vascular injuries occur, the highly contractile phenotype of mature vascular smooth muscle cells can be disrupted and those smooth muscle cells will undergo a phenotypic switch to a high rate of proliferation, migration and ECM synthesis [22].

These highly proliferating, migratory and synthetic vascular smooth muscle cells are considered undifferentiated. The vascular smooth muscle cell which has a low rate of proliferation, migration and production of ECM, but is highly contractile, is considered differentiated. Unlike the terminally differentiated skeletal and cardiac muscle, vascular smooth muscle cell phenotypes are remarkably reversible. Vascular smooth muscle cell plasticity is complicated and tightly regulated (Figure 2) [21,23,24]. Vascular smooth muscle cells undergo phenotypic changes in response to the complex integration of local environmental cues, such as cell-cell interaction, secreted growth factors, mechanical cues and ECM [21]. Among these cues, physical contact with endothelial cells is found to be critical for promoting and maintaining vascular smooth muscle cell differentiation [7].

The proper vascular smooth muscle cell phenotype is required for maintaining blood vessel functions. Dysregulated phenotypic switching is implicated in several vascular diseases, including atherosclerosis [24,25]. A lesion on the endothelial cell layer initiates atherosclerosis, and the differentiated vascular smooth muscle cells convert to a proliferating and synthetic phenotype due to the dysfunction of endothelial cells and inflammation. The injured vessel undergoes intimal hyperplasia, wherein medial smooth muscle cells proliferate and migrate into the intima. These vascular smooth muscle cells also express fewer contractile proteins, but more ECM and matrix metalloproteases (MMPs) [26]. This phenotypic transition of smooth muscle cells has a two-pronged
effect: on one hand, the synthetic smooth muscle cells produce ECM to stabilize the atherosclerotic plaques; on the other hand, these highly proliferating smooth muscle cells contribute to the stenosis of blood vessels [25]. Surgical treatment, such as angioplasty and stent placement, is the standard treatment for atherosclerosis. However, there is a high possibility of restenosis following the surgery, due to the uncontrolled dedifferentiation of vascular smooth muscle cells [25]. Thus, identifying the mechanisms that govern the phenotype of vascular smooth muscle cell is important for understanding atherosclerosis and other cardiovascular diseases.
**Notch signaling**

Notch proteins belong to an evolutionarily conserved family of cell surface receptors that transduce signals between neighboring cells [27,28]. Four members of the Notch family have been identified, NOTCH1, NOTCH2, NOTCH3 and NOTCH4. Notch receptors are single-pass transmembrane proteins which participate in specifying cell fate during development. The four Notch receptors are structural homologues including extracellular, transmembrane and intracellular regions (Figure 3) [29]. After protein synthesis, Notch receptors are cleaved at an S1 cleavage site by furin and converted into the membrane-bound full-length version during the transportation to the cell surface. Notch extracellular domain (NECD) contains a large number of epidermal growth factor-like repeats (EGFR), a cysteine-rich Notch or LIN12 (LN) domain, and a juxtamembrane region with an S2 cleavage site for protease ADAM10 or TACE. The transmembrane region has an S3 cleavage site for γ-secretase. The Notch intracellular domain (NICD) contains a RAM (RBP-Jk-associated molecule) domain, six ankyrin (ANK) repeats flanked by two nuclear localization sequences (NLSs), a transactivation domain (TAD), and a proline, glutamate, serine and threonine-rich (PEST) domain [30].

Notch receptor activation is triggered by its interaction with Delta/Serrate/Lag-2 ligands (Jagged1,2 and Delta-like ligand 1,2,4) expressed on an adjacent cell surface. Once bound to ligand, Notch receptors undergo S2 and S3 cleavages, which releases one NICD to translocate to the nucleus. The NICD binds with the transcription factor
Suppressor of Hairless/Lag1 (CSL)/Epstein-Barr virus latency C promoter binding factor (CBF)1/Recombining binding protein suppressor of hairless (RBPJ), and other cofactors, converting a repressor complex into an activator complex. This complex adapts to the CSL-binding motif on the promoter of target genes, and eventually activates downstream gene expression [29-31] (Figure 3).

Notch signaling is critical for growth regulation and cell fate determination in many cell types, including vascular cells [32,33]. In the vasculature, activation of Notch is required for arterial specification [34], and alterations in Notch signaling lead to abnormalities in vessel structure, branching and patterning [35-37].

Vascular smooth muscle cells express Notch1, Notch2 and Notch3, with Notch2 and Notch3 predominating [38]. Notch signaling is required for smooth muscle cell specification, differentiation and maturation in multiple organs during development [31]. In vitro and in vivo studies suggest Notch directly targets smooth muscle α-actin (SMA) and miR-143/145 to regulate the differentiation of vascular smooth muscle cell [39,40]. Interestingly, PDGF signaling is also shown to be positively regulated by Notch signaling, which is possibly a negative feedback loop considering PDGF is a repressor of smooth muscle cell differentiation [41]. Numerous studies have shown that different Notch receptors have distinct functions in regulating vascular smooth muscle cells in development or diseases states. Notch1 is implicated in controlling vascular smooth muscle cell phenotypic switching during vessel injury [42]. It has been shown that inhibition of Notch1 in vascular smooth muscle cells prevents the formation of neointima after vessel injury. Notch2 is implicated in the development of neural crest-derived
outflow tract and aortic arch, and the second heart field-derived pulmonary artery [43]. Inhibition of Notch2 disrupted the differentiation and patterning of vascular smooth muscle cells in these regions. In contrast to the key role of Notch2 in large arteries, Notch3 is more crucial for the small arteries [38,44]. Loss of Notch3 in vascular smooth muscle cells leads to enlarged arteries of the brain, lungs, kidneys and heart, and abnormal hemodynamics [44]. It has been shown that Notch3 is important for stability and specification of vascular smooth muscle cells, in addition to regulating angiogenesis [45-47]. Dominant mutations in NOTCH3 were found to cause cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), which is characterized by progressive loss of vascular smooth muscle cells and small infarctions in multiple brain regions [48,49].

Notch signaling is activated by the interaction of Notch receptors with the ligands expressed on the surface of adjacent cells. Several independent studies suggest that the activation of Notch signaling in vascular smooth muscle cells is initiated by their interaction with the ligand-expressing endothelial cells [50] [20]. The Lilly lab has shown that once endothelial cells physically interact with vascular smooth muscle cells, Jagged1 (Jag1) on endothelial cells activates Notch3 on smooth muscle cells. Interestingly, the upregulated Notch activity in vascular smooth muscle cells further induces Notch3 and Jag1 expression on vascular smooth muscle cells [20]. Jag1 was shown to be a direct target of Notch, and loss of Jag1 in smooth muscle cells prevented the activation of Notch signaling in outer layers of smooth muscle cells [51]. It is very likely that endothelial cells initiate Notch signaling in the inner layer of smooth muscle cells, which induces
Jag1 in these smooth muscle cells to further distribute Notch signaling to the surrounding smooth muscle cells in the blood vessel wall. As a transcription factor, the activated Notch is expected to control the expression of downstream targets and eventually regulate vascular smooth muscle function. It is important to identify those Notch downstream targets required for endothelial cell-regulated vascular smooth muscle cell function.
Syndecan family

The Syndecan family consists of four evolutionarily conserved transmembrane proteins, composed of an extracellular ectodomain, a transmembrane domain and a cytoplasmic domain (Figure 4) [52]. The ectodomains have attachment sites for heparan sulfated glycosaminoglycan chains (HS-GAGs), which facilitate interactions with extracellular matrix molecules, cytokines, chemokines, growth factors and growth factor receptors. The ectodomain can be shed from cells to sequester soluble factors and compete for cell surface binding. The cytoplasmic domain contains two conserved regions C1 and C2, which mediate binding to cytoskeletal proteins and to PDZ-containing proteins, respectively. A variable region (V) is flanked between C1 and C2. The V domain in syndecan-2 has a role in matrix assembly in fibroblasts [53] and left-right asymmetry in Xenopus [54-56]. Syndecans have been implicated in wound healing, hypoxia, obesity and angiogenesis. Furthermore, distribution and function of Syndecans have important roles in a variety of vascular diseases [52,57].

Syndecan-2 is abundantly distributed in mesenchymal tissues surrounding blood vessels, and also found in liver, kidney, lung and neural cells [58]. Moreover, it is known to be highly expressed in several cancer cell lines [58]. The broad expression of Syndecan-2 suggests it may possess a fundamental role in regulating cell growth and/or basic cellular functions. In the cardiovascular system, Syndecan-2 has been shown to mediate left-right axis formation in cardiac development [54-56]. More recently,
evidence has shown that Syndecan-2 is a vital participant in angiogenesis [59]. In a zebrafish model, Syndecan-2 knockdown led to defective angiogenic sprouting, which could be rescued by human Syndecan-2 expression [60]. When Syndecan-2 was inactivated in microvascular endothelial cells, formation of capillary tube-like structures was impaired [61]. Furthermore, overexpression of Syndecan-2 promoted membrane protrusion, migration, blood vessel formation, and cell-cell interactions [62]. Taken together, these data support the idea that Syndecan-2 plays a vital role in angiogenesis. Yet, these studies mainly focused on the function of Syndecan-2 in endothelial cells. The role of Syndecan-2 in mural cells is not clear.

One prominent characteristic of Syndecan-2 structure is the abundant glycosaminoglycan (GAG) chains attached to its extracellular domain [63-65]. These highly sulfated chains are known to work as extracellular ligand gatherers, and can interact with growth factors, extracellular matrix (ECM) molecules, and lipid-regulating enzymes [66,67]. An example of the importance of these interactions was revealed for PDGF signaling [68,69]. PDGF-BB is secreted by endothelial cells and recruits smooth muscle cells and pericytes by activation of PDGFRβ expressed on mural cells [9-11,70,71]. This process was shown to require sulfated HS chains to retain PDGF-BB and activate PDGFRβ signaling [68,69,72]. In addition to the role as PDGF coreceptor, the HS chains expressed on perivascular smooth muscle cells also support VEGF-2 signaling in adjacent endothelial cells in trans [73]. Besides binding to growth factors through GAG chains, the core protein of Syndecan-2 ectodomain is also found to directly bind with TGFβ family receptors [74]. Considering the activation of Notch signaling is
initiated by the binding of ligands and receptors and coexpression of Syndecan-2 and Notch3 in vascular smooth muscle cells, it is possible that Syndecan-2 functions as a coreceptor of Notch signaling.
MicroRNA

MicroRNAs (miRNAs) are around 22 nt RNAs which can complementarily bind to the 3’ untranslated region (3’UTR) of messenger RNA (mRNA) resulting in degradation or repression of translation [75,76]. The numerous miRNAs form a network to regulate gene expression beyond transcriptional regulation. Since first identified in C. elegans, more than 4,000 miRNAs have been found in animals, plants and viruses to date [77]. Most miRNAs are located at intergenomic regions distant from other coding genes on the genome [78-80]. These miRNAs have their own promoter elements and are regulated independently. Interestingly, some miRNAs exist in the introns of protein-coding genes and share the same promoter region [78,81,82]. After the host pre-mRNA is transcribed, the introns are spliced and processed to generate miRNAs. These miRNAs are not only co-expressed with host genes, but also functionally reinforce the host genes through synergistic or antagonistic regulating mechanisms [81,83]. It is quite common for multiple miRNAs to cluster in the genome as multi-cistronic transcripts [78,79]. These genomic-clustered miRNAs have different sequences and target different mRNAs, but can still perform similar functions [81,84]. Most miRNAs, and the genomic clusters as well, are conserved throughout related species [75,78,81,85].

The miRNA genes are first transcribed into primary microRNA transcripts (pri-miRNAs) that can be longer than 1 KB and include all of the clustered miRNAs (Figure
The level of pri-miRNAs represents the activity of miRNA transcription [78-81]. The pri-miRNAs are then cleaved by Drosha and DGCR8 to release a 60-70 nt stem-loop product, precursor miRNA (pre-miRNA) (Figure 5), which is then exported into the cytoplasm by Exportin-5 [86-91]. In the cytoplasm, the stem-loop is cleaved by Dicer and an imperfect miRNA:miRNA duplex is generated (Figure 5) [88]. Usually, only one strand of the duplex is functional [78,81,85]. The mature miRNA needs to be incorporated into an active RNA-induced silencing complex (RISC) to fulfill its functions [92-97]. In RISC, miRNA binds to the 3’UTR of targets, and either degrades mRNA or represses its translation [90,98-103]. Unlike plant miRNAs, animal miRNAs bind to a target mRNA via an imperfect match with the 6-8 nt seed site [104]. One miRNA can target up to hundreds of mRNAs, and one mRNA can be targeted by multiple miRNAs [75].

The major function of miRNAs is post-transcriptional regulation of genes, particularly, fine tuning of gene expression in different phases and different organs [75]. Although miRNAs have subtle effects on each single target, miRNAs can profoundly change cell phenotype or function via targeting multiple related proteins at the same time [75]. MiRNA was first identified as a key regulator of embryonic differentiation in DGCR8 and Dicer knockout embryos [105,106]. Without miRNAs, mouse embryonic stem cells (mESCs) were arrested in a highly pluripotent state. Further studies showed that there were two categories of miRNA regulating pluripotency of ESC [107]. One set of miRNAs are driven by pluripotent factors to repress cell-cycle repressors and hence to maintain the cell pluripotency [108]. The other set of miRNAs directly target the
pluripotent markers to induce the cell into differentiation [109-111]. Among the pluripotency repressor miRNAs, miR-145 was found to target pluripotency factors KLF4, OCT4 and SOX2 to inhibit the stem cell self-renewal and eventually differentiate to vascular smooth muscle cells [112,113]. Consistent with the role of miR-145 in regulating stem cell pluripotency, a dramatic decrease of miR-145 has been found in multiple tumor types, which was caused by the aberrant self-renewal [114-118]. The anti-tumorigenic activity of miR-145 is currently under extensive investigation for its potential therapeutic effect.

miRNAs have been highlighted in all cell lineages involved in angiogenesis [107]. Deletion of Dicer in vascular smooth muscle cell leads to early embryonic lethality due to severe hemorrhage and defective aorta [119], which suggests miRNAs are essential for vascular smooth muscle cell development. To date, several miRNAs, which play critical roles in regulating the development, differentiation, proliferation and contractility of vascular smooth muscle cell, have been discovered. In 2009, several independent groups reported that miR-145 plays an important role in regulating vascular smooth muscle cell phenotype and function [120-123]. miR-145 is clustered with miR-143 and is the most abundant miRNA in vascular smooth muscle cells [123]. miR-145 has been shown to be upregulated by myocardin, Notch signaling and TGFβ signaling, and be downregulated by PDGF signaling in vascular smooth muscle cells [40,121,124,125]. When contractile vascular smooth muscle cells dedifferentiate into a proliferative phenotype, the expression of miR-145 is dramatically decreased [123]. Loss of miR-145 does not cause embryonic lethality or severe vascular smooth muscle cell
defects, which suggests miR-145 is dispensable for vascular smooth muscle cell embryonic development and maintaining basic vascular smooth muscle cell functions [120-123]. However, miR-145 deficient adult mice do have thinner aortic walls and their smooth muscle cells bear decreased differentiation markers and increased synthetic and migratory phenotypes, suggesting miR-145 is likely responsible for fine-tuning of vascular smooth muscle cell functions [121,122]. miR-145 targets multiple genes that inhibit vascular smooth muscle cell differentiation, such as KLF4 and KLF5 [121]. miR-145 is also implicated in regulating actin polymerization [121]. In neointimal hyperplasia that occurs after wire injury of blood vessels, miR-145 was found to be dramatically decreased, and adenovirus delivery of miR-145 inhibited the neointimal formation [123]. Furthermore, the miR143/145 double knockout mice spontaneously developed neointimal lesions at 18-months of age [120]. Surprisingly, in miR-145 knockout mice, neointimal formation after wire injury is also completely inhibited [121]. This interesting phenomenon was also observed in a myocardial infarction model [126]. Restoration of miR-145 level in the late stages of coronary collateral growth (CCG) can completely restore the blood flow in rats with metabolic syndrome, but either overexpression or inhibition of miR-145 in normal rats results in a decrease of CCG and blood flow [126]. The detailed mechanisms of these complicated results remain to be further determined. Besides restenosis and myocardial infarction, miR-145 has also been found to be beneficial in pulmonary hypertension and treatment exploiting this is in preclinical trials [127]. All these findings conclude that miR-145 is a promising therapeutic target for cardiovascular diseases.
TGFβ signaling

TGFβ signaling superfamily is evolutionarily conserved and plays important roles in all organs. It regulates cell growth, cell-fate determination, differentiation, development, apoptosis, epithelial-mesenchymal transition (EMT), ECM remodeling and immune-suppression [128]. Disrupted TGFβ signaling is implicated in cancer, fibrosis, inflammation, familial primary pulmonary hypertension, Marfan syndrome and other severe diseases [129, 130].

TGFβ ligands include TGFβ, activin, nodal, bone morphogenetic proteins (BMPs), anti-mullerian hormone (AMH) and growth and differentiation factors (GDFs) [128]. After being synthesized, the ligands are cleaved by the subtilisin-like proprotein convertase (SPC) family to generate homodimeric precursors. There are three isoforms of TGFβ in humans: TGFβ1, TGFβ2 and TGFβ3. They share similar structures and signaling pathways, but have distinct functions. The latent precursor associates with the latency-associated peptide (LAP) and the latent TGFβ-binding protein (LTBP) to form a complex called the large latent complex (LLC) [128]. To release the active TGFβ, the LLC is released from the ECM, and then LAP is cleaved off. The active TGFβ is then free to bind to and activate the TGFβ receptor.

TGFβ receptors can be divided into two distinct categories: type I and type II. Seven type I receptors (ALK1-7) and five type II receptors (TGFBR2, BMPR2, AMHR2, ACVR2 and ACVR2B) exist in humans, which can be paired to form different receptor
complexes to interact with various TGFβ ligands [128,131]. TGFβ receptors have a diverse Cys-rich extracellular domain, a single-pass transmembrane domain, and a highly conserved intracellular Ser-Thr kinase domain. Once binding with the TGFβ ligands, a homodimer of type II receptors form a heterotetrameric active complex with a type I receptor dimer (Figure 6). A distinct feature of type I receptor is the 30-amino Gly-Ser rich juxtamembrane domain, “GS region”, which locates upstream of the intracellular kinase domain. The constitutively active type II receptor can phosphorylate GS region of the type I receptor in the ligand-bound complex [128,131]. Once phosphorylated by a type II receptor, the type I receptor is activated and able to propagate the signal in two separate ways, the SMAD-dependent canonical pathway and the SMAD-independent non-canonical pathways (Figure 6) [128,129,131].

In the SMAD-dependent canonical pathway, activated type I TGFβ receptors recruit and phosphorylate the receptor-specific SMADs (R-SMADs), including SMAD1, 2, 3, 5 and 8 [128,129,131]. Once phosphorylated, two R-SMADs and one common mediator, SMAD4, form a complex and translocate into nucleus. In the nucleus, active SMADs interact with cofactors on promoter regions to regulate the transcription of target genes [128,129,131]. Recently, it has been reported that R-SMADs also regulate miRNA processing [132]. At the end of the signaling process, the active R-SMADs are dephosphorylated by protein phosphatase and exported to the cytoplasm [128]. In the SMAD-independent non-canonical pathway, TGFβ receptors transmit signaling through other factors and pathways, which include the three mitogen-activated protein kinase (MAPK) isoforms (ERK, p38 and JNK), as well as phosphoinositide 3-kinase (PI3K)-
AKT pathway, RHO-ROCK pathway, TNF receptor-associated factor 4/6 (TRAF4/6) and nuclear factor-κB (NF-κB) (Figure 6) [129,130]. Activation of some pathways is not dependent on the activation of the type I receptor. The SMADs signaling and non-canonical TGFβ signaling can crosstalk by regulating each other [129]. Thus, the outcome of TGFβ signaling is extremely complex due to the dynamic combination of canonic and non-canonic signaling cascades. Additionally, TGFβ signaling is also strongly influenced by other signaling pathways, such as RAS, Hedgehog, Notch, Wnt, interferon, TNF and PI3K-AKT by crosstalk between ligands, receptors and R-SMADs [129].

TGFβ signaling is involved in a wide range of events either during embryogenesis or in adult homeostasis [131]. In humans, mutations in the TGFβ family were found to disrupt TGFβ signaling leading to aneurysms, atherosclerosis, arteriovenous malformations and other cardiovascular diseases [129,130]. Deletion of TGFβ signaling components in mice mostly results in embryonic lethality due to vascular defects [133-137]. Smooth muscle cell-specific inactivation of TGFβ signaling revealed a role in regulating vascular smooth muscle cells differentiation and recruitment [138-140]. In addition to its role in regulating the development of smooth muscle cells, TGFβ also controls the phenotypic switching of smooth muscle cells. TGFβ induces the differentiation of smooth muscle cell precursors, and the contractile phenotype of vascular smooth muscle cells by upregulating the expression of differentiation markers, such as SMA and smooth muscle myosin [141-143]. The effect of TGFβ signaling on vascular smooth muscle cell proliferation and migration is dose-dependent: it increases
vascular smooth muscle cell proliferation and migration at a low dose, but inhibits that at a high dose [144-146]. TGFβ-regulated vascular smooth muscle cell phenotype transition involves multiple cytoplasmic signaling including R-SMADs, p38 MAPK, and ERK1/2 [147-152].

TGFβ signaling also regulates the ECM production in vascular smooth muscle cells, including synthesis, deposition and degradation of a wide range of ECM molecules [153,154]. TGFβ is able to enhance the production of elastin, and type I and type III collagens by inducing their gene expression [155-161]. TGFβ also induces members of lysyl oxidase (LOX) family, which promotes the crosslinking of elastin and collagen. In addition to directly upregulating the transcription of ECM, TGFβ also induces connective tissue growth factor (CTGF) which promotes the production of ECM [162]. Along with increasing production, TGFβ suppresses the degradation of ECM as well. It can both directly inhibit the expression of several MMP genes and induce plasminogen activator-1 (PAI-1) and tissue inhibitor of metalloproteinase (TIMP) which are both inhibitors of MMPs [163-167]. However, TGFβ is also found to upregulate MMP2 and MMP9 expression [168,169]. Therefore, TGFβ can induce either production or degradation of ECM depending on the context.

The perturbation of TGFβ signaling is implicated in vascular diseases, such as Marfan syndrome, atherosclerosis, restenosis, and fibrosis. Marfan syndrome is a dominant hereditary connective tissue disorder primarily caused by defects of FIBRILLIN-1 [170]. The primary cause of morbidity in Marfan syndrome patients is ascending aorta aneurysm due to the dysfunction of smooth muscle cells and elastin.
breakdown [170]. Dysfunction of fibrillin-1 causes enhanced TGFβ signaling [170-172]. Anti-TGFβ treatment, e.g. Losartan, ameliorates aortic root dilation caused by the fibrillin-1 mutation [173]. Atherosclerosis is a chronic vascular wall disease as discussed above. Since TGFβ can suppress vascular smooth muscle cell proliferation and migration, it has been shown to have anti-atherogenic effects [174-176]. Interestingly, TGFβ family ligands and receptors were found highly upregulated in the atherosclerosis patients, suggesting that TGFβ signaling can exert pro-atherogenic effects in some contexts [177-180]. Restenosis is one major complication following angioplasty surgery for atherosclerosis. In restenosis, TGFβ signaling is found enhanced, inducing intimal hyperplasia by promoting smooth muscle cells proliferation, collagen production and fibrosis [177,181,182]. Fibrosis, characterized by aberrant accumulation of ECM, can occur in heart, lung, liver, kidney and other organs [129]. In fibrotic diseases, TGFβ signaling upregulates ECM production, inhibits collagenase expression and stimulates profibrotic factors, such as PAI-1 and CTGF [183,184]. A preclinical study showed anti-TGFβ treatment has the ability to reverse cardiac fibrosis [185]. One challenge of anti-TGFβ treatment is how to inhibit specific TGFβ-dependent cell functions without disturbing other functions. Therefore, novel and more specific anti-TGFβ treatment is under studying.
**Figure 1. Vascular formation.** Hemangioblast-derived endothelial progenitors differentiate into and endothelial cells, which coalesce to form nascent vessel. Smooth muscle cell derived from smooth muscle cell progenitors is recruited to cover the nascent tubes and turn it into a mature blood vessel. [1]
Figure 2. Vascular smooth muscle cell phenotypic switch is determined by multiple environmental cues. These highly proliferating, migratory and synthetic vascular smooth muscle cells are considered undifferentiated. The vascular smooth muscle cell which has a low rate of proliferation, migration and production of ECM, but is highly contractile, is considered differentiated. Vascular smooth muscle cells undergo phenotypic changes in response to the complex integration of local environmental cues, such as cell-cell interaction, secreted growth factors, mechanical cues and ECM. [21]
Figure 3. Notch signaling pathway. A. Structure of Notch receptors. B. Activation process of Notch signaling. [29]
Figure 4. Structure of Syndecans. [52]
Figure 5. MicroRNA biogenesis. MiRNA is first transcribed from genome to the pri-miRNA, and then cleaved to release the pre-miRNA. In the cytoplasm, the stem-loop of pre-miRNA is cleaved and an imperfect miRNA:miRNA duplex is generated. The mature miRNA is incorporated into RISC, and binds to the 3’UTR of targets to either degrade mRNA or repress its translation. [186]
**Figure 6. TGFβ signaling.** Once bound to TGFβ ligands, the constitutive active TGFβ receptor II phosphorylates TGFβ receptor I, following by the activation of downstream signaling cascades, e.g. SMAD, p38 MAPK, ERK, JNK, AKT, TRAF4/6 and ROCK, which interact and regulate other pathways. [129]
CHAPTER 2

Reciprocal Regulation of SYNDECAN-2 and Notch Signaling in Vascular Smooth Muscle Cells

Proper blood vessel formation and function is crucial for embryogenesis, wound healing, pregnancy and diseases [1,2]. The interaction of endothelial cells and vascular smooth muscle cells/pericytes is critical for assembling and maintaining blood vessels [7]. Previously, our lab and other groups showed that endothelial cells play an important role in governing vascular smooth muscle cell functions [20]. To further investigate the signaling mechanisms that govern the interaction of vascular cells, we screened for genes and microRNAs whose expression was significantly altered by the endothelial cell-smooth muscle cell interaction. One of those altered genes was SYNDECAN-2, which encodes a heparin sulfate proteoglycan (HSPG) surface receptor [52]. SYNDECAN-2 is known to be important for developmental processes including angiogenesis in vitro and in vivo [60,62], however the role of SYNDECAN-2 in vascular smooth muscle cells has not been identified. Our results show that endothelial cells induce mRNA expression of SYNDECAN-2 in smooth muscle cells by activating Notch receptor signaling. Both NOTCH2 and NOTCH3 contribute to the increased expression of SYNDECAN-2 and are themselves sufficient to promote its expression independent of endothelial cells. Syndecan family members serve as coreceptors for signaling molecules [68,69,72] [73].
and interestingly, our data show that SYND
cAN-2 regulates Notch signaling and
physically interacts with NOTCH3. Notch activity was attenuated in smooth muscle cells
made deficient in SYND
cAN-2, and this specifically prevented the expression of
differentiation marker smooth muscle α-actin (ACTA2). These results show a novel
mechanism in which Notch receptors control their own activity by inducing the
expression of SYND
cAN-2 that then acts to propagate Notch signaling by direct
receptor interaction.
**Introduction**

Proper communication between endothelial and smooth muscle cells is fundamental for formation and function of the vasculature [187, 188]. Defective interactions between these cells during development leads to vascular malformations [189], while endothelial dysfunction in mature blood vessels causes smooth muscle abnormalities associated with vascular disease [190]. Despite the obvious importance of these interactions within blood vessels, the exact signaling mechanisms that facilitate cell-cell communication remain largely undefined. One signaling pathway that has garnered much attention for facilitating vascular cell interactions is the Notch pathway [33, 191]. Notch proteins are evolutionarily conserved and critical for cell fate determination and differentiation [192, 193]. Four Notch receptors are present in mammals, Notch 1-4, and their activation is triggered by the interaction with membrane-bound ligands (Jagged-1,2/Delta-like-1,3,4). Upon binding, receptors undergo cleavage events that release NICD, which translocate to the nucleus and binds with the transcription factor CSL (CBF-1/RBP-Jk, Su(H), and Lag-1) to regulate downstream gene expression, most notably members of the Hes (hairy/enhancer of split) and Hrt (Hairy-related, also referred to as Hey, CHF, HESR) families [194]. A host of studies have examined Notch signaling in the vasculature and shown it to be important for angiogenic remodeling, arterial/venous specification, and tip cell differentiation [195]. One particularly relevant study demonstrated that endothelial-expressed Jag-1 is essential
for vascular smooth muscle differentiation [50]. Data from our own lab have shown that NOTCH3 expression is induced in smooth muscle cells when cocultured with endothelial cells [20]. We further demonstrated that differentiation of smooth muscle cells by endothelial cells was dependent upon NOTCH3. In addition to NOTCH3, in this study we show SYNDECAN-2 is another gene whose expression is induced by cocultured endothelial cells.

Like the Notch family, Syndecans are evolutionarily conserved transmembrane proteins that have been implicated in regulating a broad range of development and disease processes [57,196]. Syndecan-2 is widely expressed in developing mesenchymal tissues, including cells surrounding blood vessels [57,58]. Functional studies have demonstrated a role in left-right axis formation [54], promotion of membrane protrusions and migration [56,197,198], in addition to regulating angiogenesis. Syndecan-2 knockdown in zebrafish leads to defective angiogenic sprouting [60], and Syndecan-2 inactivation in microvascular endothelial cells causes impairments in capillary tube-like structures [61,62]. Syndecan-2 is a heparan sulfate proteoglycan with glycosaminoglycan chains attached to the extracellular domain (ectodomain) of the protein [199]. The GAG-ectodomain of Syndecan family members are known to control both cell-matrix and cell-cell interactions, and to serve as a coreceptor for the growth factors, PDGF, fibroblast growth factor (FGF), and VEGF [57,196]. Syndecan-2 acts as a coreceptor for TGFβ signaling [74]. In this report, we describe the interaction of two cell surface signaling mediators, NOTCH3 and SYNDECAN-2. We show that SYNDECAN-2 expression is induced in smooth muscle cells by coculturing with endothelial cells, and this induction relies on Notch signaling. Further, we demonstrate that SYNDECAN-2
augments Notch activity and directly binds to the NOTCH3 receptor. These data highlight the importance of crosstalk between individual signaling pathways in governing cell communication within the vasculature.
Materials and Methods

Cell Culture

Primary cultures of human aortic smooth muscle cells (HAoSMCs) and human coronary artery smooth muscle cells (HCASMCs) were purchased from Lonza and grown in Dulbecco’s Modified Eagle’s Medium (DMEM) (Mediatech, Inc.) supplemented with 10% fetal bovine serum (FBS) (HyClone), 2mM glutamine, 1mM sodium pyruvate and 100U/ml penicillin-streptomycin. Human dermal neonatal fibroblasts (HDFNs) were purchased from Cascade Biological, and cultured in DMEM supplemented as above with 5% FBS. Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza, and grown in EBM-2 supplemented with the bullet kit as recommended (Lonza). Primary cells between passages 6-9 were used for all experiments. Human hepatoblastoma (HepG2) cells and human adenocarcinoma (HeLa) cells were purchased from ATCC, and cultured in DMEM supplemented as indicated with 10% FBS. For virus production, TN-293 cells were purchased from Stratagene and cultured in 10% DMEM as above. All cultures were maintained in humidified 5% CO2 at 37°C. For coculture, 6x10^4 mural cells were plated in 12-well plates, and after adhesion, 6x10^4 HUVECs were added. To separate endothelial cells from fibroblasts and smooth muscle cells, anti-PECAM1-conjugated Dynabeads (Invitrogen) were used according to manufacturer’s instructions. All cell coculture experiments, unless indicated, were performed in media consisting of EBM-2 supplemented with all bullet kit components except FBS, VEGF
and bFGF. This media was supplemented with 1% FBS and 30ng/ml VEGF-A165 (Pepro Tech). *N*-[(3,5-Difluorophenyl)acetyl-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester DAPT (Calbiochem) was added to specified wells at the time of plating. Transwell experiments were performed as previously described [20]. Transwell inserts (12-well type) (Corning Costar) with 0.4μm pores were coated with 50μg/ml rat tail collagen I (BD Biosciences). 2x104 HDFNs or HUVECs were first plated on the outside of the polycarbonate membrane of the transwell inserts. After cell adherence, the transwell inserts were inverted and reinserted onto 12-well plates, and 2x104 HDFNs or HUVECs were plated on the top surface of the insert and cultured in a final volume of 1.3ml media (0.3ml in the insert, 1ml in the well). Following incubation for 48 hours, cells grown on the top side of the inserts were harvested by trypsinization and processed for qPCR.

**Quantitative Real-Time PCR (qPCR)**

Total RNA was isolated using TRIzol reagent following manufacturers’ instructions (Invitrogen), and reverse transcribed with M-MLV reverse transcriptase (Invitrogen) to generate cDNA. Real-time PCR was performed using a StepOne PCR system (Applied Biosystems) with SYBR Green and 50ng cDNA template. The fold difference in various transcripts was calculated using ΔΔCT method with 18S as the internal control. Primer sequences were as follows: SYNDECAN-2 For-5’-CTG GCC ACC GAC TAT GAG AA; Rev-5’-AAA ATC CAC GTG AAA AAG TTG GA; NOTCH3 For-5’-CCT AGA CCT GGT GGA CAA G; Rev-5’-ACA CAG TCG TAG CGG TTG; 18S For-5’-GTT GGT TTT CGG AAC TGA GGC; Rev-5’-GTC GGC ATC GTT TAT GGT CG; NOTCH2 For-5’-ACA GTT GTG TCT GCT CAC CAG GAT;
Rev-5’-GCG GAA ACC ATT CAC ACC GTT GAT; HRT3 For-5’-CAT ACA ATG TCC TTG TGC AGT ACA CA; Rev-5’-GCC AGG GCT CGG GCA TCA AAG AA; PDGFRβ For-5’-AGA GGC CAG GGC AGC AA; Rev-5’-CAT ACA ATG TCC TTG TGC AGT ACA CA; Smooth muscle (SM) α-ACTIN For-5’-CAA GTG ATC ACC ATC GGA AAT G; Rev-5’-GAC TCC ATC CCG ATG AAG GA; SM22α For-5’-CAA GCT GGT GAA CAG CCT GTA C; Rev-5’-GAC CAT GGA GGG TGG GTT CT; CNN1 For-5’-TGA AGC CCC ACG ACA TTT TT; Rev-5’-GGG TGG ACT GCA CCT GTG TA. RNA from yolk sacs of Notch2 [200] and Notch3 [201] mutant mice was isolated using RNeasy mini columns (Qiagen). Mouse Syndecan-2 primers were, For-5’-TCG CCT TTC GGC ATC CT; Rev-5’-GCA GTC GAT GGG TTG AAA CC.

**Immunoblotting**

Equivalent amounts of protein were run on 10% SDS-PAGE gels, transferred to immobilon–PVDF membranes (Millipore), and subjected to incubation using primary antibodies to NOTCH3 (Santa Cruz Biotechnology, sc-5593), NOTCH2 (Developmental Studies Hybridoma Bank, C651.6DbHN), and β-TUBULIN 1 (Sigma, T7816), SM α-ACTIN (Sigma, 1A4), and HA-Tag (Santa Cruz, sc-7392). Secondary antibodies conjugated to HRP (Amersham) were used for detection. Protein was detected by enhanced chemiluminescence (ECL).
RNA Interference

HAoSMCs were plated in a 12 well plate at 6x10^4 cells/well. After 12 hours, the cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen). Efficiency of knockdown was assessed using qPCR (Figure 10) and Western blot (Figure 11B). NOTCH3 siRNA was synthesized by IDT as the following sequence: 5’-AAC UGC GAA GUG AAC AUU G, and used as previously described [20]. NOTCH2 siRNA sequence: 5’-CCC CCC AUU GUG ACU UUC CAG CUC A. SYNDECAN-2 siRNA sequence: 5’-GCU GAC AUC UGA UAA AGA CAU-3’ All siRNA was transfected at 100nM. Following transfection, cells were cocultured with HUVECs for 48 hours, separated and collected for qPCR analysis and Western blotting.

Lentivirus Expression

Human NOTCH2 intracellular domain (NICD2) cDNA (a gift from Dr. Igor Prudovsky) was cloned with a HA-tag attached to the 3’ end into pCDF1-MCS2-EF1-copGFP (System Biosciences) using BamHI and EcoRI sites. NOTCH3 intracellular domain (NICD3) and dominant-negative mastermind-like 1 (DN-MAML) constructs were made as described previously [20]. The human SYNDECAN-2 open reading frame (ATCC) was amplified by PCR and cloned into pCDF1-MSC2-EF1-copGFP using XbaI and BglII sites. A HA tag was conjugated to the 3’ end of SYNDECAN-2 by PCR and cloned using XbaI and EcoRI sites. The lentivirus plasmids were transfected into TN-293 cells using Lipofectamine 2000 (Invitrogen), and the viral particles were amplified and purified as described [20]. For HDFNs and HAoSMCs infection, equal volumes of viral particles were diluted in 10% FBS in DMEM and were incubated with cells for 24
hours. The efficiency of infection was evaluated using GFP expression and qPCR. Viral particles were titrated to achieve 90% to 100% infection. Expression of NICD2, NICD3 and SYNDICAN-2 cDNAs were confirmed using qPCR and Western blot analysis.

**Plasmid Transfection and Luciferase Assays**

A 5XCBF1-luciferase plasmid was generated as described [20]. To measure the transcriptional activity, HDFNs or HAoSMCs were sequentially transfected at 80% confluency using Lipofectamine 2000 (Invitrogen) with siRNA followed by plasmids. Cells were then cocultured with an equal number of HFDNs or HAoSMCs (as control) or HUVECs for an additional 48 hours. Cells were collected and promoter activity was measured by luciferase assays using SteadyGlo reagent (Promega). To normalize the transfection efficiency, Hsp-β-galactosidase (LacZ) was cotransfected, and luciferase activities were normalized based on equivalent amount of LacZ activity. Luciferase and LacZ activity were measured as described [202], and quantified using a Molecular Devices SpectroMax luminometer. All experiments were performed in duplicate and repeated a minimum of three times.

**Immunoprecipitations**

Approximately 2×10^6 cells in a 10 cm plate were washed with ice cold KRH buffer (125 mM NaCl, 4.8 mM KCl, 2.6 mM CaCl2, 1.2 mM MgSO4, 25 mM Hepes, 5.6 mM glucose, pH 7.4), and then crosslinked by 1mM dithiobis succinimidyl propionate (DSP) (Pierce-Thermo) for 15 minutes at room temperature. After washing once, DSP was quenched by 100µl of 103mM glycine (pH 7.5) in 1% SDS. 1mg protein from cell
lysates was subjected to immunoprecipitation using magnetic beads preincubated with anti-HA antibody and mouse IgG purchased from Santa Cruz. After precipitating at 4°C for 3 hours, immunoprecipitates were eluted by elution buffer (10mM Tris (pH 7.5), 0.2M dithiothreitol and 5mM EDTA), and subjected to immunoblotting.

**Statistical Analysis**

Data analyses were performed using GraphPad Prism and comparisons between data sets were made using a Student’s t test. Differences were considered significant if P < 0.05, and data are presented as mean ± standard error of the mean (SEM). Data shown are representative of at least three independent experiments.
Results

SYNDECAN-2 is regulated by endothelial cells through Notch signaling.

Using a screen to find genes regulated by endothelial-mural cell interaction, we identified an array of genes whose expression was altered when cells of the vasculature were cocultured [203]. One of these genes was the heparan sulfate proteoglycan, SYNDECAN-2 (SDC2) [58]. Coculture of human umbilical vein endothelial cells (HUVECs) with human dermal fibroblasts (HDFNs), human aortic smooth muscle cells (HAoSMCs), or human coronary artery smooth muscle cells (HCASMCs) lead to a significant induction of SYNDECAN-2 mRNA expression in mural cells as assessed by qPCR (Figure 7A). The induction in the different mural cell subtypes ranged from approximately 10-fold in fibroblasts to 4-fold in coronary artery smooth muscle cells, which exhibited a higher level of basal SYNDECAN-2 expression. In contrast, two non-mural cell types, HepG2 and HeLa cells showed no significant induction of SYNDECAN-2 when cultured with HUVECs (Figure 7B). To determine if the induction of SYNDECAN-2 required cell-cell contact between neighboring endothelial cells and mural cells, we performed coculture experiments using transwell inserts to physically separate the cells. Compared to control wells that had endothelial cells and mural cells cultured together, when endothelial cells were separated by a 0.4 micron pore membrane, the level of SYNDECAN-2 expression remained low in the mural cells, similar to that
seen in non-cocultures (Figure 8). These data indicate that endothelial cells induce SYNDECAN-2 expression in mural cells and this is dependent upon cell-cell contact.

Our lab previously showed the importance of Notch signaling in the communication of endothelial cells and mural cells [20,203]. Given that SYNDECAN-2 induction required cell-cell contact, we tested if Notch signaling was involved using the \(\gamma\)-secretase inhibitor DAPT. In the presence of endothelial cells, 3\(\mu\)M concentration of DAPT blocked the upregulation of SYNDECAN-2 in both fibroblasts and smooth muscle cells (Figure 9A, B). We further confirmed the role of Notch signaling by using a dominant negative mastermind construct (DN-MAML), which blocks Notch transcriptional activity [204]. Similar to DAPT inhibition, SYNDECAN-2 transcript induction by endothelial cells was inhibited in the presence of DN-MAML (Figure 9C, D).

In vascular smooth muscle cells Notch2 and Notch3 are prominently expressed [42,47,205]. In HAoSMCs, both Notch2 and Notch3 can activate smooth muscle differentiation markers, NOTCH target and Notch3 using lentiviral overexpression of the intracellular domains of NOTCH2 (NICD2) and NOTCH3 (NICD3) (Figure 10A-D). To determine if one or both of these Notch receptors were responsible for the transactivation of SYNDECAN-2, we performed knockdown experiments of NOTCH2 and NOTCH3 utilizing siRNA (Figure 11). Under coculture conditions, knockdown of NOTCH2 significantly blocked SYNDECAN-2 induction by endothelial cells, while knockdown of NOTCH3 had little effect (Figure 12). siRNA inhibition of both the NOTCH2 and NOTCH3 receptors resulted in a complete abolishment of the inductive effects of endothelial cells on SYNDECAN-2 expression. To determine if the Notch receptors were
sufficient for SYNDECAN-2 gene expression, we over expressed the intracellular domains of NOTCH2 (NICD2) and NOTCH3 (NICD3) by lentiviral transduction in smooth muscle cells and measured SYNDECAN-2 expression (Figure 13). The data show that both NOTCH2 and NOTCH3 can promote SYNDECAN-2 expression in smooth muscle cells. Together, these data show that Notch signaling is sufficient, and necessary for the endothelial cell-dependent expression of SYNDECAN-2.

To determine if Syndecan-2 expression might be regulated by Notch2 and Notch3 in vivo, we examined the highly vascularized yolk sacs of embryos deficient in Notch2 [200] and Notch3 [201]. These data show that Syndecan-2 expression was significantly attenuated in yolk sacs of mouse embryos deficient in both Notch2 and Notch3, with the most pronounced decrease occurring in the absence of both Notch family members (Figure 14). These data show a dependence of Syndecan-2 gene expression on Notch signaling within the vasculature.

**SYNDECAN-2 regulates Notch signaling.**

Previously we showed that endothelial cells activate Notch signaling in neighboring mural cells to promote an autoregulatory loop resulting in NOTCH3 induction followed by NOTCH3-dependent differentiation of smooth muscle cells [20]. Because SYNDECAN-2 was a target of Notch signaling, we asked if the upregulation of SYNDECAN-2 might feedback and effect Notch signaling in smooth muscle cells. To address this, we first examined endothelial cell-activated Notch signaling using a CBF1-luciferase reporter construct, which serves as a general Notch signaling sensor. HAoSMCs were cotransfected with the CBF1-luciferase or control luciferase construct,
along with siRNA to knockdown SYNDECAN-2 expression. Notch activity was measured by luciferase assays following coculture with endothelial cells. As previously published [20], endothelial cells promote robust activity of the CBF1-reporter (Figure 15A), and cause NOTCH3 RNA and protein levels to increase (Figure 16). However, when SYNDECAN-2 is knocked down in smooth muscle cells Notch signaling is greatly attenuated (Figure 15), and furthermore, NOTCH3 expression is decreased as a likely consequence of its inability to auto activate its expression (Figure 16).

To more precisely examine the downstream effect that the loss of SYNDECAN-2 has on Notch signaling, we measured the expression of known targets of NOTCH3 in smooth muscle cells cocultured with endothelial cells. Consistent with our previous findings, expression of HRT3/HEYL, PDGFRβ, and SM-α-ACTIN, SM22α, and CALPONIN-h1 (CNN1) were upregulated in smooth muscle cells by endothelial cell coculture (Figure 17A-F). In the absence of SYNDECAN-2, however, the expression of most of these genes was abrogated. CNN1 showed a slight but not significant decrease, suggesting it is regulated differently. These data indicate that SYNDECAN-2 facilitates Notch signaling in smooth muscle cells, and is an important mediator of smooth muscle differentiation by regulating the expression of some smooth muscle genes.

To assess whether SYNDECAN-2 was sufficient to activate Notch signaling and smooth muscle gene expression, we overexpressed SYNDECAN-2 cDNA by lentivirus transduction in smooth muscle cells and measured gene expression by qPCR and Western blotting. SYNDECAN-2 was overexpressed greater than 50-fold. In smooth muscle cells cultured by themselves the overexpression of SYNDECAN-2 could not induce Notch3 expression, nor the expression of Notch signaling targets, HRT3, PDGFRβ, and SM-α-
ACTIN (Figure 18). Moreover, in cells cocultured with HUVECs, where Notch signaling is activated, overexpression of SYNDECAN-2 could not further induce any of the tested Notch targets (Figure 18).

**SYNDECAN-2 and NOTCH3 Physically Interact.**

Our data indicated that SYNDECAN-2 modulates Notch signaling and because both proteins are localized within the cell membrane, we speculated that SYNDECAN-2 was facilitating Notch signaling through direct binding to Notch receptors. Because our attempts to use commercial antibodies to detect human SYNDECAN-2 were unsuccessful, we performed co-immunoprecipitation experiments with a HA-tagged full-length SYNDECAN-2 (HA-SDC2) protein, followed by immunoblots to detect endogenous NOTCH3. HA-SDC2 was lentivirally transduced into cells, followed by immunoprecipitations with IgG or HA antibodies. NOTCH3-specific immunoblots demonstrate that NOTCH3 protein is pulled down with SYNDECAN-2 (Figure 19). The reverse experiment of immunoprecipitating endogenous NOTCH3 followed by probing for HA-tagged SYNDECAN-2 by Western blot showed a similar result (Figure 19). Thus, our data show that these two proteins physically associate in cultured cells, providing a mechanism by which SYNDECAN-2 modulates Notch signaling.
**Discussion**

The signaling events that govern the interaction of vascular cells are critical for proper formation and function of blood vessels. The data presented here provide mechanistic insight into how cells within the vasculature communicate to control the function of each other. Previously, we demonstrated a role for NOTCH3 in endothelial cell/smooth muscle cell communication [20]. Our data showed that Notch signaling is important for endothelial cell-induced differentiation of smooth muscle cells, but the mechanisms downstream of NOTCH3 were not defined. Here we show that SYND ECAN-2 is also induced by endothelial cells and is dependent upon NOTCH2 and NOTCH3 for this upregulation. Moreover, in the absence of SYND ECAN-2, the expression of downstream targets of Notch signaling is attenuated, indicating that SYND ECAN-2 acts as a facilitator of Notch receptor activity. SYND ECAN-2 is not sufficient to induce expression of NOTCH3 or Notch target genes. Notch signaling, particularly Notch3, has been shown to be important for the regulation of smooth muscle differentiation [20,47,206]. Our data suggest that SYND ECAN-2 acts to promote smooth muscle differentiation via modulation of Notch signaling. A precise role for Syndecan-2 in smooth muscle has not been reported, however studies from Syndecan family members suggest a potential function in controlling the switch between proliferation and differentiation. Deletion of Syndecan-1 causes increased intimal hyperplasia and smooth muscle proliferation particularly in response to PDGF-B [207].
Loss of Syndecan-4 limits neointimal hyperplasia and reduces smooth muscle proliferation [208], and an earlier report showed a requirement for Syndecan-4 in thrombin-induced proliferation [209]. Unpublished results from our lab indicate that in vitro SYNDECAN-2 inhibits smooth muscle proliferation, consistent with a role in governing differentiation in collaboration with Notch signaling. Whether, Syndecan-2 has functions independent of the Notch pathway in smooth muscle cells remains to be determined.

Very little is known about the regulation of Syndecan-2 gene expression. Expression levels have been reported in various cell types, and linked to certain cancers [57,58,196]. One report showed Syndecan-2 levels increase upon treatment with tumor necrosis factor-a [210]. In smooth muscle cells, FGF2 was shown to induce the expression of Syndecan-4, but not Syndecan-2 [211]. Our results show that Notch signaling regulates SYNDECAN-2 gene expression in vitro and in vivo. We show that both NOTCH2 and NOTCH3 are necessary and sufficient for SYNDECAN-2 induction in smooth muscle cells. Currently, we do not know if the Notch intracellular domain and cofactor CBF (RPB-Jk) bind directly to the SYNDECAN-2 gene to activate its transcription. Like Syndecan-2, Notch receptors are widely expressed and are known to be important for development and tumor progression [193]. Our results only examined Notch-dependent activation of SYNDECAN-2 in dermal fibroblasts, coronary artery and aortic smooth muscle cells. Given the potentially overlap of Notch receptors and Syndecan-2 expression in other cell types, its interesting to speculate that Notch receptors

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regulate Syndecan-2 expression in other tissues, and in turn Syndecan-2 acts to govern Notch signaling.

One of the most interesting finding from our study is that SYNDECAN-2 acts to reinforce Notch signaling in smooth muscle cells. SYNDECAN-2 appears to do this through direct protein-protein interaction. The Syndecan family has been reported to interact with growth factors and their receptors [57,196], and specifically Syndecan-2 has been shown to directly bind to the TGFβ type-III receptor, Betaglycan [74]. More interestingly, Pisconti et al., [212] demonstrated a direct link between Syndecan-3 and Notch1 in skeletal muscle satellite cells. These authors showed that Notch1 and Syndecan-3 directly interact, and Syndecan-3 regulates Notch1 cleavage by ADAM17/tumor necrosis factor α-converting enzyme. Thus, like growth factor receptors, Notch family members may be common targets for Syndecan regulation. Our results show for the first time that SYNDENACAN-2 is a target of Notch signaling in smooth muscle cells, and SYNDECAN-2 acts in a feed forward loop to enhance the actions of the Notch signaling through direct contact with the NOTCH3 receptor (Figure 20). Taken together, these results provide new information for the role of Syndecan-2 in smooth muscle biology.
Figure 7. Endothelial cells increase SYND ECAN-2 expression in mural cells. (A) Mural cells, (human dermal fibroblasts (HDFNs), human aortic smooth muscle cells (HAoSMCs), and human coronary artery smooth muscle cells (HCASMCs) were cultured in the presence or absence of endothelial cells (HUVECs) for 48 hours, separated using anti-PECAM1-conjugated dynabeads, and subjected to qPCR analysis for SY ND ECAN-2 transcript expression. (B) HepG2 and HeLa cells were cultured alone or cocultured as described for mural cells and the expression of SYND ECAN-2 was measured by qPCR. * P < 0.05, **P < 0.01 relative to control, n.s. not significant.
Figure 8. Physical contact is required for endothelial cells induced SYND ECAN-2 expression in mural cells. HUVECs and HDFNs were plated on either side of a transwell insert as indicated, cultured for 48 hours, and the top chamber was harvested for qPCR to examine SYND ECAN-2 mRNA expression. **P < 0.01
Figure 9. SYND ECAN-2 expression is dependent upon Notch activity. qPCR to measure SYND ECAN-2 mRNA expression in HAoS MCs (A) and HDFNs (B), which were cocultured with HUVECs in the presence or absence of γ-secretase inhibitor DAPT. Mural cells cultured alone were used as a baseline control. HAoS MCs (C) and HDFNs (D) were lentivirally transduced with GFP, as control, or DN-MAML for 48 hours, then were cultured alone or cocultured with HUVECs for an additional 48 hours. Mural cells were separated from HUVECs and processed for qPCR analysis to measure SYND ECAN-2. ** P < 0.01.
Figure 10. NICD2 and NICD3 activate Notch targets and smooth muscle marker genes. Activated forms of Notch2 (NICD2) and Notch3 (NICD3) were introduced into human aortic smooth muscle cells by lentiviral transduction, followed by qPCR to analyze gene expression. Both NICD2 and NICD3 robustly activated expression of smooth muscle genes, SMA, CNN1, and SM-MHC (A), and also activate Notch targets HEYL, HEY2, and HES1 (B), compared to a GFP-expressing control. Both NICD2 and NICD3 activated endogenous NOTCH3 expression, but not NOTCH2 expression (C). Western blot demonstrates expression of the NICD2 and NICD3 constructs with a FLAG antibody (D).
Figure 10

A

B

C

D

NOTCH2

NOTCH3

FLAG

TUBULIN
Figure 11. **SYNDECAN-2 expression is dependent upon Notch activity.** qPCR expression analysis of NOTCH2 (A) and NOTCH3 (B) after siRNA transfection. HAoSMCs were transfected with siRNA to specifically target NOTCH2 and NOTCH3 alone or together. 24 hours later, cells were cultured with/without HUVECs and samples were collected after 48 hours for analysis. **P < 0.01.**
Figure 12. NOTCH2 and NOTCH3 are required for SYNDECAN-2. (A) HAoSMCs were transiently transfected with control siRNA (siControl), NOTCH2 (siNOTCH2) or NOTCH3 (siNOTCH3) siRNA and cocultured with/without HUVECs. 48 hours later, the cocultured cells were separated by anti-PECAM1-conjugated dynabeads and total RNA was collected for qPCR to detect SYNDECAN-2 expression. (B) Western blot to demonstrate efficient knockdown of NOTCH receptors by siRNA. Tubulin expression was used as control. ** P < 0.01.
Figure 13. NOTCH2 and NOTCH3 are sufficient to drive the expression of SYNDECAN-2. The NOTCH2 (C) or NOTCH3 (D) intracellular domains were overexpressed in HAoSMCs by lentiviral infection. A lentivirus expressing GFP alone was used as control. Following infection, cells were analyzed for SYNDECAN-2 expression by qPCR. ** P < 0.01.
Figure 14. SYNDECAN-2 expression is regulated by Notch2 and Notch3 in vivo.

RNA was isolated from highly vascularized yolk sacs of mouse embryos with mutations in the Notch2 and/or Notch3 genes at embryonic day (E) 10.5. qPCR was performed to determine expression of Syndecan-2 in mice lacking one (+/-) or both (-/-) wild-type copies of Notch2 and Notch3. ** P < 0.01 versus Notch2^{+/+}; Notch3^{+/+}. 
Figure 15. SYNDECAN-2 regulates Notch activity. (A) Luciferase reporter assays were used to assess Notch signaling activity. HAoSMCs were transiently transfected with control siRNA (siCon) or SYNDECAN-2 siRNA (siSDC2) along with pGL3-promoter-luciferase (pGL3-LUC) plasmid as control, or a plasmid with 5 CBF1 binding elements upstream of the promoter (CBF-LUC). 24 hours later HUVECs were cocultured with a subset of cells, and luciferase activity was measured after 48 hours. (B) Efficient siRNA knockdown of SYNDECAN-2 was determined by qPCR. ** P < 0.01.
Figure 16. SYNDENCA-N-2 regulates NOTCH3 expression. (A) qPCR was used to assess NOTCH3 expression (autoregulation) after SYNDENCA-N-2 siRNA knockdown. (B) Western blot using NOTCH3 antibody indicates that loss of SYNDENCA-N-2 attenuates NOTCH3 protein expression. * P < 0.05.
Figure 17. SYNDECAN-2 regulates Notch target gene expression in coculture conditions. (A-E) qPCR analysis of HEYL/HRT3, PDGF-ß, SMOOTH MUSCLE (SM) α-ACTIN, SM22α, and CNN1 expression in HAoSMCs cultured with or without HUVECs after SYNDECAN-2 knockdown by siRNA. (F) Western blot analysis of SM α-ACTIN, showing loss of SYNDECAN-2 attenuates the induction of SM α-ACTIN by cocultured endothelial cells. * P < 0.05, ** P < 0.01.
Figure 17

A. HRT3

B. PDGFRβ

C. SM α-ACTIN

D. SM22α

E. CNN1

F. Western blot analysis of SM α-ACTIN and TUBULIN
Figure 18. Overexpression of SYNDEN-2 is not sufficient to induce Notch target genes. (A-E) qPCR analysis of SYNDEN-2, NOTCH3, HEYL/HRT3, PDGF-ß and SMOOTH MUSCLE (SM) α-ACTIN expression in HAoSMCs overexpressing SYNDEN-2 and cultured with or without HUVECs. (F) Western blot analysis of NOTCH3 and SM α-ACTIN showing absence of induction by SYNDEN-2 overexpression. * P < 0.05, ** P < 0.01.
Figure 18

A

SYNDENAN-2

B

NOTCH3

C

HRT3

D

PDGFRβ

E

SM α-ACTIN

F

NOTCH3
SM α-ACTIN
TUBULIN

GFP
SYNDENAN-2
HUVECS
+ - + -
- + - +

Relative expression

0 20 40 60 80 100 120 140

Relative expression

0 1 2 3 4 5

Relative expression

0 1 2 3 4 5

Relative expression

0 1 2 3 4 5
Figure 19. SYNDECAN-2 and NOTCH3 physically interact. A HA-tagged SYNDECAN-2 cDNA (HA-SDC2) was transduced into HDFNs and co-immunoprecipitations (IP) were performed by pull down with a HA antibody or IgG (as control), followed by Western blot to detect NOTCH3 (top panel). Reverse IP was performed by pull down of endogenous NOTCH3 or IgG control followed by Western blot to detect HA-tagged SYNDECAN-2 (lower panel). Input amount is 1/40 of the total protein used for the immunoprecipitation.
Figure 20. Model illustrating feed forward loop to amplify Notch signaling by employment of SYNDECAN-2. 1) Endothelial cells activate Notch signaling in smooth muscle cells by presenting a membrane bound Notch ligand. 2) SYNDECAN-2 expression is increased by Notch activation (NICD) resulting in more SYNDECAN-2 protein at the cell surface. 3) SYNDECAN-2 cooperates with Notch receptors at the membrane surface to enhance Notch activity leading to an increase in Notch-dependent gene expression.
CHAPTER 3

miR-145 regulates TGFBR2 expression and matrix synthesis in vascular smooth muscle cells

MicroRNA miR-145 has been implicated in vascular smooth muscle cell differentiation [40,121,213], but its mechanisms of action and downstream targets have not been fully defined. Our lab previously showed that endothelial cells promote smooth muscle differentiation, and here we show that the miR-143/145 gene cluster is induced in smooth muscle cells by coculture with endothelial cells. Endothelial cell-induced expression of miR-143/145 is augmented by Notch signaling and accordingly expression is reduced in Notch receptor-deficient cells. To further define the function of miR-145 in smooth muscle cells, we screened putative target genes to identify over represented signaling pathways. Our results revealed that the TGFβ pathway has a significantly high number of miR-145 target genes, and we show that TGFβ receptor II (TGFBR2) is a direct target of miR-145. Extracellular matrix (ECM) genes, which are regulated by TGFBR2, were attenuated by miR-145 overexpression, and miR-145 mutant mice exhibit an increase in ECM synthesis. Furthermore, activation of TGFβ signaling via angiotensin II infusion revealed a pronounced fibrotic response in the absence of miR-145. These data demonstrate a specific role for miR-145 in the regulation of matrix gene expression in smooth muscle cells, and suggest that miR-145 acts to suppress TGFβ-dependent ECM synthesis.
accumulation and fibrosis, while promoting TGFβ-induced smooth muscle cell differentiation. Our findings offer evidence to explain how TGFβ signaling exhibits distinct downstream actions via its regulation by a specific microRNA.
**Introduction**

MicroRNAs have been cast as modulators of gene expression, whose fundamental function is to fine-tune cellular phenotypes in response to intrinsic signals or environmental stress [214]. In the vasculature, the ability of cells within the vessel wall to adjust to a range of cues is critically important for maintaining proper flow and pressure. While the endothelial cells serve as the primary sensor of blood vessels, smooth muscle cells act as the essential workhorse, by providing stability and contraction as needed. Smooth muscle cells are dynamic cells that can exist in a range of phenotypes. In addition to being contractile, these cells can be proliferative and/or exist in a synthetic state, where they secrete extracellular matrix (ECM) that is needed for the blood vessel wall [21]. The ability of smooth muscle cells to undergo phenotypic transitions is essential for vascular development and remodeling associated with changes in blood flow; however in vascular disease, phenotypic modulation can have detrimental impact.

A host of mediators have been implicated in the control of phenotypic modulation [21]. The transcription factor tandem, serum response factor (SRF) and Myocardin are master regulators of smooth muscle differentiation that drive cells towards a contractile phenotype [215]. Notch signaling has been shown to be important for smooth muscle development and differentiation, and plays a specific role in endothelial cell-dependent maturation [50,216]. Two opposing growth factor signaling pathways, platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β have been shown to drive
cells towards a proliferative and differentiated phenotype, respectively [21]. Additionally, the TGFß pathway can induce matrix synthesis under certain conditions, suggesting that this pathway might have a dual role in smooth muscle cell programming [153,154]. With the discovery of microRNAs that are enriched in smooth muscle cells, an obvious question is how they might contribute to phenotypic modulation by intervening with these established regulatory pathways. Not surprisingly, several microRNAs have been associated with the regulation of smooth muscle phenotypes [217-219]. For example, miR-1 was shown to be induced by Myocardin, can inhibit proliferation and is reduced in a carotid artery ligation model [217]. miR-21 also exhibits a pro-differentiation profile by being regulated by BMP and TGFß and is important for TGFß-mediated smooth muscle maturation [218]. In contrast, miR-221 is induced by PDGF signaling and can drive vascular smooth muscle cells towards a dedifferentiated state, partially through downregulation of Myocardin [219].

In 2009, a series of publications highlighted the importance of the miR-143/145 microRNA cluster in the regulation of smooth muscle cell phenotypes [120-123,220]. The results showed that microRNA-143/145 are highly expressed in contractile smooth muscle and are reduced in proliferative conditions. Data indicated that genetic loss of these microRNAs in mice, while not lethal, caused a decrease in smooth muscle stress fiber formation and an increase in rough endoplasmic reticulum, both indicators of a less differentiated and more synthetic phenotype. Attempts to identify targets of this miR cluster revealed they had a hand in the regulation of proliferation, actin remodeling, and contractility genes [221,222]. Despite the consensus that miR143/145 contribute to a
differentiated phenotype, inconsistencies in the data using different experimental models strongly suggested that miR-143/145 function is context-dependent.

In this study we show that miR-145 is induced in smooth muscle cells by endothelial cell signaling. Endothelial cells promote the increase in miR-145 expression through Notch signaling, consistent with a differentiated phenotype. Examination of putative miR-145 target genes revealed that miR-145 regulates TGFβ receptor II (TGFBR2) expression and governs the expression of downstream matrix genes in smooth muscle cells. Our results suggest that miR-145 functions to modulate TGFβ signaling in smooth muscle cells as a mechanism to suppress matrix gene expression, while sparing smooth muscle-specific differentiation genes. These actions of miR-145 may have implications in disease progression, where suppression of detrimental matrix synthesis by miR-145 could be used to alleviate vascular fibrosis.
Material and Methods

Cell culture

Primary cultures of human aortic smooth muscle cells (HAoSMCs) were purchased from Vasculife and grown in Dulbecco’s Modified Eagle’s Medium (DMEM) (Mediatech, Inc.) supplemented with 10% fetal bovine serum (FBS) (Hyclone), 2mM glutamine, 1mM sodium pyruvate and 100U/ml penicillin-streptomycin. Human mesenchymal stem cells (HMSCs) were purchased from Sciencell, and cultured in DMEM supplemented as above with 5% FBS. Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza, and grown in EBM-2 supplemented with the bullet kit as recommended (Lonza). Primary cells between passages 7-8 were used for all experiments. For virus production, TN-293 cells were purchased from Stratagene and cultured in DMEM supplemented as above with 10% FBS. Mouse embryo fibroblasts (MEFs) were isolated from embryonic day 10.5 mouse embryos and cultured in DMEM supplemented as above with 5% FBS [223]. All cultures were maintained in humidified 5% CO2 at 37°C. For coculture, 3x10⁴ mural cells were seeded in 12-well plates, and after adhesion, 3x10⁴ HUVECs were added. To separate endothelial cells from HAoSMCs and HMSCs, anti-PECAM1-conjugated Dynabeads (Invitrogen) were used according to manufacturer’s instructions. All cell coculture experiments, unless indicated, were performed in media consisting of EBM-2 supplemented with the bullet kit. NOTCH inhibitor, DAPT (anil-2-phenyl]glycine-1,1-dimethylethyl ester, Calbiochem) was added
to specified wells at the time of plating at 10µM. BMP inhibitor, LDN193189 (Reagentsdirect) was added at a 100nM, and TGFβ inhibitor, SB431542 (Reagentsdirect) was added at a 1µM. For TGFβ1 treatment, cells were serum starved for 24 hours before TGFβ1 (Peprotech) was added at a 10ng/ml concentration. For conditioned media assays, after 24 hours conditioning, media from HUVECs or HAoSMDs was transferred to HAoSMDs. For transwell assays, 4x10^4 HAoSMDs were plated on 12-well plates, and 0.4µm pore-size transwell inserts (Corning Costar) were inserted containing 2x10^4 HUVECs or HAoSMDs.

**Primary mouse aorta smooth muscle cell culture**

Mice were euthanized at 4–5 weeks of age and a midsternal thoracotomy was performed. The thoracic aorta was isolated and adventitia was carefully removed in cold PBS (Phosphate Buffered Saline). Aorta was digested with 1mg/ml Collagenase II (Sigma, C6885) and 100µg/ml Elastase (Sigma, E0127) at 37°C for 40 minutes. After digestion, cells were pelleted and plated in DMEM with 10% FBS. The next morning, cells were washed with PBS 3-4 times, followed by media refresh every 48 hours. Primary cells at passage 2 were used for experiments.

**Quantitative Real-Time PCR (qPCR)**

Mouse tissue was homogenized using TissueLyzer II (Qiagen). Total RNA was isolated using TRIZol reagent following manufactures’ instructions (Invitrogen), and reverse transcribed with M-MLV reverse transcriptase (Promega) to generate cDNA. Real-time PCR was performed using a StepOne PCR system (Applied Biosystems) with
SYBR Green and 50ng cDNA template. The fold difference in various transcripts was calculated using ΔΔCT method with 18S as the internal control. The primer sequences are attached in Table 1. To detect microRNA, RNA was reverse transcribed with TaqMan MicroRNA Reverse Transcription Kit (ABI) and analyzed with Taqman micromna assay (ABI). The fold difference was normalized with U6 as the internal control.

**Immunoblotting**

Equivalent amounts of protein were run on 10% SDS-PAGE gels, transferred to Nitrocellulose membranes (Millipore), and subjected to incubation using primary antibodies to TGFBR2 (Cell Signaling, 3713), PAI-1 (BD Transduction, 612025), Fibronectin (BD, 610078), Collagen I (Abcam, ab292), Elastin (Abcam, ab77804), Calponin (Sigma, C2687), SM α-ACTIN (Sigma, 1A4), and GAPDH (Novus Bio, NB300-221). Secondary antibodies conjugated to HRP (Amersham) were used for detection. Protein was detected by enhanced chemiluminescence (ECL).

**RNA Interference**

HAoSMCs were plated in a 12-well plate at 3x10⁴ cells/well. After 12 hours, the cells were transected with miR-145 or control RNA mimic at 40nM using Lipofectamine RNAiMAX (Invitrogen). Efficiency of transfection was assessed using qPCR.

**Lentivirus Expression**

Mouse NICD1 cDNA was cloned into pCDF1-MCS2-EF1-copGFP (System Biosciences) in front of the CMV promoter using BamHI and EcoRI sites. NICD2,
NICD3 and DN-MAML constructs were made as described previously [224]. The lentiviral plasmids were transfected into TN-293 cells using Polyjet (SignaGen), and the viral particles were amplified and purified as described [20]. For HAoSMCs infection, equal volumes of viral particles were diluted in 10% FBS in DMEM and were incubated with cells for 24 hours. The efficiency of infection was evaluated using GFP expression and qPCR. Viral particles were titrated to achieve 90% to 100% infection. Expression of cDNAs were confirmed using qPCR and Western blot analysis.

**Plasmid Transfection and Luciferase Assays**

psi-CHECK2-TGFB2 3’UTR plasmid was obtained from Addgene plasmid 31882 [225]. miR-145 seed site was mutated with Phusion polymerase (Promega). HEK293 cells were plated in a 12 well plate at 3x10^5 cells/well. After 12 hours, the cells were transfected with 500ng plasmid and RNA mimics at 100nM concentration. 24 hours later, Dual Luciferase Assay was performed to measure the Firefly luciferase activity normalized to Renilla luciferase activity followed the instruction of manufacturer (Promega).

**Collagen Secretion Assay**

Cell culture medium was incubated with 25% (NH4)2SO4 at 4°C overnight. The secreted collagen was pelleted by centrifugation at maximal speed and resuspended in 950μl of 50μM Sirius Red at room temperature. The stained collagen was centrifuged down and dissolved in 0.1M KOH. The absorbance was determined in spectrophotometer of 540nm wavelength.
miR-145 knockout animals and angiotensin II infusion

The mouse studies were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee at the Research Institute at Nationwide Children’s Hospital. miR-145 knockout mice, referred to here as miR-145/-, were generated and generously provided by Dr. Eric Olson [121], and maintained in C57Bl/6 background. miR-145+- mice were crossed to generate wildtype and miR-145/- mice. For Angiotensin II (Ang II) infusion, wild-type and miR145/- mice (10-weeks old) were randomly divided into two treatment groups: one group (n = 5 per group) received vehicle (0.9% saline) and the other group (n = 6 per group) was administered Ang II (500 ng/kg/day, Sigma, St. Louis, MO) via Alzet mini osmotic pumps (Durect Corporation, Model 2004, Cupertino, CA). Briefly, mini-pumps were filled with either vehicle or Ang II and allowed to prime for 48-hours prior to surgical implantation according to the manufacturer’s instructions. Pumps were implanted subcutaneously under 2% isoflurane anesthesia using aseptic technique, after which they were given ibuprofen for pain in drinking water and monitored until ambulation. After 14 days of treatment, mice were sacrificed and tissues were harvested for RNA isolation or sectioning.

Ex vivo culture of mouse aorta

Thoracic aortas were dissected from 4-week old mice, and the endothelial layer was carefully removed by scraping with scalpel. After cutting into two equal halves, aorta was cultured in EBM-2 with 10% FBS for 24 hours and then serum starved in DMEM
with 0.25% FBS for additional 24 hours. After starvation, ex vivo cultured aorta was treated with or without TGFβ1 for 24 hours.

**Immunohistochemistry**

After fixation in 4% paraformaldehyde, tissues were processed, embedded in paraffin, and sectioned at 8 µm. For immunohistochemistry, sections were baked at 60°C for 1 hour, cleared in xylene, rehydrated through a descending concentration of ethanol for 2 minutes each ending in distilled water. Antigen retrieval was done in citrate buffer (0.01 M, PH=6.0) using pressure cooker for 30 minutes. Sections were cooled to room temperature and blocked with 5% goat serum diluted in PBS with 0.5% Triton-X-100 for 1 hour at room temperature. Sections were then incubated with primary antibodies, SMA (1:1000, SIGMA, Cat: A2547), TGFBR2 (1:100, Santa Cruz, sc-400) overnight at 4 °C. After washing in PBT, sections were incubated with appropriate Alex Flour–conjugated secondary antibody (1:1000 Invitrogen) for 1 hour at room temperature, counterstained with DAPI, and mounted in Vectashield mounting medium (Vector Laboratories, H-1400). Pictures were taken using fluorescence microscope (OLYMPUS, 1X51). Primary smooth muscle cells were cultured on chamber slides and fixed with 4% PFA at room temperature for 1 hour. Slides were washed with PBS and treated with 0.5% Trixton-X-100 for 1 hour and blocked with 3% BSA in PBS for 1 hour at room temperature. Slides were incubated with primary antibodies in 3% BSA in PBS for 1 hour at room temperature, incubated with secondary antibody and costained with DAPI during mounting. The intensity of fluorescence was quantified using ImageJ. Fluorescence from same area was quantified and normalized to DAPI intensity. Masson’s trichrome staining
was performed on sections using a kit purchased from Sigma following kit instructions. Quantification of trichrome staining was performed using Image-Pro Plus software.

**Statistical Analysis**

Data analyses were performed using GraphPad Prism and comparisons between data sets were made using a Student’s t test. Differences were considered significant if P < 0.05, and data are presented as mean ± standard error of the mean (SEM). Data shown are representative of at least three independent experiments.
Results

**miR-143/145 is induced in smooth muscle cells by endothelial cells.**

Endothelial cells can regulate the phenotype of smooth muscle cells and our lab previously demonstrated that cocultured endothelial cells promote vascular smooth muscle cell differentiation [20]. To examine the extent of this modulation by endothelial cells, we measured the expression of the microRNA gene cluster miR-143/145, which has been linked to governing smooth muscle differentiation [120-122]. Coculture of human umbilical vein endothelial cells (HUVECs) with human aortic smooth muscle cells (HAoSMCs) caused an increase in precursor microRNA pri-miR-143/145 transcript as well as the individual mature microRNAs (Figure 21A-C). The expression of both the precursor and mature microRNAs was observed at high levels after 48 hours of incubation and was sustained, albeit at reduced levels up to six days in coculture (Figure 22A-C). Smooth muscle α-actin showed a similar expression profile in cocultured aortic smooth muscle cells, compared to miR143/145 (Figure 22D). Expression of miR-143/145 could also be induced in smooth muscle cells cocultured with microvascular endothelial cells (Figure 23), and was also upregulated in cocultured mesenchymal stem cells (Figure 24). Thus, miR-143/145 shows an increase in expression consistent with the smooth muscle differentiation phenotype induced by endothelial cells.
miR-143/145 is regulated by Notch signaling.

To determine whether the induction of miR-143/145 required cell-cell contact between endothelial cells and smooth muscle cells, we first treated smooth muscle cells with conditioned media. miR-143/145 levels remained low in smooth muscle cells treated with HUVEC-conditioned media, similar to those treated with HAoSMC-conditioned media, whereas the control cocultured cells showed robust induction (Figure 25A). We also utilized a transwell assay to physically separate cocultured cells. Like the conditioned media experiments, smooth muscle expression of miR-143/145 was not increased by endothelial cells that were separated by a transwell membrane (Figure 25B). Our data suggested that miR-143/145 expression required cell-cell contact, and given that membrane-bound Notch signaling is known to be critical for endothelial cell-induced smooth muscle differentiation [20], we tested the role of Notch signaling in miR-143/145 expression.

Using the chemical inhibitor, DAPT and a lentiviral-delivered dominant-negative-mastermind (dn-MAML) protein [204] to block Notch signaling, we measured the induction of miR-143/145 in smooth muscle cells. Both strategies to block Notch signaling resulted in an almost complete absence of miR-134/145 induction by endothelial cells (Figure 26A,B). In contrast, inhibitors to TGFβ and BMP, both which have been previously shown to induce miR-143/145 expression did not block the upregulation (Figure 27) [124,125]. Further, overexpression of the intracellular domains of Notch1, Notch2, or Notch3 promoted robust expression of miR-143/145 in smooth muscle cells (Figure 28). To test if Notch signaling regulates miR-143/145 expression in vivo, we isolated ascending and descending aortas from Notch3-deficient mice [201], and
examined expression. The data show that miR-143/145 levels are decreased in the Notch3 null mice (Figure 29). This was further confirmed using mouse embryonic fibroblasts (MEFs) deficient in both Notch2 [200] and Notch3 (Figure 30). These data are consistent with previous published in vitro findings [40], and show that Notch signaling regulates miR-143/145 expression in vivo.

**miR-145 targets the TGFβ signaling pathway.**

Expression and deletion analysis of miR-143/145 have suggested a role in the modulation of smooth muscle cell phenotypes, and although targets for these miRs have been identified, the analysis is incomplete. In an attempt to identify key signaling pathways that miR-143/145 may influence we performed a computational screen with the PANTHER classification system [226,227] using data derived from TargetScan [228]. TargetScan, identified 717 putative target genes for miR-143 and 717 possible targets for miR-145. These target genes were screened as a group using PANTHER to identify pathways, which were preferentially targeted by these individual miRs. miR-143 targeted pathways unrelated to miR-145 (not shown), Interestingly, miR-145 preferentially targeted two opposing signaling pathways in smooth muscle, PDGF and TGFβ (Table 2). The PDGF pathway had been previously shown to be a miR-145 target and suppressor of miR-145 expression [213], while TGFβ was shown to be upstream activator of miR-145 expression [124,125], suggesting that miR-145 may exist in a negative feedback loop to dampen TGFβ signaling. Overexpression of a miR-145 mimic in smooth muscle cells caused a decrease in TGFβ readout genes, PAI-1 and SMAD7 [229,230], suggesting a
decrease in TGFβ signaling (Figure 31). A cursory examination of the 16 putative target genes within the TGFβ pathway (Table 2) showed that 7 of the 16 exhibited a significant decrease in transcript expression in the presence of a miR-145 mimic in smooth muscle cells (Figure 32). The most significantly downregulated of these genes was the TGFβ receptor II (TGFBR2). Examination of the 3’ untranslated region (UTR) of the TGFBR2 gene revealed a highly conserved miR-145 target sequence (Figure 33). To test if this element was a functional seed sequence, the 3’UTR was cloned into a luciferase expression vector to evaluate its response to miR-145 [225]. Cotransfection of the luciferase reporter with the miR-145 mimic showed the 3’UTR conveyed decreased expression, whereas a mutated version of the 3’UTR that could not be recognized by miR-145 was not affected (Figure 34). The ability of miR-145 to regulate TGFBR2 was further confirmed by Western blot, where overexpression of the miR-145 mimic reduced TGFBR2 protein in smooth muscle cells (Figure 35). In addition, both mRNA and protein of TGFBR2 are downregulated by coculturing with endothelial cells (Figure 36).

miR-145 regulates TGFβ-pathway genes and matrix gene expression in smooth muscle cells.

In smooth muscle cells, TGFβ signaling and miR-145 are pro-differentiation mediators [120-122,231]; yet, our data indicate that miR-145 suppresses the expression of certain TGFβ genes. Therefore, we sought to explore how miR-145 would influence TGFβ-dependent smooth muscle gene expression. Overexpression of the miR-145 mimic in smooth muscle cells in the presence and absence of TGFβ1 ligand showed, as before, a decrease in TGFBR2 and PAI-1 RNA and protein expression (Figure 37A, Figure 38). As
expected, the smooth muscle marker genes, SM-a-actin, CNN1, and SM22a all were increased with TGFβ stimulation and miR-145 mimic alone, and together appeared to exhibit an additive response, suggesting independent mechanisms of action (Figure 37B, Figure 38). Together these data indicate that miR-145 has a selective effect on TGFβ signaling and may facilitate unique downstream events. Deletion analysis of TGFBR2 in smooth muscle cells of mice revealed an important role in the regulation ECM genes, like elastin, collagens, and the matrix crosslinking genes, Lox and Lox1 [232,233]. We examined expression of these matrix genes in the presence of the miR-145 mimic, and similar to TGFBR2 expression, we showed a decrease in expression at the both RNA and protein levels (Figure 37C, Figure 38). This data demonstrate that in smooth muscle cells, miR-145 selectively regulates TGFβ signaling and blocks matrix synthesis, while permitting expression of smooth muscle differentiation genes.

**Loss of miR-145 causes increased TGFβ signaling in smooth muscle cells in vivo.**

To investigate whether miR-145 regulates TGFβ-dependent matrix genes expression in vivo, we utilized miR-145-deficient (miR-145^-/-) mice [121]. miR-145 null mice are viable with deficits in smooth muscle function. We isolated ascending aortas from adult wild-type and miR-145^-/- mice and performed immunostaining to detect TGFBR2 and SM-a-actin expression (Figure 39A-C). Expression of TGFBR2 in the miR-145^-/- aortas was increased compared to wild-type, while SM-α-actin expression showed slight but insignificant difference between wild-type and miR-145-deficient mice. Ascending aorta tissue was isolated and cultured ex vivo with or without TGFβ1 and matrix gene expression was measured by qPCR (Figure 40). While there were no
significant differences in the basal level of expression between wild-type and miR-145 null mice, after TGFβ1 challenge there was a greater induction in the absence of miR-145. We additionally isolated aortic smooth muscle cells from wild-type and miR-145 null mice and measured matrix gene expression cultured cells. Expression of some matrix and matrix synthesis genes was increased at basal levels in the absence of miR-145, and all showed an increased level of expression in response to TGFβ1 (Figure 41A,B). Collagen secretion was also measured from the culture media and showed an increase in the miR-145 null mice (Figure 42). Immunostaining of cultured cells to detect TGFBR2 and SM-a-actin showed consistent results (Figure 43). Thus, these findings demonstrate that miR-145 functions to suppress matrix gene expression and likely acts to govern aberrant ECM deposition.

**Loss of miR-145 exacerbates angiotensin II-induced fibrosis**

The data indicate that miR-145 regulates TGFBR2 and matrix synthesis and this is most evident when the TGFβ pathway is robustly activated. Fibrotic diseases are associated with excess TGFβ signaling in activated fibroblasts, and given that miR-145 also is expressed in fibroblasts, we wondered if it might act as a suppressor of pathological fibrosis REF. To test this, we infused angiotensin II (AngII) into mice to induce TGFβ-dependent cardiac fibrosis [234,235]. Infusion of AngII (500ng/kg/day) [236] by osmotic pumps for 14 days in wild-type and miR-145-deficient mice revealed data consistent with our previous results. AngII caused increases in Col1A and Elastin expression in wild-type mice, and this expression was significantly increased in the miR-145 knockout animals (Figure 44). Examination of fibrosis in the hearts of these mice by
Masson’s tri-chrome staining revealed a much more pronounced collagen deposition in the miR-145 null mice (Figure 45). Thus, these data demonstrate that miR-145 has the capacity to regulate TGFβ-dependent responses in pathological conditions, and acts to selectively regulate matrix synthesis independent of smooth muscle differentiation genes.
Discussion

Previous studies demonstrated a role for miR-145 in regulating smooth muscle differentiation [120,121,125,213]. Collectively the data indicate that miR-145 acts to drive smooth muscle-specific gene expression in a vast regulatory loop that includes activation of Myocardin and inactivation of dedifferentiation mediator KLF4. Our data support this pro-differentiation notion, as we show that miR-143/145 is induced by endothelial cells during Notch-regulated smooth muscle differentiation. TGFβ is a well-described smooth muscle differentiation inducer, and studies have shown that TGFβ activates miR-145 expression [124,125]. Our initial finding that miR-145 preferentially targeted TGFβ signaling genes suggested it might exist in a negative feedback loop. Though this may be the case, further analysis in this study revealed that miR-145 targeted distinct subsets of TGFβ-dependent genes. We show that in smooth muscle cells TGFβ activates both matrix genes and smooth muscle-specific genes, but miR-145 specifically attenuated the expression of the matrix genes, leaving the smooth muscle-specific genes unaffected. This selective effect on TGFβ target genes implies that miR-145 functions to control the actions of TGFβ and define smooth muscle cell phenotypes. A proposed model is illustrated in (Figure 46), where TGFβ causes an increase in both matrix genes and smooth muscle differentiation genes. Under conditions that drive a contractile phenotype, miR-145 expression is high causing a TGFβ to preferentially activate smooth muscle differentiation genes and suppress matrix. Under conditions in which a synthetic
phenotype is warranted, miR-145 levels are reduced, which allows for TGFβ-dependent matrix gene expression to ensue.

Our data demonstrate that TGFBR2 is a direct target of miR-145 and this finding lead us to examine matrix gene expression because of the established link to this receptors activity. TGFBR2 is essential for normal vascular development and mouse knockout have shown a role in the regulation of matrix synthesis, elastogenesis, and aortic wall homeostasis [232,233]. Human mutations found in this receptor cause Loeys-Dietz and Marfan syndrome type 2 [237,238]. The ability of miR-145 to decrease TGFBR2 levels in smooth muscle cells is one mechanism through which it regulates matrix, but we expect other direct targets of the TGFβ pathway and matrix synthesis also contribute to this outcome. Indeed, we demonstrated that additional genes in the TGFβ pathway are decreased by miR-145, but whether these are direct targets is currently not known. TGFβ signaling is a complex pathway that involves autoregulation of both a positive and negative nature. Mutations in TGFβ components have lead to paradoxical increases in TGFβ signaling [238]. Our attempts to quantify changes in TGFβ signaling under differing miR-145 levels by measuring SMAD and MAP kinase phosphorylation did not reveal any consistent changes. Thus, we are currently unable to explain the exact mechanism through which miR-145 suppresses matrix gene expression. Furthermore it is unclear if suppression of matrix genes by miR-145 is entirely through regulation of the TGFβ pathway.

Finally, data presented here indicate that miR-145 might serve as a critical checkpoint in the development of TGFβ-associated diseases. TGFβ signaling is linked to a range of cardiovascular diseases, many of which are based on inappropriate ECM
deposition [184,234]. Our results demonstrate that cardiac fibrosis is increased in the absence of miR-145, thus finding ways to increase or maintain miR-145 in fibrotic diseases may have beneficial consequences. A recent report analyzing miR-145 in cardiac fibroblasts indicated that just as in smooth muscle cells, miR-145 promoted a contractile phenotype, and consistent with our results, loss of miR-145 increased scaring in response to injury [239]. Given that miR-145 can preferentially down regulate TGFß-dependent matrix synthesis, while leaving other TGFß-responsive pathways unaffected could serve as a valuable treatment strategy.
**Table 1. Primer sequence for qPCR analysis.** All sequences list from 5'-end to 3'-end.

For: Forward, Rev: Reverse.

<table>
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<td>FIBRONECTIN For</td>
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**Mouse genes**

<p>| GAPDH For          | GACGGGCCGCATCTTCTTGT |
| GAPDH Rev          | CACACCGACCTTCACCATTT |
| Pri-miR-143/145 For| GGACCGCAGCGAGAAGGT |
| Pri-miR-143/145 Rev| GGACCTGTCGACGATTATTAATAA |
| PAI-1 For          | CCCTGGAACAAAGAATGAGATCAG |
| PAI-1 Rev          | CTCTAGGTCCGGCTGGACAA |
| CTGF For           | AAAGTGCAATCCGGACACCTAA |
| CTGF Rev           | TGCAGCCAGAAAGCTCAAACT |
| COL1A1 For         | CTTACACAGCCACCCCTG |
| COL1A1 Rev         | TGAAGTTGCTCCTCCACCT |
| ELASTIN For        | CTTTGGACCTTTTCTCCCATTATCC |
| ELASTIN Rev        | GGTCCCCAGAAGATCACTTTCTC |
| LOX For            | TGAGAGTTGCGGGAACAAAG |
| LOX Rev            | GGTCGGCTCTCCTTGGTTT |
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| LOXL1 Rev          | CGCGGGATCGTAGTCTCAT |</p>
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**Table 2. Pathway analysis of miR-143 and miR-145 targets.** 406 miR-143 targets and 717 miR-145 targets predicted by TargetScan were subjected to PANTHER pathway analysis to identify over represented pathways.
Figure 21. Coculturing of endothelial cells and vascular smooth muscle cells induce miR-143/145 expression in vascular smooth muscle cells. Human umbilical vein endothelial cells (HUVECs) and human aortic smooth muscle cells (HAoS MCCs) were cocultured for 48 hours, separated using anti-PECAM1-conjugated dynabeads, and subjected to qPCR analysis for (A) pri-miR143/145 transcript, (B) mature miR-143 and (C) mature miR-145. **P < 0.01 relative to control without endothelial cells.
Figure 22. Endothelial cells induce miR-143/145 expression in vascular smooth muscle cell. Human aortic smooth muscle cells (HAoSMCs) were cocultured with human umbilical vein endothelial cells (HUVECs) for 2, 4 and 6 days, separated using anti-PECAM1-conjugated dynabeads, and subjected to qPCR analysis for (A) pri-miR143/145 transcript, (B) mature miR-143, (C) mature miR-145 and (D) SM α-actin expression. * P < 0.05, **P < 0.01.
Figure 23. Human microvascular endothelial cells induce miR-143/145 expression in vascular smooth muscle cell. HAoSMCs were cocultured with human microvascular endothelial cells (HMECs) for 2 days, followed by separation and qPCR analysis for pri-miR143/145 transcript, miR-143 and miR-145. * P < 0.05, **P < 0.01.
Figure 24. Endothelial cells induce miR-143/145 expression in human mesenchymal stem cells. Human mesenchymal stem cells (HMSCs) were cocultured with HUVECs for indicated days, separated using anti-PECAM1-conjugated dynabeads, and subjected to qPCR analysis for pri-miR143/145 transcript (A), miR-143 (B), miR-145 (C) and SM α-actin (D) expression. * P < 0.05, **P < 0.01.
Figure 25. Endothelial cells induce miR-143/145 expression in vascular smooth muscle cells through physical contact. (A) HAoSMCs were treated with conditioned media collected from HAoSMCs or HUVECs for 48 hours. (B) HAoSMCs were cultured with HUVECs seeded in a transwell insert as indicated for 48 hours. Cocultured cells were used as a positive control. Expression of pri-miR-143/145 was analyzed by qPCR. **p < 0.01, n.s. not significant.
Figure 26. Endothelial cells induce miR-143/145 expression in vascular smooth muscle cells through Notch signaling. (C) HAoSMCs were cultured with HUVECs in the presence or absence of Notch inhibitor DAPT for 48 hours. (D) HAoSMCs were lenti-virally transduced with GFP, as control, or dnMAML for 48 hours, and then cultured alone or with HUVECs for an additional 48 hours. Expression of pri-miR-143/145 was analyzed by qPCR. **P < 0.01.
Figure 27. Endothelial-cells-dependent miR-143/145 expression in vascular smooth muscle cells is not regulated by TGFβ signaling. HAoSMCs were cultured with HUVECs in the presence or absence of BMP inhibitor, LDN193189 and TGFβ inhibitor, SB431542 for 48 hours. Expression of pri-miR-143/145 was analyzed by qPCR. n.s. not significant.
Figure 28. Notch signaling activates miR-143/145 expression. The Notch1 (NICD1), Notch2 (NICD2) or Notch3 (NICD3) intracellular domains were overexpressed in HAoSMCs by lenti-viral infection for 96 hours. A GFP-expressing virus was used as control. Expression of pri-miR-143/145 was analyzed by qPCR. * P < 0.05, **P < 0.01.
Figure 29. miR-143/145 expression decreases in Notch3 mutant mice. Ascending aorta and descending aorta were isolated from Notch3\textsuperscript{+/−} and Notch3\textsuperscript{−/−} mice to isolate RNA for miR-143/145 analysis. Expression of pri-miR-143/145 was analyzed by qPCR. **P < 0.01.
Figure 30. miR-143/145 expression decreases in MEFs with Notch mutations. RNA was collected from mouse MEFs isolated from embryos with mutations in the Notch2 and Notch3 genes. Expression of pri-miR-143/145 was analyzed by qPCR. * P < 0.05, **P < 0.01.
Figure 31. miR-143/145 overexpression inhibits TGFβ signaling. HAoSMCs were transiently transfected with control RNA mimic or a miR-145 mimic and isolated RNA was tested for expression of known TGFβ-dependent genes PAI-1 and SMAD7 by qPCR. * P < 0.05, **P < 0.01.
Figure 32. The 16 predicted miR-145 targets found in the TGFβ signaling pathway were measured by qPCR following overexpression of the miR-145 mimic. * P < 0.05, **P < 0.01.
Figure 33. The seed sequence of miR-145 on TGFBR2 3’UTR is highly conserved across species.
Figure 34. TGFβ receptor II (TGFB2) is a direct target of miR-145. Wildtype or mutant 3’UTR of TGFB2 gene was cloned following Renilla gene. Wildtype, mutant TGFB2-3’UTR or empty vector was transfected into HEK293 cells with the presence or absence of miR-145, followed by a luciferase assay to measure Renilla luciferase. **P < 0.01, n.s. not significant.
Figure 35. TGFBR2 protein level is downregulated by miR-145. Graph shows quantification from 6 separate experiments. * P < 0.05.
Figure 36. TGFBR2 expression is downregulated by coculturing with endothelial cells. (A) HAoSMCs were cocultured with HUVECs for 48 hours and qPCR was performed to analyze the mRNA level of TGFBR2. (B) HAoSMCs were cocultured with HUVECs for 2 days, 4 days and 6 days. Westernblot was performed to measure the protein level of TGFβ receptor II expression. **P < 0.01.
Figure 37. miR-145 regulates TGFβ-dependent matrix synthesis genes. HAoSMCs were transfected with miR-145 mimic in the presence or absence of TGFβ1. (A) qPCR was performed to measure the expression of TGFBR2 and PAI-1, (B) smooth muscle differentiation genes and (C) matrix genes. * P < 0.05, ** P < 0.01, n.s. not significant.
Figure 38. miR-145 regulates TGFβ-dependent gene protein. Western blot to analyze the protein expression of smooth muscle and matrix genes.
Figure 39. TGFBR2 expression in ascending aorta increases in miR-145 null mice.

(A) Ascending aortas were isolated from wild-type and miR-145 mutant mice and stained for TGFBR2 (red), SM α-actin (green) and DAPI (blue). (B, C) Intensity of TGFBR2 and SM-α-actin were quantified and normalized to DAPI. **P < 0.01, n.s. not significant.
Figure 40. Matrix genes expression in ascending aorta increases in miR-145 null mice. Thoracic aortas were isolated from wild-type and miR-145 mutant mice, and cultured in the presence or absence of TGFβ1. RNA was extracted after 24 hours and subjected to qPCR analysis. * P < 0.05, **P < 0.01.
Figure 41. Matrix genes expression increases in miR-145 deficient aorta smooth muscle cells. Aortic smooth muscle cells were isolated from wild-type and miR-145 mutant mice and treated with or without TGFβ1 for 48 hours. (A) qPCR was performed to measure the expression TGFβ signaling downstream targets and matrix synthesis genes. (B) Western blot was performed to analyze Fibronectin and Collagen I protein expression. * P < 0.05, **P < 0.01.
Figure 42. Collagen secretion increases in miR-145 deficient aorta smooth muscle cells. Aortic smooth muscle cells were isolated from wild-type and miR-145 mutant mice and treated with or without TGFβ1 for 48 hours. Media was collected and collagen content was measured using a picosirius red assay. **P < 0.01.
Figure 43. TGFBR2 increases in miR-145 deficient aorta smooth muscle cells. Fluorescent immunostaining was performed to detect expression of TGFBR2 (red), SM α-actin (green) and DAPI (blue).
Figure 44. Loss of miR-145 increase matrix gene expression in angiotensin II-infused mouse hearts. Expression of Col1A1 (A) and Elastin (B) mRNA from heart tissue of wildtype or miR-145-deficient mice infused with angiotensin II or control saline for 14 days. * P < 0.05, **P < 0.01.
Figure 45. Loss of miR-145 exacerbates angiotensin II-induced fibrosis. (C) Masson’s trichrome staining of representative heart sections highlighting collagen deposition in blue. (D) Quantification of trichrome staining to measure collagen in the different groups. 

**P < 0.01.
Figure 46. Loss of miR-145 switches TGFβ-regulated contractile phenotype to synthetic phenotype in smooth muscle cells.
Feeding-forward Notch signaling loop regulates endothelial cell / smooth muscle cell interactions

Blood vessel formation is a tightly regulated process that serves a critical role in both health and disease [1,2]. The interactions between endothelial cells and mural cells are required for proper blood vessel formation [6,7]. The major function of vascular smooth muscle is to maintain contractility and stretch in arteries. Smooth muscle surrounding the endothelial cell tube receives cues from endothelial cells and then provides contractility to maintain vessel tone, blood pressure and blood flow distribution [21]. Recently, Notch signaling has been implicated in the process of endothelial cell-guided smooth muscle cell differentiation.

Notch proteins belong to an evolutionarily conserved family of cell surface receptors that transduce signals between neighboring cells [27,28]. Activation of Notch signaling in smooth muscle cells is initiated by the interaction with the ligand-expressing endothelial cells. The Lilly lab has shown that once endothelial cells physically interact with vascular smooth muscle cells, the Jag1 ligand on endothelial cells activates Notch3 on smooth muscle cells. Interestingly, the upregulated Notch activity in vascular smooth muscle cells further induces Notch3 and Jag1 expression on vascular smooth muscle cells.
[20]. It is very likely that endothelial cells initiate Notch signaling in the inner layer of smooth muscle cells, which induces Jag1 expression and further distributes Notch signaling to the outer layers of smooth muscle cells in the blood vessel wall. Later, the Epstein Lab showed that loss of Jag1 in neural crest cells resulted in deficient smooth muscle cell differentiation, and Jag1 is a direct downstream target of Notch [51].

Taken together, all these data suggest that endothelial cells initiate Notch signaling, which is propagated by a Notch/Jag1 induction pathway from the inner layer of vascular smooth muscle cells to the outer layer, until Notch signaling is activated through the whole vascular walls. The interaction of endothelial cells and smooth muscle cells more likely happens at the earlist embryonic stages, since the basemembrane separates the endothelial layer from smooth muscle layers. A prominent question is how Notch signaling remains activated in smooth muscle cells. I hypothesize that Notch upregulates certain downstream target proteins which function to facilitate the amplifying loop of Notch signaling in smooth muscle cells.

**Syndecan-2: a coreceptor of Notch signaling**

Using a screen to find genes regulated by endothelial-mural cell interactions, our lab identified an array of genes whose expression was altered when cells of the vasculature were cocultured [203]. One of these genes was the heparan sulfate proteoglycan, SYNDECAN-2 (SDC2) [58]. Syndecan-2 is abundantly distributed in mesenchymal tissues surrounding blood vessels, and Syndecan-2 is a vital participant in angiogenesis [59]. One prominent characteristic of Syndecan-2 structure is the abundant
glycosaminoglycan (GAG) chains attached to its extracellular domain [63-65]. These highly sulfated chains of Syndecan family members are known to control both cell-matrix and cell-cell interactions, and to serve as a coreceptor for the growth factors, PDGF, fibroblast growth factor (FGF), and VEGF [57,196].

I show that SYNDECAN-2 is induced by endothelial cells and is dependent upon Notch signaling. NOTCH2 and NOTCH3 are necessary and sufficient for the proper induction of SYNDECAN-2 in vitro and in vivo. Further exploring SYNDECAN-2 shows that the expression of NOTCH3 and downstream targets of Notch signaling are attenuated in the absence of SYNDECAN-2. Moreover, SYNDECAN-2 and NOTCH3 physically associate in smooth muscle cell. The most interesting finding from this study is that SYNDECAN-2 acts to reinforce Notch signaling in smooth muscle cells. Considering that syndecan family can function as a coreceptor, I propose a feed forward loop formed by SYNDECAN-2 and Notch signaling (Figure 20). Initially, endothelial cells activate Notch signaling in smooth muscle cells by presenting a membrane bound Notch ligand. SYNDECAN-2 expression is increased by Notch activation (NICD) resulting in more SYNDECAN-2 protein at the cell surface. Increased SYNDECAN-2 binds with Notch receptors to facilitate its interaction with ligands leading to further activation of Notch signaling. Previously, we showed that endothelial-cell dependent Notch activation results in upregulation of NOTCH3 receptor in smooth muscle cells [20]. Here we show that loss of SYNDECAN-2 partially inhibits NOTCH3 upregulation, but overexpression of SYNDECAN-2 is not sufficient to induce NOTCH3 expression. Taken together, Notch signaling induces SYNDECAN-2 as an important mediator facilitating its further amplification. My results only examined Notch-dependent
activation of SYNDECAN-2 in dermal fibroblasts, coronary artery and aortic smooth muscle cells. Given the potential overlap of Notch receptors and Syndecan-2 expression in other cell types, its interesting to speculate that Notch receptors regulate Syndecan-2 expression in other tissues, and in turn Syndecan-2 acts to govern Notch signaling.

**Complex role of miR-145**

MicroRNAs (miRNAs) are ~22 nt RNAs which can bind to the 3’ untranslated region (3’UTR) of messenger RNA (mRNA) resulting in degradation or repression of translation [75]. Several miRNAs have been discovered regulating vascular smooth muscle cell function, miR-145 being the most impressive one. miR-145 clustered with miR-143, is the most abundant miRNA in vascular smooth muscle cells. It has been shown to be upregulated by myocardin, Notch signaling and TGFβ signaling, and downregulated by PDGF signaling in vascular smooth muscle cells [40,121,124,125,213]. Therefore, miR143/145 regulates smooth muscle phenotype as a converging node of multiple signaling pathways. Previously, our lab showed that heterotypic contact of endothelial cell and smooth muscle cell activates Notch signaling, which is critical for smooth muscle differentiation [20].

Here I show that coculture with endothelial cells induces miR-143/145 expression in smooth muscle cells. This induction is dependent on activated Notch signaling in smooth muscle cells. Since miR-143/145 has been shown to promote smooth muscle differentiation, endothelial-cell dependent miR-143/145 expression might be one of the mechanisms of inducing smooth muscle differentiation by heterotypic interaction with endothelial cells. Furthermore, canonical Notch signaling directly activates transcription
of target genes, so miR-143/145 can function as a repressive mediator of Notch signaling to inhibit genes which need to be silenced in angiogenesis. microRNAs usually target hundreds of genes simultaneously, so one microRNA may target multiple genes involved in the same signaling pathway and result in a profound effect. Previously, miR-143/145 has been shown to suppress smooth muscle cell migration by targeting PDGF signaling[213]. In this report, miR-145 is found to suppress matrix synthesis by targeting TGFβ signaling. Therefore, Notch signaling is likely using miR-145 to regulate other signaling pathways, such as PDGF and TGFβ signaling.

Consistent with the function of these signaling pathways, miR-145 is able to induce vascular smooth muscle cells differentiation, and repress migration as well [40,121,213]. However, loss of miR-145 does not cause embryonic lethality or severe smooth muscle defects, which suggests miR-145 is dispensable for embryonic development and maintaining basic function of smooth muscle [121]. Further investigation of the role of miR-145 in the context of vascular injury discovered some intriguing discrepancies. After wire injury of blood vessels, miR-145 was found to be dramatically decreased, and restoration of miR-145 with adenovirus delivery inhibited the neointimal hyperplasia [123]. It was expected that loss of miR-145 would result in more dramatic neointimal formation. Paradoxically neointimal hyperplasia, secondary to vessel injury, was completely inhibited after the wire injury in miR-145 knockout mice [121]. Similarly, in a myocardial infarction model, the restoration of miR-145 restored the blood flow by promoting the growth of collateral coronary arteries [126]. These
apparently paradoxical findings indicate that miR-145 may exert some unveiled functions in smooth muscle cells.

**miR-145: a TGFβ signaling inhibitor?**

Smooth muscle cells are characterized by their high plasticity, which is tightly regulated by local environmental cues [21,23,24]. In adult arteries, the endothelial-cell tube is covered by multiple layers of highly differentiated, contractile and quiescent vascular smooth muscle cells. To build and maintain a functional vessel, endothelial cells instruct vascular smooth muscle cell differentiation through heterotypical cell contact and secreted factors [6,7]. In certain pathological scenarios, vascular smooth muscle cells undergo dedifferentiation, converting to a proliferating, migratory and synthetic phenotype [184]. This phenotypic switch can be either beneficial such as repopulating the injury sites, or harmful, in restenosis for example. Of the many factors regulating smooth muscle differentiation, TGFβ signaling plays a critical role, as revealed by transgenic mouse studies [133-137]. TGFβ signaling superfamily is an evolutionarily conserved cytokine family and has multifaceted roles in regulating cell function. Once bound to TGFβ ligands, the constitutive active TGFβ receptor II phosphorylates TGFβ receptor I, following by the activation of downstream signaling cascades, e.g. SMAD, p38 MAPK, ERK and JNK, which interact and regulate other pathways [128-131]. Mutations in TGFβ family have been associated with vascular diseases, such as aneurysm, atherosclerosis, arteriovenous malformations (AVM) and other cardiovascular diseases [129,130]. Furthermore, TGFβ signaling is also implicated in regulating synthesis,
deposition and degradation of ECM [153,154]. On one hand, TGFβ directly induces elastin, type I and type III collagen expression and crosslinking [155-161]. On the other hand, TGFβ also can upregulate MMP2 and MMP9, which degrades the ECM [168,169]. The complexity of TGFβ signaling pathways indicates that the functional outcome of TGFβ signaling in smooth muscle cells is largely dependent on its surrounding context.

In this study, I show that miR-145 directly targets TGFBR2, which has been found involved in regulating matrix synthesis in the smooth muscle [232,233]. The ECM genes induced by TGFβ were attenuated by the overexpression of miR-145. Interestingly, the smooth muscle differentiation markers induced by TGFβ were further upregulated by miR-145. This selective regulation of TGFβ signaling could be partially explained by its repressive effect on TGFBR2. Like the other components of TGFβ signaling, loss of TGFBR2 resulted in decreased differentiation markers in smooth muscle cells. However, TGFBR2 mutant mice have decreased expression of matrix genes [232,233]. Therefore, miR-145 may specifically repress matrix genes by targeting TGFBR2. An alternative explanation is that the miR-145 directly promotes smooth muscle differentiation by bypassing its inhibitory effect on TGFβ signaling. It has been shown that miR-145 represses KLF4 and KLF5 to remove the inhibition of smooth muscle differentiation, and the repression mechanism does not depend on TGFβ signaling [113,123,125]. Therefore, miR-145 and TGFβ may have an additive effect on smooth muscle differentiation. One common characteristic of miRNA is the huge number of their targets and their subtle effects on each individual target. It is very likely that miR-145
directly mediates genes which regulate matrix synthesis. I compared the phosphorylation level of SMAD2 and p38 MAPK in primary aorta smooth muscle cells isolated from wildtype or miR-145 mutant mice, and revealed no significant difference (Figure 46). This result supports the notion that miR-145 may directly exert its function on the matrix genes without changing the activity of the TGFβ pathway.

TGFβ has been known to have double-edged effects in many scenarios [129,240]. For example in carcinogenesis, TGFβ inhibits primary tumor growth at early stages, but stimulates tumor progression at later stages [241,242]. In the context of blood vessels, aberrant TGFβ activity has been implicated in vascular diseases, such as aneurysm and restenosis [130,240]. In my model, the progression of pathological phenotypic switching of smooth muscle cells, TGFβ plays a beneficial role at early stages by increasing differentiation and inhibiting proliferation (Figure 48). At this stage, smooth muscles still express a high level of miR-145. Removing miR-145 expression at this early stage releases the TGFβ signaling to further prevent smooth muscle phenotypic switching. This may explain why the miR-145 knockout mice do not form neointimal hyperplasia after the vascular injury. When the disease further progresses, TGFβ signaling becomes detrimental by promoting smooth muscle cell migration and matrix deposition (Figure 48). At this late stage, miR-145 level is low, and restoration of it can both inhibit the TGFβ signaling and directly induce the differentiation of smooth muscle cells. My study demonstrates the role of miR-145 in the regulation of TGFβ signaling in smooth muscle cells. Considering the complex role of TGFβ in smooth muscle cells, miR-145 may be a promising tool to selectively regulate TGFβ-related diseases,
especially by suppressing detrimental ECM accumulation caused by aberrant TGFβ signaling.

To evaluate whether miR-145 regulates matrix synthesis is involved in diseases, a cardiac fibrosis mouse model was utilized. Low concentration of AngII was infused to mice for a short time (2 weeks), and there was no obvious change of mean arterial pressure after AngII infusion. Any phenotype observed here was not secondary to the change of smooth muscle cell contraction. Although miR-145 expression is low in heart, it has been shown to have similar effects in cardiac fibroblasts compared to vascular smooth muscle cells [239]. Here, we show that loss of miR-145 significantly increased collagen deposition in heart (Figure 44). Meanwhile, there was no change of differentiation markers. These results suggest that miR-145 specifically regulates TGFβ-dependent matrix synthesis in heart. Considering AngII induces ECM synthesis and deposition by triggering TGFβ signaling independent of TGFBR2 [234], miR-145 is very likely directly regulating matrix synthesis through unidentified targets. My initial data indicate that miR-145 can function as a specific inhibitor of TGFβ-dependent matrix synthesis in cardiovascular diseases. miR-145 has been found limiting tumor growth, I will be interesting to investigate whether miR-145 regulates TGFβ signaling that contributes to its anti-tumor effects.

**Notch signaling guides vascular smooth muscle cell function**

Upon the heterotypic cell-cell interaction, Notch signaling is activated in vascular smooth muscle cells. As a key regulator of the smooth muscle function, activated Notch signaling controls multiple downstream targets to fulfill its roles. Once Notch signaling is
activated, SYNDECAN-2 is upregulated. This Notch-dependent SYNDECAN-2 functions as a coredceptor for Notch ligand and receptor, and facilitates the further amplification of Notch signaling. Therefore, Notch using SYNDECAN-2 forms a feed-forward loop to maintain activated Notch signaling.

Moreover, Notch induces miR-143/145 which has been shown to induce smooth muscle cell differentiation, and this is very likely one of the mechanisms how interaction with endothelial cells induces smooth muscle cell differentiation. Beyond regulating differentiation, miR-145 specifically inhibits TGFBR2 and matrix synthesis in vascular smooth muscle cells in vitro and in vivo. Furthermore, activation of TGFß signaling via angiotensin II infusion revealed a pronounced fibrotic response in the absence of miR-145. These data suggest that Notch-regulated miR-145 acts to suppress TGFß-dependent ECM accumulation and fibrosis without changing TGFß-induced smooth muscle cell differentiation.
Figure 47. TGFβ downstream signaling is slight changed in miR-145 mutant smooth muscle cells. Primary smooth muscle cells from wildtype and miR-145 mutant mice were serum starved for 24 hours and followed by TGFβ1 treatment for 0, 20 minutes, 1 hour, 6 hours and 24 hours. Western blot was performed to detect the expression pattern of phospho-Smad2 and phosphor-P38.
Figure 48. Model illustrating miR-145 regulating TGFβ signaling during the smooth muscle cell phenotypic switching process.
References


