An Investigation of Two Notch Regulated Genes in the Cardiovascular System

Undergraduate Research Thesis

Presented in partial fulfillment of the requirements for graduation *with honors research distinction* in Chemistry in the undergraduate colleges of The Ohio State University

By

Kristen Dammeyer

The Ohio State University
May 2018

Project Advisor: Dr. Brenda Lilly, Department of Pediatrics

Introduction:

Notch Signaling in the Vasculature

The Notch Signaling Pathway

The Notch signaling pathway plays a role in vascular development and physiology, including a pivotal role in the regulation of vascular smooth muscle cell phenotypes[1]. Notch receptors are evolutionarily conserved cell surface receptors that interact with ligands on nearby cells via direct cell-to-cell contact[2,3]. Mammals express four Notch receptors (Notch1-4) and five Notch ligands (Jagged1 and 2, Delta-like ligand 1, 3, and 4). Both ligands and receptors are Type-1 transmembrane proteins containing long extracellular domains consisting of epidermal growth-factor like repeats. The extracellular domain serves as a binding platform and is the site of receptor-ligand interactions. In the vasculature, Notch2 and Notch3 in smooth muscle cells and Jagged1 in endothelial cells are the predominantly expressed receptors and ligands, respectively[4,5].

The Notch signaling pathway operates through juxtacrine signaling. Ligands present on the cell membrane of endothelial cells interact with Notch receptors located on the cell membrane of smooth muscle cells. Ligand- receptor interactions result in proteolytic cleavage of the Notch intracellular signaling domain catalyzed by the gamma secretase complex. Cleavage frees the Notch intracellular signaling domain which, due to the presence of a nuclear localization signal, translocates to the nucleus. Nuclear translocation of the intracellular signaling domain results in the formation of a complex with the RBPJ DNA binding protein; this formation process displaces the transcriptional repressors Ski-interacting protein, CBF1-interacting corepressor, KyoT2, SMRT-and HDAC-associated repressor, and histone deacetylases[6]. The resulting Notch intracellular signaling domain-RBPJ complex can then recruit transcriptional activators

such like MAML1 and acetyltransferase to activate the expression of Notch target genes. Thus, the Notch intracellular signaling domain acts as a transcriptional co-factor. Notch-activated genes that are highly expressed include beta helix-loop-helix proteins like those of the HES/ HEY family which act as transcriptional repressors[7].

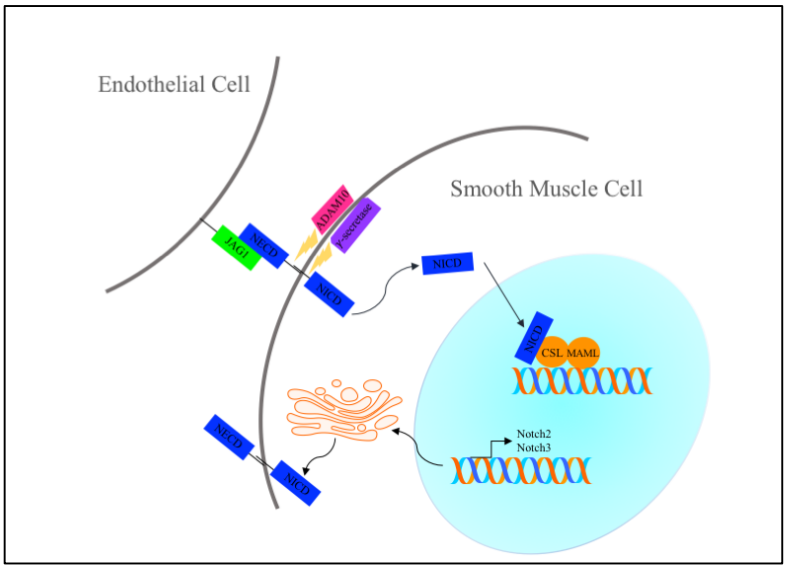

Figure 1. Depiction of the canonical Notch signaling pathway.

The cardiovascular system consists of the heart and an expansive network of blood vessels[8]. Blood vessels in the cardiovascular system are composed of an inner endothelial cell tube surrounded by one or more layers of vascular smooth muscle cells. Endothelial cells regulate the exchange between the bloodstream and surrounding tissues. Endothelial cells also translate hemodynamic cues into signals sent to smooth muscle cells, which then respond accordingly – by constriction or dilation[8].

Communication between endothelial cells and smooth muscle cells is vital for proper formation and function of the vasculature, and the Notch signaling pathway is one such method of communication. Generally, the roles of Notch signaling include regulation of artery-vein specification and differentiation, regulation of endothelial tip cell differentiation during angiogenesis, influence over tumor angiogenesis, and regulation of physiological responses of vascular smooth muscle cells[9]. Mutations of components of the Notch signaling pathway have also been associated with a myriad of diseases

including Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy, Alagille Syndrome, Infantile myofibromatosis, and childhood pulmonary arterial hypertension[10–16]. Most pertinent to the current study is the role of Notch signaling in controlling vascular smooth muscle cell phenotypes. Vascular smooth muscle cells serve to control blood flow and regulate vessel permeability, among other functions. For example, endothelial cells respond to hemodynamic cues and signal smooth muscle cells to contract or dilate accordingly. Uniquely, vascular smooth muscle cells are not terminally differentiated. Rather, these cells can adapt their expression patterns and resultant activities in response to a variety of cues. The ability of vascular smooth muscle cells to have a variety of different functions in response to external cues is known as phenotype switching or plasticity[17–19].

In adult mammals, the vasculature must actively maintain homeostasis as well as be able to respond to vascular injury. Plasticity of vascular smooth muscle cells, as regulated at least in part by the Notch signaling pathway, is key to homeostasis and injury response in the vasculature. There is a spectrum of states in which the vascular smooth muscle cell can exist in order to achieve proper vessel function. A differentiated smooth muscle cell is thought to express contractile proteins and to be quiescent. A proliferative vascular smooth muscle cells is mitotically active and is nominally considered dedifferentiated[17]. The synthetic vascular smooth muscle cell is characterized by its contribution to extracellular matrix synthesis through production of proteins such as collagen[20,21]. Notch signaling also influences vascular smooth muscle cell survival and apoptosis in response to stressors as well as migration during vessel development and response to vascular injury[1,22]. The current study will investigate how

the protein Cytoglobin and the micro RNA miR-145 are regulated by the Notch signaling pathway to influence vascular smooth muscle cell phenotype and contribute to cardiovascular form and function.

Cytoglobin

Cytoglobin is a hexacoordinate hemoglobin that is widely expressed in a myriad of tissues. As a member of the globin family, it is able to bind gaseous ligands such as oxygen, carbon monoxide, and nitric oxide. The ability to bind gaseous ligands, much like the other globin family members hemoglobin and myoglobin, suggests an important role for Cytoglobin in maintaining cellular homeostasis. Cytoglobin has recently been implicated in vascular function and stress regulation, and has potential roles in the modulation of nitric oxide metabolism. Nitric oxide is central to maintaining normal blood vessel physiology via its role in regulating smooth muscle cells tone, signaling between endothelial cells and smooth muscle cells, and conducting physiological responses. Given the potential implications of Cytoglobin in vascular smooth muscle cell function, this study sought to examine the regulation and function of Cytoglobin. We identified Cytoglobin as a gene induced in vascular smooth muscle cells by co-cultured endothelial cells. Our data show that Cytoglobin expression is induced in smooth muscle cells through endothelial cell-dependent Notch signaling and that smooth muscle cell receptors are necessary for Cytoglobin expression. Examining the function of Cytoglobin in smooth muscle cells indicates a link to managing vascular-derived stress. Our findings support the notion that endothelial cells induce Cytoglobin expression in smooth muscle cells as a means to regulate nitric oxide bioavailability in blood vessels. In all, our results reveal a novel mechanism in which Notch signaling

contributes to vascular function through regulation of a gene implicated in nitric oxide availability.

miR-145

Cardiac fibrosis, or cardiac scar tissue, develops as a result of unremitting, pathological tissue repair following cardiovascular insult or injury. Nearly all forms of cardiovascular disease are associated with cardiac fibrosis. A major cellular player in cardiac fibrosis is the cardiac fibroblast. The cardiac fibroblast is a major cell type in the heart that provides structural support and works to maintain homeostasis of the myocardial extracellular matrix. Upon stress, injury, environmental stimuli, or pressure overload, the cardiac fibroblast is activated and transdifferentiates into the myofibroblast. Myofibroblasts secrete and remodel the extracellular matrix as a part of a normal healing regime after cardiac injury or insult. When myofibroblasts accumulate, persist, and deposit excessive extracellular matrix, the process becomes pathological leading to fibrosis and progression of heart disease. Several signaling pathways have been implicated in myofibroblast activation. We propose that miR-145 functions in cardiac fibroblasts to suppress cardiac fibrosis by inhibiting various mediators of the fibrotic cellular phenotype such as Transforming Growth Factor Beta (TGF-ß) and p38 Mitogen Activated Protein Kinase Signaling (MAPK). Interest in miR-145's role in cardiac fibrosis developed in the Lilly Lab as a result of findings demonstrating that loss of miR-145 exacerbates angiotensin II-induced fibrosis. These studies focused on miR-145 expression in smooth muscle cells. Induction of miR-145 in smooth muscle cells occurred upon co-culture with endothelial cells, was augmented by Notch signaling, and was reduced in Notch receptor deficient cells. The current study seeks to characterize

the phenotype of novel RFP-miR-145 transgenic mice, which will enable the studied mice strains to be used to study the role that miR-145 plays in modulating cardiac fibrosis via posttranscriptional regulation the cardiac fibroblast.

Cytoglobin Expression is Induced in Vascular Smooth Muscle Cells Through Endothelial Derived Notch Signaling:

Implications of a Protective Role in Blood Vessel Function

Introduction

Cytoglobin, so named for its ubiquitous expression in a variety of tissues[23], is the newest member of the globin family, a class of proteins implicated in the storage and transportation of oxygen. Specifically, Cytoglobin is a hexacoordinate heme protein with the ability to bind gaseous ligands such as oxygen, nitric oxide, and carbon monoxide. It is detected ubiquitously in a variety of tissues, but it is predominantly found in fibroblast-related cell lineages[24–26]. Primarily, Cytoglobin is a cytoplasmic protein, although it has been detected in the nucleus as well[27]. Although the precise functional role of Cytoglobin is not yet well defined, the hemoprotein is generally thought to play a cytoprotective role, participating in oxygen storage and transport and nitric oxide scavenging to protect the cell from oxidative stress[28]. Cytoglobin has been found to be a stress related gene that is up-regulated during oxidative stress. Additionally, it is known that Cytoglobin protects cells from injury induced by oxidative stress and from the harmful consequences of excess reactive oxygen species in the cell[23].

Several diseases have been associated with Cytoglobin, including multiple modalities of vascular injury, fibrosis, and cancer[29–32]. In vascular injury models, it has been demonstrated that Cytoglobin regulates the hyperplastic response[(1)] by providing protection from unnecessary programmed cell death through a redox sensitive signaling network[30]. Further, Cytoglobin is implicated in the regulation of vascular tone through its activity as an NO dioxygenase[(2)] and through its role in the management of NO metabolism[31]. Supporting Cytoglobin's connection to NO and ROS, Cytoglobin knockout

---

[(1)] Hyperplasia is an increase in the number of normal cells in a tissue or organ.
[(2)] An enzyme that catalyzes the conversion of nitric oxide to nitrate.

mice experienced multiple spontaneous organ abnormalities in both young and aged mice along with increased NO metabolites and ROS production in the serum[32]. The apparent ability of Cytoglobin to regulate the consumption and metabolism of NO and ROS indicates an important role for the hemoprotein in vascular homeostasis that could have clinical implications and calls for a closer look at its regulation.

Cytoglobin is widely expressed, but evidence indicates that its expression is tightly regulated and responds to environmental cues. Analyses of the Cytoglobin promoter region reveal that a GC rich promoter region is located between -1113 and -10 relative to the translation start site. The promoter region contains binding sites for c-Ets-1 and Sp1. C-Ets-1 is a member of the Ets family and plays a critical role in the gene expression regulation and development of hematopoietic cells. Sp1 is member of family of proteins that are essential for transcription activation and therefore expression regulation of housekeeping, tissue specific and viral genes[33]. Further investigation of Cytoglobin's promoter region resulted in the identification of hypoxia response element motifs in the 5' untranslated region. Hypoxia-inducible factor-1 (HIF-1) is able to bind to this hypoxia response element. Under hypoxic conditions, the HIF-1a protein is stabilized, allowing it to bind to HIF-1b and form the HIF-1 transcription complex. It is these elements rather than the c-Ets-1 and Sp1 elements that are proposed to be responsible for the activation of Cytoglobin expression under hypoxic conditions[34].

Other reports demonstrate that Cytoglobin is transcriptionally regulated by oxidative stress, with upregulation occurring under hypoxic conditions possibly mediated by the HIF-1a transcription factor[23,35]. HIF-1a, along with other transcription factors such as AP-1 and NFAT, has an evolutionarily conserved binding element in the upstream

region of the Cytoglobin gene, indicating a role in regulation. AP-1 and NFAT transcription factors are linked to calcineurin, a calcium-calmodulin activated phosphatase involved in the signaling pathways that regulate cardiac genes and subsequent processes such as cardiac remodeling, hypertrophy, apoptosis and metabolism. Both hypoxia and manipulations of calcineurin alter AP-1 and NFAT interactions with Cytoglobin resulting in modulation of its expression[36]. Cytoglobin has also been shown to be transcriptionally regulated by ΔNp63, an indispensable factor for maintaining a cell's proliferative abilities[37].

Beyond transcriptional regulation, Cytoglobin expression has been found to be under the influence of growth factors as well. One such growth factor is fibroblast growth factor 2, which was demonstrated to be a strong inducer of Cytoglobin expression in hepatic stellate cells[38]. Returning to the vasculature, Cytoglobin is expressed in mature smooth muscle cells. Interestingly, Cytoglobin is expressed in contractile smooth muscle cells but experiences downregulation upon dedifferentiation[30]. Despite evidence of these expression patterns in vascular smooth muscle cells, vasculature-specific transcriptional mediators have not been thoroughly investigated.

The vasculature is comprised of two different types of cells, endothelial cells and smooth muscle cells. Proper communication between these cells is vital for ensuring correct blood vessel function and formation throughout development and into adulthood[39,40]. The Lilly Lab has extensively studies this communication and has demonstrated that the Notch signaling pathway plays a key role in smooth muscle cell-endothelial cell interaction. In vitro assays done in the lab show that the Notch signaling

pathways is activated in smooth muscle cells cocultured with endothelial cells. Various proteins have been identified as being upregulated under these coculture conditions as well. Cytoglobin is one such protein. This project sought to test the central hypothesis that Cytoglobin is regulated by Notch signaling and functions in vascular homeostasis. Evaluation of the function and regulation of Cytoglobin will contribute to the current understanding of cellular communication mechanisms and consequences in the vasculature and may provide new insight into therapies for vascular diseases.

Specifically, the current study aims to investigate the regulation of Cytoglobin by the interactions of endothelial and smooth muscle cells. The role of the Notch signaling pathway on Cytoglobin expression in smooth muscle cells via endothelial cells contact is directly tested. In addition, the function of Cytoglobin in vascular smooth muscle cells is explored.

In this study, we demonstrate that Cytoglobin expression is induced in smooth muscle cells by endothelial cells in coculture. We further demonstrate that the endothelial dependent induction of Cytoglobin is dependent on the Notch signaling pathway and Notch signaling is required to maintain Cytoglobin expression in smooth muscle cells. Additionally, we show that Cytoglobin is linked to hypoxia as is consistent with previous research, and that modulation of Cytoglobin levels can result in the modulation of nitric oxide levels in the cell. Taken together, these data uncover a novel mechanism by which endothelial cells regulate the expression of Cytoglobin in smooth muscle cells, and reveal a putative function for this regulation – influencing smooth muscle cell function via mediators of vascular reactivity.

Materials and Methods

Cell Culture

Primary cultures of human aortic smooth muscle cells (HAoSMCs) and human coronary artery smooth muscle cells were purchased from Lonza and grown in Dulbecco's Modified Eagle's Medium (DMEM) (Mediatech, Inc.) and were supplemented with 5% fetal bovine serum (FBS) (Hyclone), 2mM glutamine, 1 mM sodium pyruvate and 100 U/ml penicillin-streptomycin. Human aortic endothelial cells (HAECs) and human coronary artery endothelial cells (HCAECs) were purchased from Lonza and cultured in EBM-2 supplemented with the bullet kit as recommended (Lonza). Primary cells between passages 6 and 10 were used for all experiments. All cultures were maintained in a humidified 5% CO_2 incubator at 37°C.

For coculture smooth muscle cells were plated at a density of 1 x 10^5 cells/ well in a 6-well plate or at a density of 5 x 10^4 cells/ well in 12-well dish. After adhesion, endothelial cells were added at 1 x 10^5 cells/ well in a 6-well plate or at 5 x 10^5 cells/ well in a 12- well plate. Cells were then incubated 24 to 48 hours prior to cell separation and processing. All co-culture and alone control experiments were conducted in EBM-2 media supplemented with the bullet kit. To separate HAoSMCs from HAECs, anti-PECAM1-conjugated Dynabeads (Invitrogen) were used according to the manufacturer's instructions[40]. All co-culture experiments were performed in EBM-2 media supplemented with the bullet kit. Notch Inhibitor, DAPT (*N*-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester, Calbiochem) was added to specified wells after cell adhesion. Donor was added to specified wells at

a final concentration of 0.05 micromolar; CPITO, an NO inhibitor, at 0.013 micromolar; H_2O_2 at 0.1 micromolar; and $CoCl_2$, a hypoxia mimic, at 0.05 micromolar.

Quantitative Real-Time PCR (qPCR)

Total RNA was isolated from cells using RiboZol RNA Extraction Reagent (Ampresco) according to the manufacturer's instructions. RNA was reverse transcribed with M-MLV Reverse Transcriptase (Promega) to generate cDNA. Real-Time PCR was performed using the StepOne Real Time PCR system by Applied Biosystems. The reporter dye was the Power SYBR Green PCR Mastermix and 50 ng of cDNA template was used. The fold difference in mRNA levels of the target gene was calculated using the $\Delta\Delta$CT method with normalization to RPL13A mRNA levels. The following are the primer sequences: CYGB, 5'-AAC ACT GTC GTG GAG AAC CTG CAT -3' (forward) and 5' –TGA AGT ACA CCG GTT CCA CCT TGT -5' (reverse); NOTCH3, 5'- GAG CCA ATG CCA ACT GAA GAG -3' (forward) and 5'-GGC AGA TCA GGT CGG AGA TG -3' (reverse); NOTCH2, 5'-ACA GTT GTG TCT GCT CAC CAG GAT -3' (forward) and 5'-GCG GAA ACC ATT CAC ACC GTT GAT -3' (reverse); RPL1A, 5'-CTT GTG AGT GGG GCA TCT G -3' (forward) and 5'- CCC TGT GTA CAA CAG CAA GC -3' (reverse); NOX4, 5' –CTGCTGACGTTGCATGTTTC – 3' (forward) and 5'-CTGAGAGCTGGTTCGGTTAAG -3' (reverse).

siRNA Transfection

HAoSMCs were plated in either a 6-well plate at 1 x 10^5 cell/ well or in a 12-well plate at 5 x 10^4 cells/well. After 24 hours, cells were transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen). Efficiency of knockdown was assessed using qPCR and Western Blot. Control Silencer Select Negative Control siRNA was

purchased from Invitrogen. NOTCH3 siRNA was synthesized by IDT: 5'-AAC UGC GAA GUG AAC AUU G, and used as previously described. NOTCH2 siRNA was purchased from Qiagen (Assay ID:GS4853) and CYGB siRNA from ThermoFisher (Assay ID:s41571). All siRNAs were transfected at 100 nM. After a 24-hour transfection period, cells were co-cultured with endothelial cells for 48 hours, separated, and collected for qPCR analysis and Western blotting.

Western Blotting

Cells were homogenized with RIPA buffer containing 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% SDS, and protease inhibitors (Sigma). Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Fischer). Equal amounts of total cellular protein were loaded onto a 12.5% SDS-Polyacrylamide (SDS-PAGE) gels and were transferred to nitrocellulose blotting membranes (GE Healthcare). Membranes were incubated for 1 hour at room temperature in 5% nonfat dry milk in 1x PBS. Membranes were then incubated overnight at 4° C with primary antibody against CYGB (Rabbit PolyAb Anti-CYGB, 1:1000) (Proteintech 13317-1-AP) in 2.5% nonfat dry milk in 1x PBS. Membranes were incubated for one hour at room temperature with primary antibody against β-TUBULIN 1 (Mouse Anti-tubulin, 1:20,000) (Sigma, T7816). Secondary antibodies were incubated 1 hour at room temperature (Donkey anti-Rabbit IR-Dye 680RD, Donkey anti-Mouse IR-Dye 800CW; LI-COR). Proteins were detected by LI-COR Odyssey Infrared Imaging System and quantified using Image Studio Software by normalizing to β-TUBULIN 1 expression.

Statistical Analysis

Data analyses were performed using GraphPad Prism. Comparisons between data set were made using One-way ANOVA. Differences with $P < 0.05$ were considered significant. Data are representative of at least three independent experiments.

Results

Cytoglobin is Induced in Smooth Muscle Cells by Cocultured Endothelial Cells

In order to examine the functional relationship between vascular smooth muscle cells and endothelial cells, our laboratory previously examined expression profiles of genes implicated in vascular structure and function[20,40–43]. In the current study, we measured the expression of Cytoglobin in smooth muscle cells that were either cultured alone or in the presence of endothelial cells. The culturing of smooth muscle cells and endothelial cells in direct contact is referred to as coculture in this study. Human aortic and coronary artery smooth muscle cells were cultured for 48 hours followed by cell separation using endothelial cell-specific Pecam-1 conjugated beads. Quantitative (q)PCR was performed to measure levels of Cytoglobin RNA (Figure 1A). Transcript levels of Cytoglobin increased approximately 10-fold in human aortic smooth muscle cells that were cocultured with human aortic endothelial cells. Likewise, human coronary artery smooth muscle cells demonstrated a significant, yet less pronounced, increase in Cytoglobin expression after coculture with human coronary artery endothelial cells. As seen in Figure 1A, the difference in fold-increase in Cytoglobin between aortic and coronary smooth muscle cells may be explained by the greater basal expression of Cytoglobin in coronary smooth muscle cells cultured independently of endothelial cells. This suggests that, in coronary artery smooth muscle cells, expression of Cytoglobin is

endothelial cell independent to a considerable extent. For a more complete analysis, Cytoglobin expression was analyzed via detection of Cytoglobin protein by western blot in human aortic smooth muscle cells. The pattern of protein expression detected was consistent with the observed endothelial cell-dependent induction of Cytoglobin transcript. An approximately 3-fold increase in Cytoglobin protein was observed after 24-hour co-culture, which was increased to approximately 6-fold after 48 hours (Figure 1B, C). These data demonstrate that endothelial cells are capable of inducing the expression of Cytoglobin RNA and protein in vascular smooth muscle cells, suggesting a novel means through which endothelial cells govern vascular function.

To further investigate the mechanism by which Cytoglobin expression is induced in smooth muscle cells by contact with endothelial cells[(3)], a Notch inhibitor was utilized. Smooth muscle cells were incubated in the presence of DAPT which is a Notch inhibitor that blocks the activity of gamma secretase, the enzyme responsible for cleaving the Notch intracellular signaling domain. Effectively, DAPT prevents the Notch intracellular signaling domain from translocating to the nucleus where it acts as a transcriptional cofactor[1]. Incubation with DAPT resulted in the loss of induction of Cytoglobin by cocultured endothelial cells (Figure 2). This result suggests that the endothelial-cell dependent expression of Cytoglobin in smooth muscle cells is regulated by Notch signaling.

It is known that Cytoglobin is regulated by hypoxia[23]. Nitric oxide levels and nitric oxide signaling have a role in cellular responses to hypoxia[44]. Because endothelial cells

---

[(3)] Transwell experiments were performed in order to demonstrate that induction was mediated by cell-cell contact rather than by an endothelial cell-derived secreted factor[56].

produce ample nitric oxide, we tested whether it might contribute to Cytoglobin expression in smooth muscle cells. Neither a nitric oxide donor (DETA-NONOate) nor a nitric oxide inhibitor (CPITO) had a significant effect on Cytoglobin expression compared to controls (Figure 3). Cobalt chloride is a hypoxia mimic through its stabilization of HIF (Hypoxia Inducible Factor), a protein hallmark of hypoxia that is stabilized under hypoxic conditions. Upon stabilization, HIF acts as a transcription factor for genes involved in angiogenesis, cell survival, metabolism and cell migration[45,46]. Upon culturing smooth muscle cells with the hypoxia mimic cobalt chloride, there was significant induction of Cytoglobin expression, as predicted.

Cytoglobin Expression in Smooth Muscle Cells is Regulated by Notch Signaling

Although Notch signaling has been implicated in smooth muscle cell differentiation[7], the role of Notch is much less defined in the context of vascular homeostasis. To explore this and the contribution of Notch receptors in Cytoglobin expression, we utilized siRNA to specifically knockdown Notch2 and Notch3 in human aortic smooth muscle cells (Figure 4A, B). Notch2 and Notch3 are the predominantly expressed Notch receptors in the vasculature and are important for normal vascular smooth muscle cell formation and function. Under coculture conditions, we observed that Notch2, but not Notch3, was necessary for endothelial cell-induced Cytoglobin expression (Figure 4C). Because Notch signaling itself promotes the expression of Notch receptors, expression of Notch2 and Notch3 were examined to confirm the efficacy of knockdown[(4)].

[(4)] Sufficiency of Notch2 and Notch3 to activate Cytoglobin expression was demonstrated via lentiviral expression of the Notch intracellular signaling domains NICD2 and NICD3 in the absence of endothelial cells[56].

Induction of Cytoglobin by Endothelial Cells Modulates Nitric Oxide

Within intact blood vessels, endothelial cells produce nitric oxide that diffuses to neighboring smooth muscle cells and asserts control over vascular functions such as proliferation and tone[47]. Recently, Cytoglobin has been implicated in nitric oxide metabolism[31,48,49]. It can be postulated that endothelial cells activate Cytoglobin expression in smooth muscle cells to modulate nitric oxide levels. In order to test whether or not Cytoglobin affects nitric oxide levels in smooth muscle cells, we first demonstrated the ability to knock down Cytoglobin expression by Cytoglobin specific siRNA in aortic smooth muscle cells cultured either alone or in the presence of endothelial cells. Efficacy of Cytoglobin ablation was confirmed at the transcript and protein level (Figure 5). This knockdown process was later used to investigate the difference between nitric oxide levels in smooth muscle cells cultured alone or in coculture with endothelial cells in the absence of Cytoglobin[(5)].

[(5)] Nitric oxide levels were tracked using the DAR-4M reactive dye. Results indicated that ablation of Cytoglobin resulted in increased nitric oxide, pointing to a role for Cytoglobin in regulating nitric oxide availability and thus contributing to nitric oxide function[56].

Figures

Figure 1

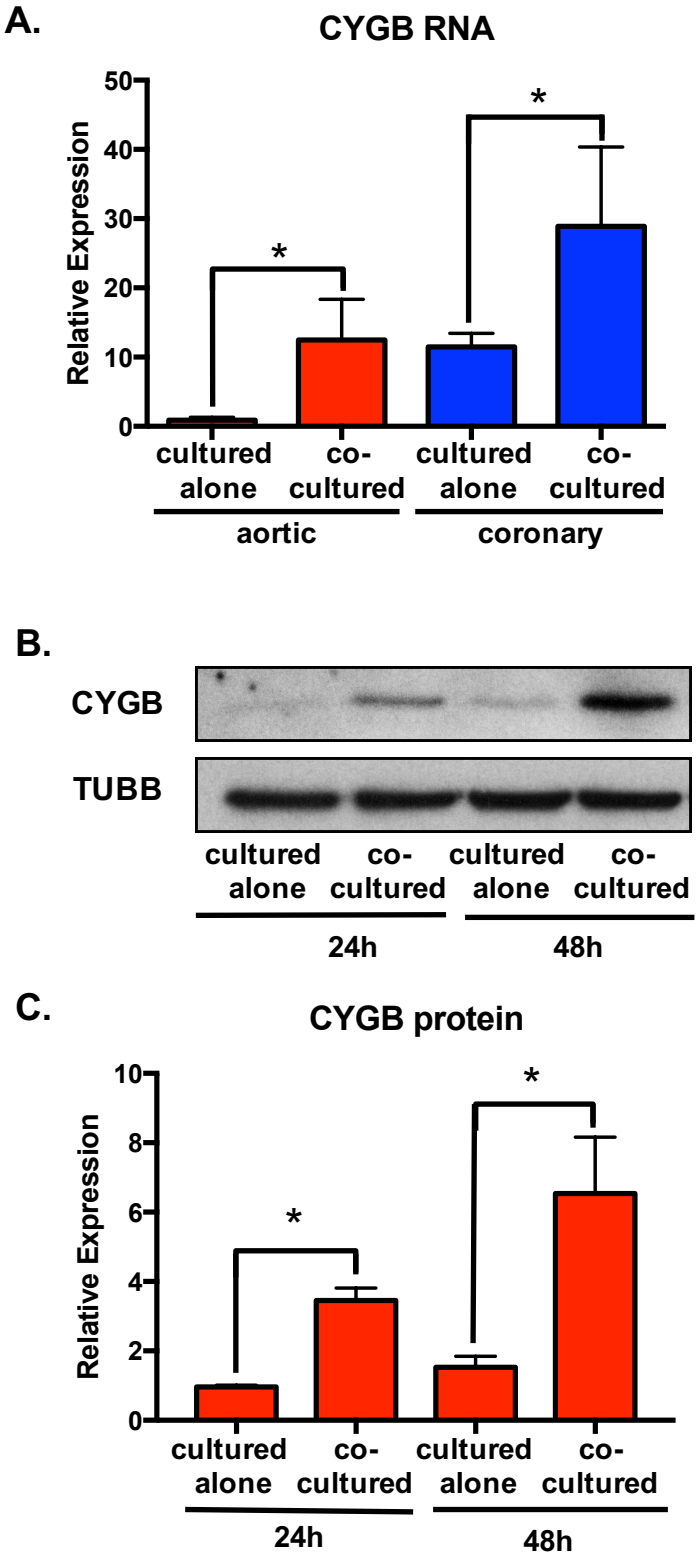

Figure 1. Cytoglobin expression is induced in smooth muscle cells by endothelial cells. (A) Cytoglobin expression in human aortic smooth muscle cells and human coronary artery smooth muscle cells in response to coculture with endothelial cells. Smooth muscle cells were cultured either alone or in coculture with aortic/ coronary endothelial cells. After 48 hours cell separation was performed to remove endothelial cells, RNA was isolated subjected to qPCR. (B) Protein lysate was isolated from human aortic smooth muscle cells that were cultured either alone or in coculture with aortic endothelial cells. After 24 and 48 hours, smooth muscle cells were separated and western blots were performed to detect Cytoglobin and Tubulin (TUBB) protein. (C) Cytoglobin protein quantified from three independent western blots. Cytoglobin protein levels were standardized with respect to Tubulin. *P < 0.05.

Figure 2

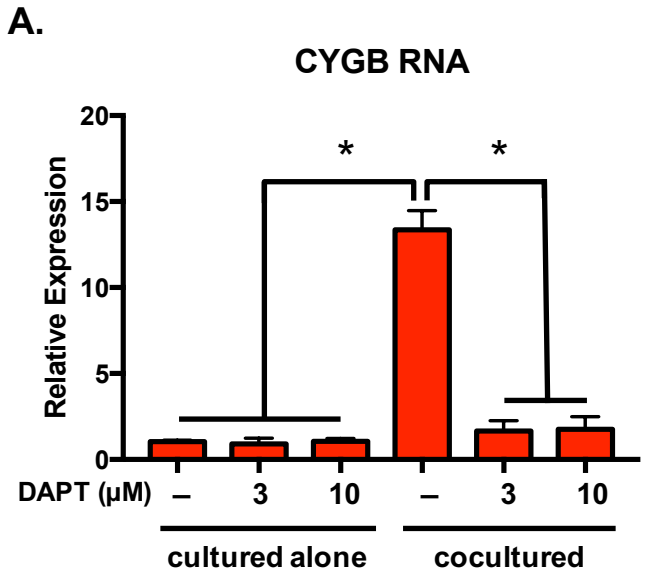

Figure 2. Cytoglobin expression is blocked by Notch inhibition (A) CYGB expression when treated with DAPT. Human aortic smooth muscle cells were cultured either alone or in co-culture with human aortic endothelial cells and in the presence of 3 μM, 10 μM or 0 μM DAPT. Smooth muscle cells were separated from endothelial cells prior to RNA isolation and qPCR was performed in order to determine the relative expression of CYGB RNA transcripts. * P < 0.05

Figure 3

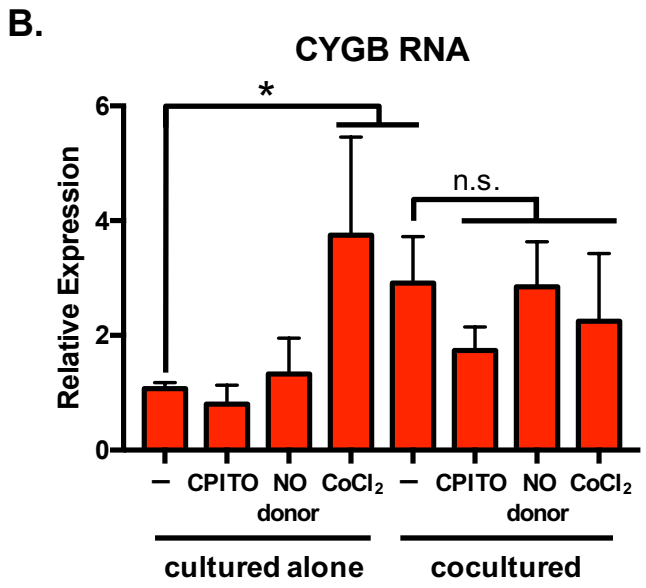

Figure 3. **Cytoglobin expression is influenced by reactive oxygen species and hypoxia mimics.** RNA was isolated from human aortic smooth muscle cells that were cultured either alone or in coculture with human aortic endothelial cells. Cells were not separated prior to analysis. Cells were subjected to treatment with nitric oxide donor (DETA-NONOate) and nitric oxide inhibitor (CPITO), and the hypoxia mimic $CoCl_2$. * $P < 0.05$, n.s. = not significant.

Figure 4

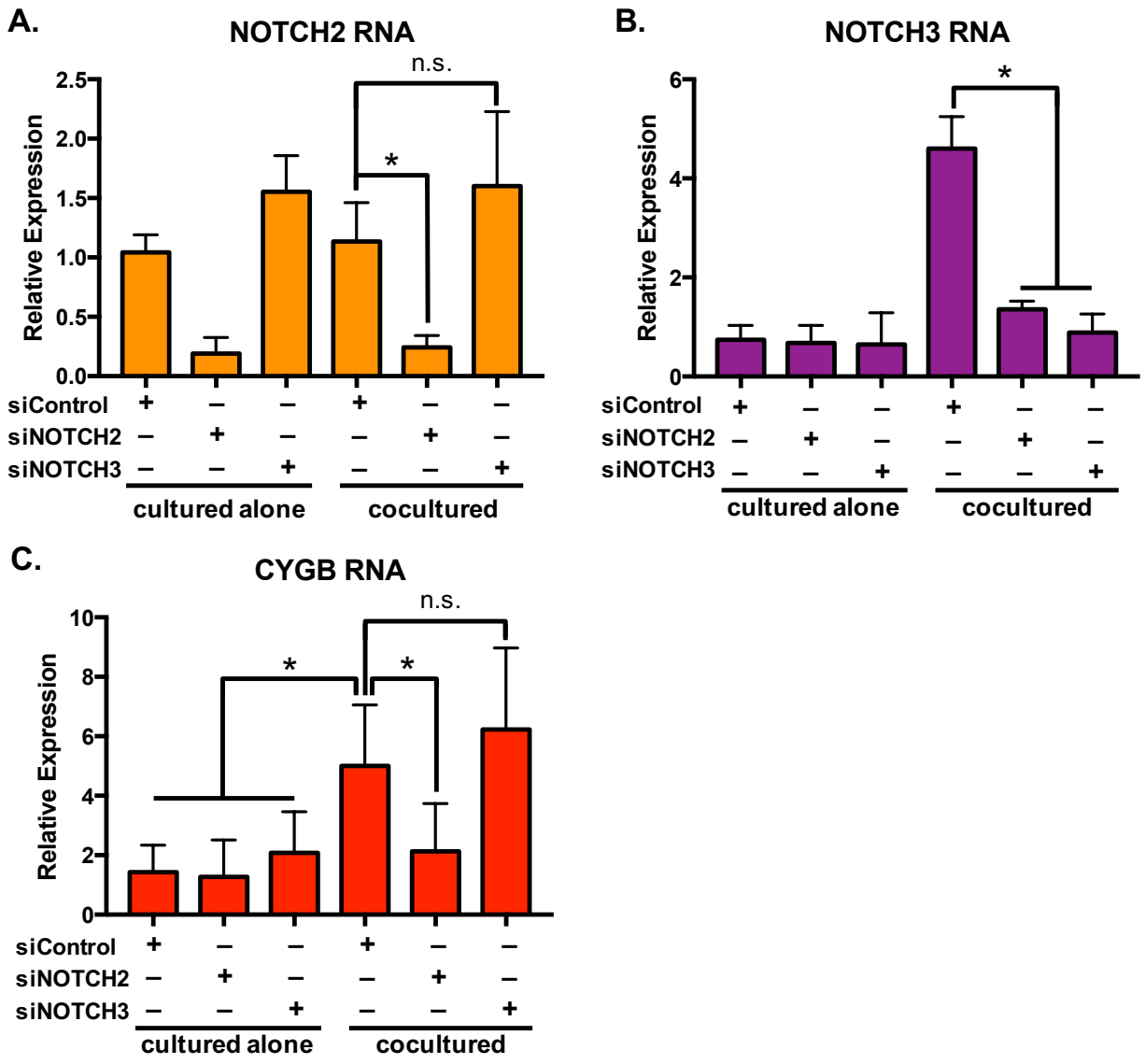

Figure 4. Cytoglobin expression is inhibited by siRNA. siRNA for Notch2 and Notch3 transcripts transfected into human aortic smooth muscle cells cultured alone or in coculture with endothelial cells. After 48 hours, cells were separated and RNA was isolated from smooth muscle cells. qPCR analysis was performed to assess Notch2 (A), Notch3 (B), and Cytoglobin (C), transcript levels under the different conditions. Notch2 and Notch3 were examined to assess efficiency of knockdown. Notch3 is known to be regulated by Notch signaling itself; therefore, a decrease is Notch3 transcript levels upon Notch2 as well as Notch3 siRNA knockdown is anticipated. Notch2, on the other hand, is not subject to the same regulation by the Notch signaling pathway. *P < 0.05, n.s. = not significant.

Figure 5

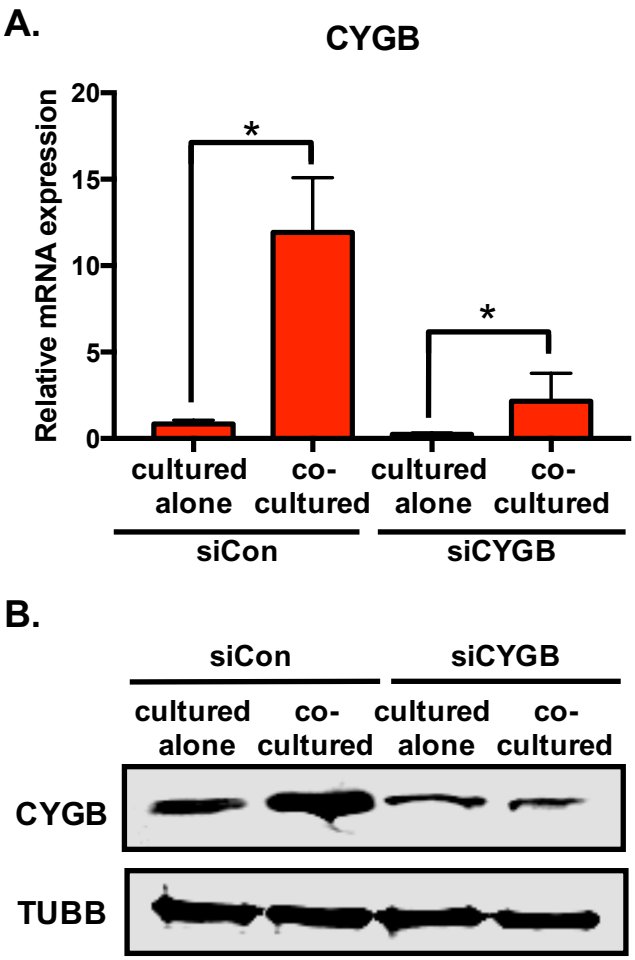

Figure 5. Knockdown of Cytoglobin expression by siRNA. Human aortic smooth muscle cells were cultured alone or in coculture with endothelial cells. Cells were transfected with either control or Cytoglobin specific siRNA. After 48 hours, smooth muscle cells were separated. (A) Cytoglobin transcript levels were measured by qPCR from isolated RNA. *P < 0.05. (B) Protein lysates were isolated from the separated smooth muscle cells and western blots were performed to asses Cytoglobin protein levels, with Tubulin (TUBB) as an internal control.

Discussion

Cytoglobin is a hexacoordinate hemoprotein and a member of the ancestral globin family which consists of respiratory heme-containing proteins. With other globin family members, Cytoglobin shares the ability to bind gaseous ligands such as oxygen, carbon monoxide and nitric oxide[24,27,28]. Cytoglobin expression has been demonstrated

in vascular smooth muscle cells, and Cytoglobin has been implicated in the processing of nitric oxide, a molecule that plays a critical role in vascular function[29]. The second major cell type comprising functional blood vessels, endothelial cells, is a major source of nitric oxide, primarily through the actions of endothelial nitric oxide synthase[47]. Nitric oxide is a key regulator of vascular tone, but the mechanisms by which it regulated have yet to be clearly delineated[47].

Our study found that endothelial cells can induce Cytoglobin expression in smooth muscle cells in coculture. These results demonstrate a regulatory role for endothelial cells in adjusting smooth muscle cell Cytoglobin levels. Further, we show that the Notch signaling pathway is specifically responsible for Cytoglobin induction in smooth muscle cells via endothelial cell contact.

The Lilly lab has previously demonstrated that endothelial cells possess the ability to alter smooth muscle cell gene expression profiles[40,42,43,50]. There are likely several processes and pathways that facilitate this communication between vascular endothelial and smooth muscle cells, but the Notch signaling pathway emerges as a key contributor. Data demonstrates that endothelial cell derived Notch signaling induces the expression of Notch3, syndecan-2, the microRNA 143/145 gene cluster, and smooth muscle cell contractile markers such as smooth muscle alpha actin, calponin, and smooth muscle 22[40,42,43,50–52]. The current study identifies Cytoglobin as another protein whose expression profile in smooth muscle cells is altered by endothelial cells. Results show that Notch signaling is necessary for the induction of Cytoglobin expression. Interestingly, specific knockdown of Notch2 and Notch3 identifies a unique role for the Notch2 receptor in the induction of Cytoglobin expression. There is no clear explanation

or significance of the differential role of the Notch2 versus the Notch3 receptor; however, previous studies have identified unique functions of the receptors in vascular smooth muscle cells. Specifically, the previous research suggests that Notch2 plays an anti-proliferative and Notch3 a pro-proliferative and pro-survival role in smooth muscle cell function[1,53].

In general, endothelial cells promote a differentiated, quiescent, and contractile smooth muscle cell phenotype. Evidence from this study reveals that endothelial cells induce Cytoglobin protein expression in smooth muscle cells through the Notch signaling pathway. Based on data shown presently, other experiments performed in our lab, and previous research, the ability of endothelial cells to induce Cytoglobin expression suggests that endothelial cells are able to influence the vascular smooth muscle cell's ability to process nitric oxide. For example, previous studies have identified a significant increase in nitric oxide metabolites in Cytoglobin deficient mice, linked the dysregulation of nitric oxide metabolism to Cytoglobin deficiency, and postulated the role of Cytoglobin in facilitating the vasodilatory function of nitrite under hypoxic conditions[32,54,55]. Consistent with this, other experiments performed in our lab show via direct measurement with DAR-4M reactive dye that nitric oxide levels are significantly increased in Cytoglobin deficient smooth muscle cells[56]. Consequently, endothelial cells appear to be able to mediate the capability of smooth muscle cells to metabolize nitric oxide. This finding is consistent with endothelial cells' important role in communicating with smooth muscle cells in the vasculature in order to ensure homeostasis and proper vascular function in response to environmental stimuli. Further studies are required to identify whether or not there exists a precise role for Cytoglobin

in contributing to vascular pathologies, and whether endothelial-cell derived Notch signaling contributes to smooth muscle cell pathologic phenotype modulation and disease progression.

miR-145:

Characterization of a Novel miR-145 Transgenic Mouse Line: Defining the Role of miR-145 in Cardiac Fibrosis

Introduction

Cardiac Fibrosis and the Cardiac Fibroblast

Heart failure is the final step in the progression of many cardiovascular diseases, and heart failure is characterized by interstitial fibrosis, chamber remodeling, and reduced ventricular compliance[57]. Heart disease is a significant global health problem in terms of morbidity and mortality as well as health care costs, with average annual costs estimated to be greater than $300 billion[58]. Nearly all etiologies of cardiovascular disease involve the dysfunction of cardiac fibroblasts, which engage in persistent, pathological tissue repair resulting in excessive extracellular matrix deposition and maladaptive scar tissue formation[57]. Upon injury or stress to the heart, cardiac fibroblasts transdifferentiate into a myofibroblast phenotype[59]. Myofibroblasts are essentially a fibroblast-smooth muscle cell hybrid characterized by their ability to secrete extracellular matrix, their ability to remodel tissue, and their contractile properties[60]. The activated myofibroblasts in the injured heart begin to accumulate and secrete extracellular matrix. Extracellular matrix deposition is proposed to act to preserve myocardial structure and functional integrity, which may otherwise be compromised due to the loss of injured and necrotic cardiomyocytes[61]. This normal and essential cardiac remodeling becomes pathologic when myofibroblasts accumulate and persist in depositing extracellular matrix beyond what is necessary for fibrotic scar formation. Pathologic cardiac remodeling by myofibroblasts can result in fibrosis and the progression of heart failure[62].

While persistent fibrotic healing is known to progress heart disease, it is challenging to treat due to an incomplete understanding of the molecular regulatory

pathways that govern fibrosis. The cardiac fibroblast has been implicated as having a major role in the pathologic fibrotic healing process, but more research must be done to understand the precise mechanistic role of cardiac fibroblasts. There are currently many signaling pathways under investigation for their role in regulating myofibroblast transformation. One such pathway is the canonical Transforming Growth Factor Beta (TGF-ß) signaling pathway involving the SMAD family transcription factors. In TGF-ß signaling, SMAD2/3 becomes phosphorylated and interacts with SMAD4. The complex then translocates to the nucleus where it is thought to induce transcription of myofibroblast genes[60]. Another pathway is the non-canonical TGF-ß signaling pathway which acts through Mitogen Activated Protein Kinase K (MAPKK) to affect the Serum Response Factor (SRF) transcription factor. SRF serves to mediate transcription of myofibroblast genes[60]. Additionally, roles for Angiotensin II signaling, Endothelin-1 signaling, the SRF MRTF-RhoA-GTPase pathway, and receptor protein channels have been implicated as summarized in Figure 1[60,63].

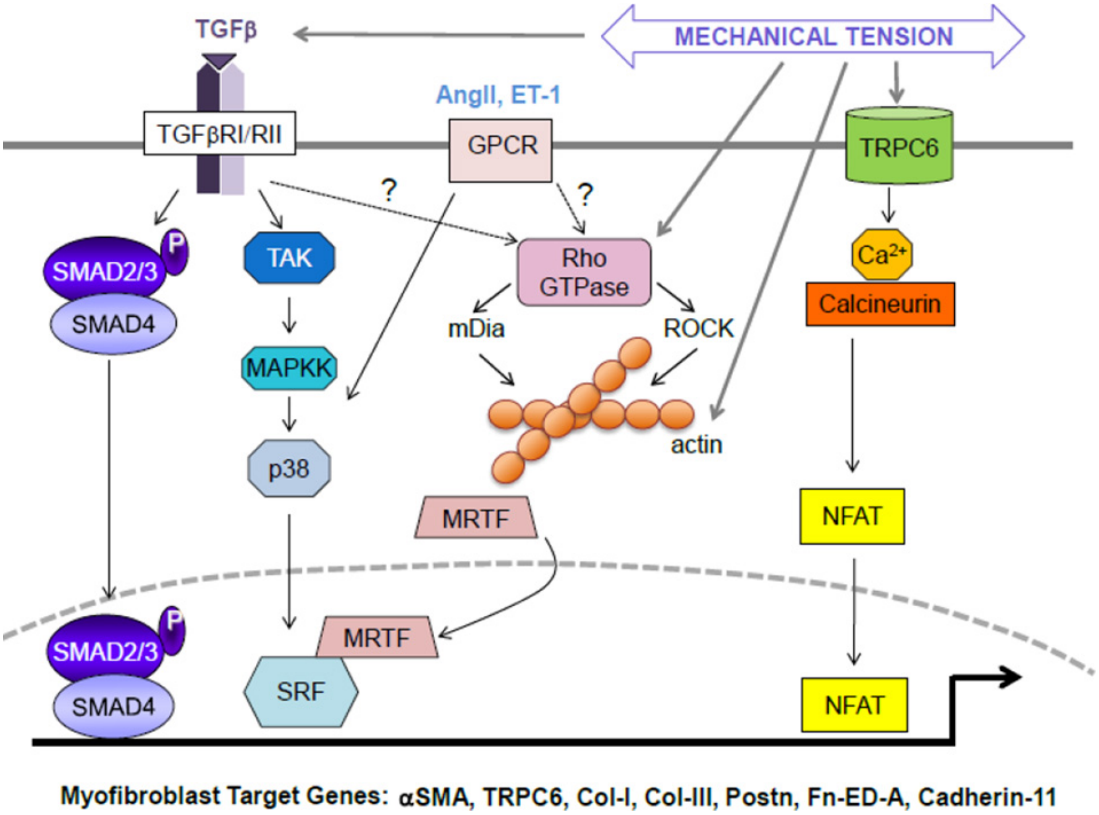

Figure 1. From Davis & Molkentin; 2014[60].

miR-145

MicroRNA miR-145 is part of a class of non-coding RNA molecules that participate in post-transcriptional gene regulation. MicroRNA's are proposed to engage in precise regulation of gene expression with the primary function of dialing-in cellular phenotype during processes such as development and differentiation[64]. Most of the current research on miR-145 has involved its expression and function in smooth muscle cells, where it has been shown to regulate smooth muscle cells phenotypes[65–69]. As previously noted, smooth muscle cells are not terminally differentiated and can exist in multiple phenotypes ranging from contractile to proliferative or synthetic. Switching between phenotypes occurs in response to cues or changes from the local environment[19,70]. Collectively, results from established studies demonstrate that miR-145 (and miR-143, present in the same gene cluster) is highly expressed in contractile smooth muscle cells and reduced under proliferative conditions[65–69].

Our lab developed an interest in exploring the molecular pathways that govern myofibroblast transformation and cardiac fibrosis after an investigation of the role miR-145 in vascular smooth muscle cell differentiation. In experiments focusing on miR-145 expression in smooth muscle cells, it was discovered that induction of miR-145 in smooth muscle cells occurred upon co-culture with endothelial cells, was augmented by Notch signaling, and was reduced in Notch receptor deficient cells. It was also discovered that loss of miR-145 promotes angiotensin-II induced collagen deposition in the coronary arteries. Essentially, loss of miR-145 exacerbated induced fibrosis[42]. Further, results from this study showed that TGF-ß is a direct target of miR-145. Overexpression of miR-145 resulted in attenuation of extracellular matrix genes

regulated by TGF-ß Receptor II while decreased expression of miR-145 resulted in increased extracellular matrix production[42]. Finally, in an attempt to quantify changes in TGF-ß signaling, it was found that miR-145 deficient mouse aortas had a significant increase in p38 MAP kinase relative to wild-type[42]. Together, these findings provide the rationale for the central hypothesis of the current study. The central hypothesis is that miR-145 represses cardiac fibrosis via posttranscriptional inhibition of mediators of the fibrotic pathway in cardiac fibroblasts (Figure 2)[71]. The rationale for this hypothesis is that miR-145 has been found to regulate the TGF-ß pathway, the p38 pathway, and the expression of extracellular matrix genes in smooth muscle cells. Here, the cardiac fibroblast is studied because fibroblasts are the key cell type involved in fibrosis, and the effects of miR-145 are proposed to be similar in cardiac fibroblasts compared to smooth muscle cells.

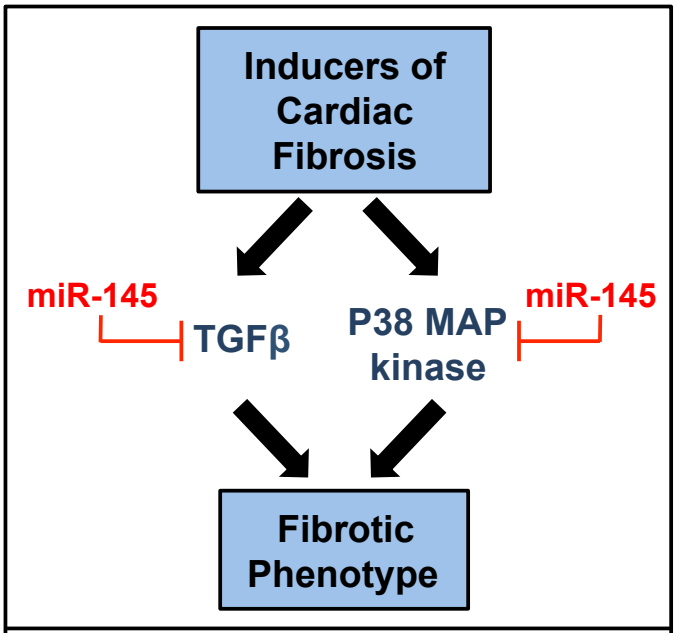

Figure 2. Central Hypothesis. Figure via Dwitya Sawant[71].

Taken with the findings that miR-145 targets TGF-ß signaling to reduce extracellular matrix gene expression and that loss of miR-145 exacerbates angiotensin II mediated fibrosis, it is reasonable to conclude that miR-145 suppresses TGF-ß-dependent fibrosis and promotes smooth muscle cell differentiation. In this proposal, this reasoning is expanded to suggest a mode of action for miR-145 in cardiac fibroblasts that is similar to the mode of action for miR-145 in smooth muscle cells.

Thus, it is hypothesized that miR-145 represses cardiac fibrosis via posttranscriptional inhibition of mediators of the fibrotic pathway, specifically TGF-ß and p38 MAP Kinase.

Generation of RFP-miR-145 Transgenic Mice

In order to explore the role of miR-145 in cardiac fibrosis, novel RFP-miR-145 transgenic mice were generated in the Lilly Laboratory. These mice conditionally express an RFP-tagged miR-145 transgene through Cre-mediated recombination. The transgene construct consists of a CAG promoter, LoxP sites, RFP, and miR-145 (Figure 3)[71]. The CAG promoter consists of the cytomegalovirus enhancer fused to a chicken beta-actin promoter, and is a strong synthetic promoter capable of driving high levels of gene expression[72,73]. The Lox-Stop-Lox cassette consists of a stop codon flanked by loxP sites, and is used in order to prevent expression except in the case of cre-mediated recombination. Upon the expression of the Cre protein, recombination results in removal of the stop codon, allowing for expression of both RFP and miR-145.

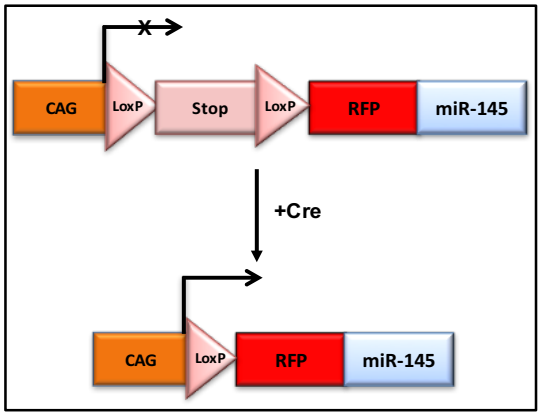

Figure 3. Transgene construct – A Cre-Lox system is used to generate expression of RFP and miR-145. via Dwitya Sawant[71].

Generation of the transgenic mouse by random knock-in of the genetic construct resulted in a variety of lines. Characterization of the lines began with phenotypic observations when the transgenic mice were crossed with non-inducible myocardin-Cre mice. Myocardin-Cre served to turn on expression of miR-145

Line	RFP-miR-145TG/+	RFP-miR-145TG/+; MCC/+	% RFP-miR-145TG/+; MCC/+	Total pups
AA	12	7	21.9%	32
AB	10	6	20.7%	29
AC	13	4	13.3%	30
BA	15	0	0.0%	57
BB	11	0	0.0%	29
DA	14	0	0.0%	48
DC	5	7	20.6%	34
EB	14	0	0.0%	28
EA	10	0	0.0%	47

Figure 4. Phenotypic observations of RFP-miR-145 transgenic mice crossed with Myocd Cre mice. Data from Jeremy Baeten[74].

specifically in cardiac and smooth muscle cells. Viability results are displayed in Figure 4[74]. The lines highlighted in yellow demonstrated in embryonic lethality. Then, expression of miR-145 was examined via embryo gross fluorescence and immunostaining. Lines were discarded that showed expression of miR-145 without the presence of Cre, that showed inconsistent expression of miR-145, and that lacked robust expression. The lines of mice chosen for study based on phenotypic and gene expression data were the BB, DA, and AA lines. The BB line was chosen for its embryonic lethality, which demonstrates high expression of miR-145 and an important role for miR-145 in embryonic development, and its high expression of mature miR-145 as examined via TaqMan analysis. The AA line was chosen because AA x Myocd (Figure 5)[71] mice survived until two weeks of age, suggesting a more moderate expression of miR-145 that could be more physiologically relevant. The DA line served as an intermediate between the BB and AA line, displaying embryonic lethality yet a more moderate expression of miR-145 than the BB line. Further study demonstrated inconsistency in RFP and miR-145 expression in the BB line, leaving the AA and DA lines for characterization here.

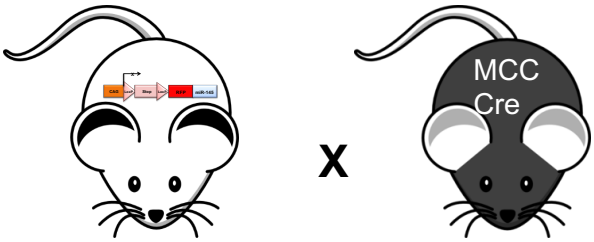

Figure 5. Example Cross of RFP-miR-145 transgenic mouse with Cre expressing mouse[71].

A variety of Cre expressing mouse lines were employed to characterize the miR-145 transgenic mice, including Myocardin (Myocd) Cre and inducible Transcription Factor 21 (TCF21) Cre. Highlighted here are the Myocd Cre and the TCF21 Cre. Myocd Cre is a valuable tool for characterizing the phenotypes and expression patterns of the transgenic mice, and TCF21 is particularly valuable is assessing the function of miR-

145 in a fibrosis disease model due to the fact that it can be induced after birth of the pups.

Mice of the Myocd Cre line express Cre protein under the influence of the Myocardin promoter. Myocardin is a transcription co-factor of the SRF transcription factor, and aids in the regulation of cell proliferation, differentiation and development of the cardiovascular system[75]. This transcription co-factor is expressed in cardiac and smooth muscle cells and is necessary for induction of cardiac and smooth muscle gene expression. For example, cardiac and smooth muscle cells markers transgelin, Atrial Natriuretic Factor, and smoothelins are dependent upon myocardin expression and activity for induced or increased expression[76–78]. In terms of tissue, Myocardin mRNA is apparent in cardiac forming tissues at embryonic day 7.5 and in smooth muscle cell tissues at embryonic day 9.5, the time point of murine embryogenesis at which vascularization begins[78]. Myocardin's early and specific expression patterns make the Myocd Cre mice a good option for characterizing the phenotype and expression patterns of the AA and DA RFP-miR-145 transgenic mouse lines.

The Myocd Cre line, however, is not inducible and results in either embryonic lethality or lethality at a mere two weeks of age, making it unsuitable for miR-145 functional studies in fibrosis disease models. The TCF21 Cre mouse, on the other hand, allows for inducible expression of Cre recombinase via the ingestion of tamoxifen and is thus better suited for studying the functional role of miR-145. Transcription Factor 21 (TCF21) is a mesoderm-specific member of the class II bHLH family of transcription factors, which are involved in cell fate specification, differentiation, and morphogenesis of certain tissues[79]. One study used reporter activity to examine cell populations

possessing Cre activity following induction of Cre recombinase by tamoxifen. Induction of Cre activity at post-natal days 5 and 7 resulted in reporter expression in the vast majority of interstitial cells, or the cardiac fibroblasts. Hence, inducible TCF21 Cre lines can be used to induce RFP-miR-145 expression in cardiac fibroblasts postnatally.

Characterization of RFP-miR-145 Transgenic Mice

The aim of this study is to characterize the role of miR-145 in vascular biology and to define the function of miR-145 in a fibrosis disease model. To begin to address these aims, we sought to characterize RFP-miR-145 transgenic mouse lines AA and DA generated in the Lilly laboratory. The purpose of characterization is to not only delineate the role of miR-145 in cardiac development and vascular biology, but also the ensure consistent, reproducible expression of the transgene before conducting studies on the role miR-145 has in fibrosis.

We sought to characterize the transgenic mice by examining phenotype and RFP/ miR-145 expression when crossed with Myocd Cre mice. Both DA and AA embryos were collected at various time points (E9.5-E11.5), and AA mice hearts were collected upon death at approximately two weeks (DA embryos die in utero). Collected tissues were processed and examined. The RFP mRNA and Cre mRNA levels were determined via qPCR and miR-145 mature micro RNA expression levels were determined via TaqMan analysis. The hearts of two-week-old AA mice were collected, processed, and examined via microscopy to examine morphological differences between miR-145 expressing and non-expressing mice. We hypothesize that ectopic expression of miR-145 would disrupt normal vascular development. Our preliminary results reveal expression of the transgene and phenotypically abnormal mutant hearts.

Materials and Methods

Quantitative RT-PCR (qPCR)

Embryos were collected by Dwitya Sawant with assistance[80]. Total RNA was isolated from embryos using RiboZol RNA Extraction Reagent (Ampresco) according to the manufacturer's instructions. RNA was reverse transcribed with M-MLV Reverse Transcriptase (Promega) to generate cDNA. Real-Time PCR was performed using the StepOne Real Time PCR system by Applied Biosystems. The reporter dye was the Power SYBR Green PCR Mastermix and 50 ng of cDNA template was used. The fold difference in mRNA levels of the target gene was calculated using the ΔΔCT method with normalization to RPL13A mRNA levels. The following are the primer sequences: RPL13A, 5'- TCC CTG CTG CTC TCA AGG -3' (forward) and 5'- GCC CCA GGT AAG CAA ACT T -3' (reverse); 1084 Cre, 5' – GCG GCT TGG CAG TAA AAA CTA TC -3' (forward) and 1085 Cre, 5'- GTG AAA CAG CAT TGC TGT CAC TT -3'; RFP 145, 5'- CCA CCA CCT GTT CTC GTA AT -3' (forward) and 5'- CGC TGT ATA CAC CCT CTT C -3' (reverse).

TaqMan Assays

Embryos were collected by Dwitya Sawant with assistance[80]. Total RNA was isolated from embryos in the manner described in the qPCR methodology. RNA was transcribed with Multiscribe Reverse Transcriptase (Applied Biosystems) using the TaqMan MicroRNA Reverse Transcription Kit to generate cDNA. Real-Time PCR was performed using the StepOne Real Time PCR system by Applied Biosystems. The reporter dye was TaqMan Gene Expression Master Mix (Applied Biosystems). The fold difference in mRNA levels of the target gene was calculated using the ΔΔCT method

with normalization to U6 snRNA levels. The primers were obtained from TaqMan microRNA assays kit by Applied Biosystems: miR-145 (Catalog #002278) and U6 snRNA (Catalog #001973).

Mouse Lines, Genotyping, and Crosses

All strains were maintained in a C57Bl/6 background. Genotyping of mice and embryos was carried out by PCR using the following primers: CreA, 5'- TGC CAC GAC CAA GTG ACA GC -3' and CreB, 5'- CCA GGT TAC GGA TAT AGT TCA TG -3'; Internal Control Forward, 5'- CTA GGC CAC AGA ATT GAA AGA TCT -3' and Internal Control Reverse, 5'- GTA GGT GGA AAT TCT AGC ATC ATC C -3'; GT-RFP145-F1, 5'- TCC CAC AAC GAG GAC TAC A -3' and GTRFP145-R1, 5'- GCT AAG CCA TGA CCT CAA GAA -3'; TCF21F, 5' TTC TCC AGG CTC AAG ACC AC -3' and TCF21R, 5' -CAA ACC CTA GCA CAA ATC ACT CGC -3' and TCF21MerP, 5' –GCT TCC GAT ATC CAG ATC CAG AC -3'; miR 145 Forward, 5' -GCA CGT GCT GAA GGC ATC TCT C -3' and miR-145 Reverse, 5' –CCT AGA CAG AGG GAG GGT GGG -3'.

Imaging

Tissues were fixed in 4% paraformaldehyde, processed, embedded in paraffin and sectioned at 8 micrometers. Sections were treated by hematoxylin and eosin staining (Sigma) according to the manufacturer's protocol. Tissue fixation, processing, embedding, sectioning, and staining procedures were performed by Dwitya Sawant with assistance[80]. Stained sections were imaged using the Zeiss Axio Observer Microscope.

Results and Discussion

Characterization of the AA Line

Analysis of gene expression in RFP and Myocd positive mice revealed an induction of Cre and RFP expression in double mutant mice compared to wild type mice. Similarly, mature miR-145 transcripts demonstrated an approximately 10-fold induction in RFP;Myocd mutants relative to wild type mice. Data from a representative litter of AA x Myocd mice is shown in Figure 2. Overall, the AA line demonstrated consistent expression of miR-145 when crossed with Myocd expressing mice. The AA line mice are viable until approximately two weeks when expressing Myocd. At a consistent time point of two weeks and two days, mice expressing both RFP/miR-145 and Myocd die. The hearts of RFP;Myocd mice have noticeably enlarged atria relative to their wild type counterparts (Figure 2A-B). Both the aortic valves (Figure 2C) and the mitral valves (Figure 2D) demonstrate visibly thinner valve leaflets when compared to wild type valve leaflets. Further exploration is required to more accurately characterize the phenotype of AA pup hearts.

Characterization of the DA Line

The most obvious phenotype of the DA line, when crossed with Myocd, is embryonic lethality of RFP/miR-145 and Myocd positive embryos. Gene expression analysis of the DA line at embryonic day 9.5 via qPCR and TaqMan analysis produced inconsistent results. While both Cre and RFP are highly expressed in mutant strains, this expression is not always consistently observed in all mice of the RFP/miR-145 and Myocd positive mice. Likewise, miR-145 expression does not appear to be consistently

or efficiently induced. Data from a representative litter of DA x Myocd mice is displayed in Figure 3.

Summary and Future Directions

Overall, the AA line demonstrates a more consistent expression of the miR-145 transgene relative to the DA line. Moving forward, we will continue to characterize both the AA and DA lines using a variety of different lines of Cre mice, including MYH11 Cre and TCF21 Cre. Development of the AA mouse hearts, examined at two weeks and two days of age, appeared abnormal. Mouse strains that were both RFP/miR-145 and Myocd positive demonstrated enlarged atria and thinning of aortic and mitral valve leaflets. These abnormalities will be investigated further to analyze the extent and prevalence of the phenotype. Further characterization will also include investigating the cellular composition of the valves and examining expression of the RFP transgene in embryonic hearts and 2-week old hearts by immunostaining. Based on characterization thus far, the most likely candidate in which the role of miR-145 in cardiac fibrosis will be tested is the AA miR-145 transgenic mouse line but more data will contribute to this decision.

Figures

Figure 1

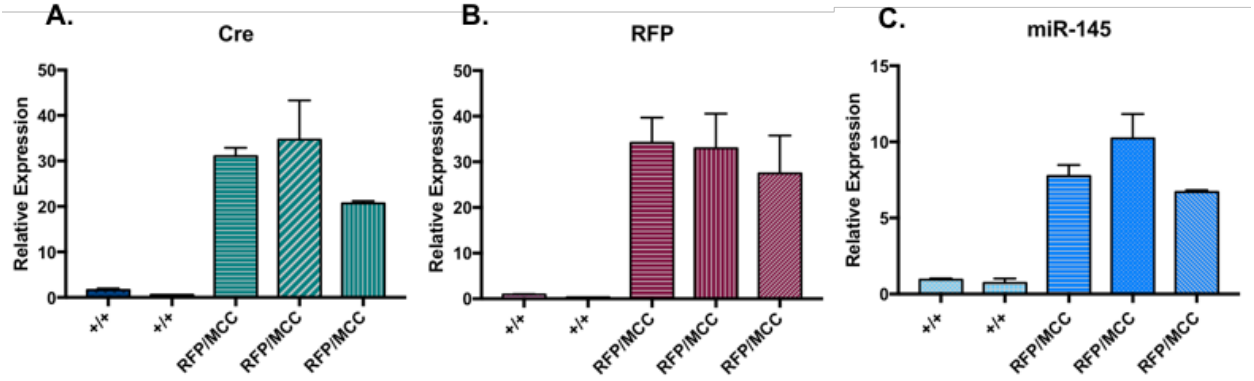

Figure 1. Expression of AA line x Myocd mouse embryos. Embryos from a cross between AA and Myocd mice were dissected at embryonic day 9.5[80]. Data from a representative trial is displayed above. The symbol +/+ represents a wild type genotype while RFP/Myocd mice are positive for both RFP and Myocd mutant alleles. RNA transcripts were isolated from embryonic hearts. Expression of Cre (A) and RFP (B) were assessed using qPCR. Expression of mature miR-145 (C) was assessed via TaqMan assay.

Figure 2

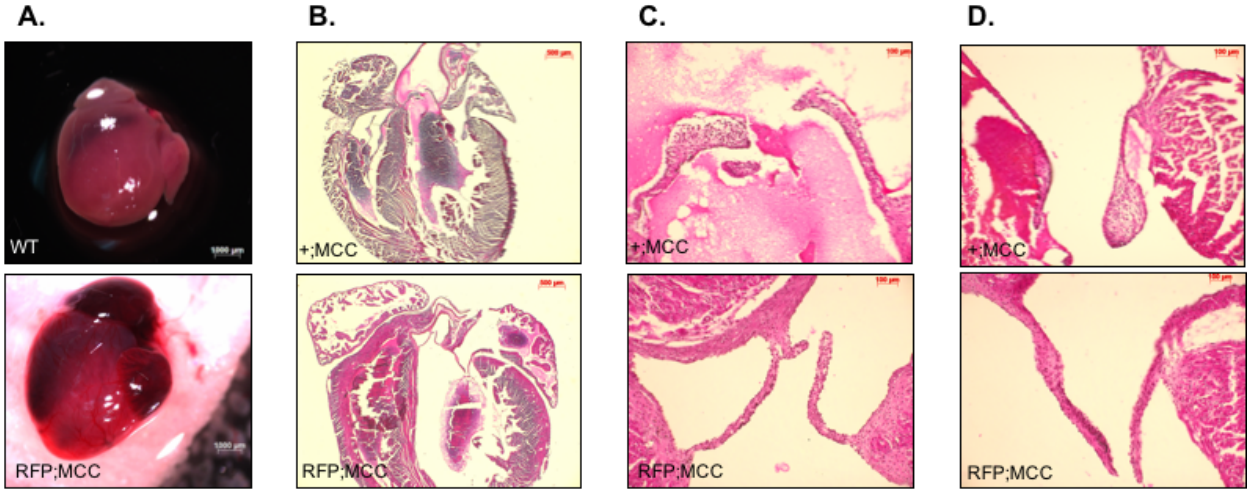

Figure 2. Expression of AA line x Myocd mouse embryos. Pups from a cross between AA and Myocd mice were dissected at postnatal day 16[80]. Data from a representative trial is displayed above. The symbol +/+ represents a wild type genotype, +; Myocd represents mice positive for Myocd but not RFP/miR-145, and RFP/Myocd represents mice are positive for RFP/miR-145 and Myocd mutant alleles. Full hearts were imaged prior to fixation[80] (A) and after fixing, sectioning, and staining with H&E (B). Aortic valves (C) and mitral valves (D) were imaged after fixing, sectioning, and staining with H&E.

Figure 3

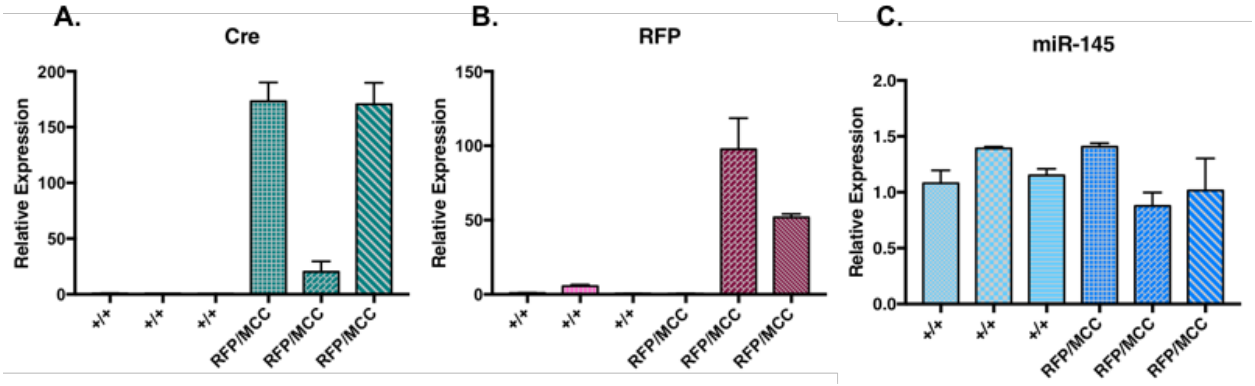

Figure 3. Expression of DA line x Myocd mouse embryos. Embryos from a cross between DA and Myocd mice were dissected at embryonic day 9.5[80]. Data from a representative trial is displayed above. The symbol +/+ represents a wild type genotype while RFP/Myocd mice are positive for both RFP and Myocd mutant alleles. RNA transcripts were isolated from embryonic hearts. Expression of Cre (A) and RFP (B) were assessed using qPCR. Expression of mature miR-145 (C) was assessed via TaqMan assay.

Conclusion:

Cytoglobin and miR-145, Two Notch Regulated Genes in the Cardiovascular System

Conclusion

The Notch signaling pathway is an important regulator of vascular development and physiology, playing roles in vital processes such as angiogenesis, artery-vein differentiation, and regulation of the smooth muscle cell phenotype[9]. Notch signaling operates via cell-to-cell contact, which manifests in the vasculature as ligands on endothelial cells interacting with receptors on smooth muscle cells to affect downstream signaling. The Lilly Lab, in which I have completed this work, studies the Notch signaling pathway to better understand the fundamentals of blood vessel development and smooth muscle cell differentiation and phenotype switching.

Previous research in the Lilly Lab revealed that the expression of certain genes in smooth muscle cells is altered when cocultured with endothelial cells. One of those genes was Notch, and it was later uncovered that the Notch signaling pathway operates between endothelial cells and smooth muscle cells and contributes to proper angiogenesis and phenotypic modulation of smooth muscle cells. Another gene up-regulated in co-culture was Cytoglobin. The current study aimed to investigate the regulation of Cytoglobin by the interactions of endothelial cells and smooth muscle cells and to explore the function of Cytoglobin in vascular smooth muscle cells. We demonstrate that coculture with endothelial cells induces the expression of Cytoglobin in smooth muscle cells. This endothelial cell-derived expression is dependent on the Notch signaling pathway, and Notch signaling is required for activation of Cytoglobin expression in cocultured smooth muscle cells. Further, our studies revealed a role for Cytoglobin in enabling smooth muscle cells to regulate nitric oxide bioavailability and to

manage vascular derived stress. This cytoprotective role is mediated by endothelial cells.

Interactions between endothelial cells and smooth muscles cells sparked interest in the Lilly Lab in another biomolecule, microRNA miR-145. Previous Lilly Lab findings detail the endothelial-derived induction of miR-145 in smooth muscle cells, a process which is augmented by Notch signaling and reduced in cells lacking Notch receptors. The study also found that miR-145 deficient mice experienced exacerbated angiotensin II-induced fibrosis. Turning this result around, we hypothesized that over-expression of miR-145 would attenuate angiotensin II-induced fibrosis. Thus, a novel miR-145 transgenic mouse was created to explore the relationship between miR-145 and cardiac fibrosis. The current study aimed to characterize two lines of miR-145 transgenic mice – the AA line and the DA line. Although work is still in progress, the AA line presents promising results. Embryonic expression of mature miR-145 in mice expressing both RFP/ miR-145 and Myocd Cre is increased relative to wild type. Additionally, two-week-old AA pups demonstrate phenotypic abnormalities of the heart. Moving forward, we will continue to characterize the AA and DA lines and investigate the effect that overexpression of miR-145 has on angiotensin II-induced cardiac fibrosis. In doing so, we may reveal yet another sector of the cardiovascular system in which the Notch signaling pathway exerts its influence.

References

1. Baeten, J. T. & Lilly, B. Notch Signaling in Vascular Smooth Muscle Cells. *Adv. Pharmacol.* **78,** 351–382 (2017).
2. Artavanis-Tsakonas, S., Rand, M. D. & Lake, R. J. Notch signaling: cell fate control and signal integration in development. *Science* **284,** 770–6 (1999).
3. Egan, S. E., St-Pierre, B. & Leow, C. C. Notch receptors, partners and regulators: from conserved domains to powerful functions. *Curr. Top. Microbiol. Immunol.* **228,** 273–324 (1998).
4. High, F. A. *et al.* An essential role for Notch in neural crest during cardiovascular development and smooth muscle differentiation. *J. Clin. Invest.* **117,** 353–363 (2007).
5. Tang, Y. *et al.* Notch and Transforming Growth Factor-β (TGFβ) Signaling Pathways Cooperatively Regulate Vascular Smooth Muscle Cell Differentiation. *J. Biol. Chem.* **285,** 17556–17563 (2010).
6. Borggrefe, T. & Oswald, F. The Notch signaling pathway: Transcriptional regulation at Notch target genes. *Cell. Mol. Life Sci.* **66,** 1631–1646 (2009).
7. Gridley, T. Notch signaling in the vasculature. *Curr. Top. Dev. Biol.* **92,** 277–309 (2010).
8. Patel-Hett, S. & D'Amore, P. A. Signal transduction in vasculogenesis and developmental angiogenesis. *Int. J. Dev. Biol.* **55,** 353–63 (2011).
9. Gridley, T. Notch signaling in vascular development and physiology. *Development* **134,** 2709–18 (2007).
10. McDaniell, R. *et al.* NOTCH2 Mutations Cause Alagille Syndrome, a Heterogeneous Disorder of the Notch Signaling Pathway. *Am. J. Hum. Genet.* **79,** 169–173 (2006).
11. Chabriat, H., Joutel, A., Dichgans, M., Tournier-Lasserve, E. & Bousser, M.-G. CADASIL. *Lancet Neurol.* **8,** 643–653 (2009).
12. Joutel, A. *et al.* Strong clustering and stereotyped nature of Notch3 mutations in CADASIL patients. *Lancet* **350,** 1511–1515 (1997).
13. Joutel, A. *et al.* Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. *Nature* **383,** 707–710 (1996).
14. Joutel, A. *et al.* The ectodomain of the Notch3 receptor accumulates within the cerebrovasculature of CADASIL patients. *J. Clin. Invest.* **105,** 597–605 (2000).
15. Lee, J. Mutations in PDGFRB and NOTCH3 are the first genetic causes identified for autosomal dominant infantile myofibromatosis. *Clin. Genet.* **84,** 340–341 (2013).
16. Chida, A. *et al.* Mutations of *NOTCH3* in childhood pulmonary arterial hypertension. *Mol. Genet. Genomic Med.* **2,** 229–239 (2014).
17. Owens, G. K., Kumar, M. S. & Wamhoff, B. R. Molecular Regulation of Vascular Smooth Muscle Cell Differentiation in Development and Disease. *Physiol. Rev.* **84,** 767–801 (2004).
18. Gomez, D. & Owens, G. K. Smooth muscle cell phenotypic switching in atherosclerosis. *Cardiovasc. Res.* **95,** 156–164 (2012).
19. Alexander, M. R. & Owens, G. K. Epigenetic Control of Smooth Muscle Cell Differentiation and Phenotypic Switching in Vascular Development and Disease.

Annu. Rev. Physiol. **74,** 13–40 (2012).
20. Lilly, B. & Kennard, S. Differential gene expression in a coculture model of angiogenesis reveals modulation of select pathways and a role for Notch signaling. *Physiol. Genomics* **36,** 69–78 (2009).
21. Lin, C. H. & Lilly, B. Notch signaling governs phenotypic modulation of smooth muscle cells. *Vascul. Pharmacol.* **63,** 88–96 (2014).
22. Hellström, M., Kalén, M., Lindahl, P., Abramsson, A. & Betsholtz, C. Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development* **126,** 3047–55 (1999).
23. Chakraborty, S., John, R. & Nag, A. Cytoglobin in tumor hypoxia: Novel insights into cancer suppression. *Tumor Biol.* **35,** 6207–6219 (2014).
24. Kawada, N. *et al.* Characterization of a Stellate Cell Activation-associated Protein (STAP) with Peroxidase Activity Found in Rat Hepatic Stellate Cells *. **276,** 25318–25323 (2001).
25. Burmester, T., Ebner, B., Weich, B. & Hankeln, T. Cytoglobin: A novel globin type ubiquitously expressed in vertebrate tissues. *Mol. Biol. Evol.* **19,** 416–421 (2002).
26. Trent, J. T. & Hargrove, M. S. A ubiquitously expressed human hexacoordinate hemoglobin. *J. Biol. Chem.* **277,** 19538–19545 (2002).
27. Oleksiewicz, U., Liloglou, T., Field, J. K. & Xinarianos, G. Cytoglobin: biochemical, functional and clinical perspective of the newest member of the globin family. *Cell. Mol. Life Sci.* **68,** 3869–3883 (2011).
28. Rahaman, M. M. & Straub, A. C. The emerging roles of somatic globins in cardiovascular redox biology and beyond. *Redox Biol.* **1,** 405–410 (2013).
29. Halligan, K. E., Jourd'heuil, F. L. & Jourd'heuil, D. Cytoglobin is expressed in the vasculature and regulates cell respiration and proliferation via nitric oxide dioxygenation. *J. Biol. Chem.* **284,** 8539–8547 (2009).
30. Jourd, F. L. *et al.* The Hemoglobin Homolog Cytoglobin in Smooth Muscle Inhibits Apoptosis and Regulates Vascular Remodeling. (2017). doi:10.1161/ATVBAHA.117.309410
31. Liu, X. *et al.* Cytoglobin regulates blood pressure and vascular tone through nitric oxide metabolism in the vascular wall. *Nat. Commun.* **8,** (2017).
32. Thuy, L. T. T. *et al.* Absence of cytoglobin promotes multiple organ abnormalities in aged mice. *Sci. Rep.* **6,** 24990 (2016).
33. Guo, X., Philipsen, S. & Tan-Un, K. C. Characterization of human cytoglobin gene promoter region. *Biochim. Biophys. Acta - Gene Struct. Expr.* **1759,** 208–215 (2006).
34. Guo, X., Philipsen, S. & Tan-Un, K. C. Study of the hypoxia-dependent regulation of human CYGB gene. *Biochem. Biophys. Res. Commun.* **364,** 145–150 (2007).
35. Oleksiewicz, U. *et al.* Cytoglobin has bimodal : tumour suppressor and oncogene functions in lung cancer cell lines. **22,** 3207–3217 (2017).
36. Singh, S. *et al.* Calcineurin activates cytoglobin transcription in hypoxic myocytes. *J. Biol. Chem.* **284,** 10409–10421 (2009).
37. Latina, A. *et al.* ΔNp63 targets cytoglobin to inhibit oxidative stress-induced apoptosis in keratinocytes and lung cancer. *Oncogene* 1–11 (2015). doi:10.1038/onc.2015.222

38. Sato-matsubara, M. *et al.* Fibroblast growth factor 2 (FGF2) regulates cytoglobin expression and activation of human hepatic stellate cells via JNK signaling. **2,** 18961–18972 (2017).
39. Liu, H., Zhang, W., Kennard, S., Caldwell, R. B. & Lilly, B. Notch3 is critical for proper angiogenesis and mural cell investment. *Circ. Res.* **107,** 860–870 (2010).
40. Liu, H., Kennard, S. & Lilly, B. NOTCH3 expression is induced in mural cells through an autoregulatory loop that requires Endothelial-expressed JAGGED1. *Circ. Res.* **104,** 466–475 (2009).
41. Pajaniappan, M. *et al.* Endothelial cells downregulate apolipoprotein D expression in mural cells through paracrine secretion and Notch signaling. *AJP Hear. Circ. Physiol.* **301,** H784–H793 (2011).
42. Zhao, N. *et al.* MicroRNA miR145 regulates TGFBR2 expression and matrix synthesis in vascular smooth muscle cells. *Circ. Res.* **116,** 23–34 (2015).
43. Zhao, N., Liu, H. & Lilly, B. Reciprocal regulation of syndecan-2 and notch signaling in vascular smooth muscle cells. *J. Biol. Chem.* **287,** 16111–16120 (2012).
44. Hendrickson, M. D. & Poyton, R. O. Crosstalk between nitric oxide and hypoxia-inducible factor signaling pathways : an update. *Res. Reports Biochem.* 147–161 (2015). doi:10.2147/RRBC.S58280
45. Li, J. & Wang, H.-M. Effects of cobalt chloride on phenotypes of normal human saphenous vein smooth muscle cells. *Int J Clin Exp Med* **7,** 4933–4941 (2014).
46. Wu, D. & Yotnda, P. Induction and Testing of Hypoxia in Cell Culture. *J. Vis. Exp.* 4–7 (2011). doi:10.3791/2899
47. Vanhoutte, P. M., Zhao, Y., Xu, A. & Leung, S. W. S. Thirty Years of Saying NO. 375–397 (2016). doi:10.1111/apha.12646
48. Liu, X. *et al.* Characterization of the function of cytoglobin as an oxygen-dependent regulator of nitric oxide concentration. *Biochemistry* **51,** 5072–5082 (2012).
49. Liu, Xiaoping; Tong, Jianjing; Zweier, Joseph R; Follmer, Douglas; Hemann, Craig; Ismail, Raed S; Zweier, J. L. Differences in oxygen-dependent nitric oxide metabolism by cytoglobin and myoglobin account for their differing functional roles. *FEBS* **280,** 3621–3631 (2013).
50. Lin, C. & Lilly, B. Endothelial Cells Direct Mesenchymal Stem Cells Toward a Smooth Muscle Cell Fate. **23,** 2581–2590 (2014).
51. Boucher, J., Gridley, T. & Liaw, L. Molecular Pathways of Notch Signaling in Vascular Smooth Muscle Cells. *Front. Physiol.* **3,** 81 (2012).
52. Boucher, J. M., Peterson, S. M., Urs, S., Zhang, C. & Liaw, L. The miR-143/145 Cluster Is a Novel Transcriptional Target of Jagged-1/Notch Signaling in Vascular Smooth Muscle Cells. *J. Biol. Chem.* **286,** 28312–28321 (2011).
53. Boucher, J. M., Harrington, A., Rostama, B., Lindner, V. & Liaw, L. A Receptor-Specific Function for Notch2 in Mediating Vascular Smooth Muscle Cell Growth Arrest Through Cyclin-dependent Kinase Inhibitor 1B. *Circ. Res.* **113,** 975–985 (2013).
54. Liu, T. *et al.* Role of blood and vascular smooth muscle in the vasoactivity of nitrite. *AJP Hear. Circ. Physiol.* **307,** H976–H986 (2014).
55. Van Thuy, T. T., Thuy, L. T. T., Yoshizato, K. & Kawada, N. Possible Involvement

of Nitric Oxide in Enhanced Liver Injury and Fibrogenesis during Cholestasis in Cytoglobin-deficient Mice. *Sci. Rep.* **7,** 1–14 (2017).
56. Lilly, B. Unpublished. (2018).
57. Travers, J. G., Kamal, F. A., Robbins, J., Yutzey, K. E. & Burns, C. Cardiac Fibrosis: The Fibroblast Awakens. - PubMed - NCBI. **118,** 1021–1040 (2017).
58. Benjamin, E. J. *et al. Heart Disease and Stroke Statistics'2017 Update: A Report from the American Heart Association*. *Circulation* **135,** (2017).
59. Kawaguchi, M. *et al.* Inflammasome Activation of Cardiac Fibroblasts Is Essential for Myocardial Ischemia/Reperfusion Injury. *Circulation* **123,** 594–604 (2011).
60. Davis, J. & Molkentin, J. D. Myofibroblasts: Trust your heart and let fate decide. *J. Mol. Cell. Cardiol.* **70,** 9–18 (2014).
61. van den Borne, S. W. M. *et al.* Myocardial remodeling after infarction: the role of myofibroblasts. *Nat. Rev. Cardiol.* **7,** 30–37 (2010).
62. Weber, K. T. Fibrosis and hypertensive heart disease. *Curr. Opin. Cardiol.* **15,** 264–72 (2000).
63. Molkentin, J. D. *et al.* Fibroblast-Specific Genetic Manipulation of p38 Mitogen-Activated Protein Kinase in Vivo Reveals Its Central Regulatory Role in Fibrosis. *Circulation* **136,** 549–561 (2017).
64. Sevignani, C., Calin, G. A., Siracusa, L. D. & Croce, C. M. Mammalian microRNAs: A small world for fine-tuning gene expression. *Mamm. Genome* **17,** 189–202 (2006).
65. Boettger, T. *et al.* Acquisition of the contractile phenotype by murine arterial smooth muscle cells depends on the Mir143 / 145 gene cluster. *J. Clin. Invest.* **119,** 2634–2647 (2009).
66. Cheng, Y. *et al.* MicroRNA-145, a novel smooth muscle cell phenotypic marker and modulator, controls vascular neointimal lesion formation. *Circ. Res.* **105,** 158–166 (2009).
67. Xin, M. *et al.* MicroRNAs miR-143 and miR-145 modulate cytoskeletal dynamics and responsiveness of smooth muscle cells to injury. *Genes Dev.* **23,** 2166–2178 (2009).
68. Cordes, K. R. *et al.* NIH Public Access. *Nature* **460,** 705–710 (2010).
69. Elia, L. *et al.* NIH Public Access. *Cell* **16,** 1590–1598 (2011).
70. Owens, G. K., Kumar, M. S. & Wamhoff, B. R. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol. Rev.* **84,** 767–801 (2004).
71. Sawant, D. & Lilly, B. Unpublished. (2018).
72. CAG promoter Sequence and Map. Available at: http://www.snapgene.com/resources/plasmid_files/basic_cloning_vectors/CAG_promoter/. (Accessed: 1st March 2018)
73. Alexopoulou, A. N., Couchman, J. R. & Whiteford, J. R. The CMV early enhancer/chicken β actin (CAG) promoter can be used to drive transgene expression during the differentiation of murine embryonic stem cells into vascular progenitors. *BMC Cell Biol.* **9,** 2 (2008).
74. Baeten, J. T. & Lilly, B. Unpublished. (2018).
75. Espinoza-Lewis, R. A. & Wang, D.-Z. Generation of a Cre knock-in into the Myocardin locus to mark early cardiac and smooth muscle cell lineages. *Genesis*

52, 879–87 (2014).
76. Wang, Z. *et al.* Myocardin and ternary complex factors compete for SRF to control smooth muscle gene expression. *Nature* **428,** 185–189 (2004).
77. Wang, Z., Wang, D.-Z., Pipes, G. C. T. & Olson, E. N. Myocardin is a master regulator of smooth muscle gene expression. *Proc. Natl. Acad. Sci.* **100,** 7129–7134 (2003).
78. Wang, D. *et al.* Activation of cardiac gene expression by myocardin, a transcriptional cofactor for serum response factor. *Cell* **105,** 851–62 (2001).
79. Acharya, A. *et al.* Eficient inducible Cre-Mediated recombination in Tcf 21 cell lineages in the heart and kidney. *Genesis* **49,** 870–877 (2011).
80. Sawant, D., Lowe, M., Dammeyer, K. & Lilly, B. Unpublished. (2018).