Oh My Beating Heart: The Effect of Sympathetic Innervation on Cardiac Myocyte Development

Senior Thesis

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by
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ACKNOWLEDGMENTS

Having done research throughout my undergraduate career, I now understand that progress is the culmination of many people's combined effort, and I cherish everyone, past and present, for their advice, support, and help. But I would like to take a couple of words to thank several people without whom I could never have written this thesis.

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Finally, I would like to thank past members of the Birren Lab, specifically Jason Baade, Mareshia Donald, and Justin Dore for welcoming me into the lab and showing me the ropes.

ABSTRACT

In fetal life heart growth is driven by cardiac myocyte cell division, but soon after birth, these cells withdraw from the cell cycle and further heart grow takes place via cellular hypertrophy. Sympathetic innervation of the heart takes place concomitantly with this key developmental transition in myocytes. We therefore investigated whether sympathetic signaling regulates myocyte development using an *in vitro* culture system containing neonatal sympathetic neurons and cardiac myocytes. Our data showed a strong trend at 2 days *in vitro*, with myocytes cultured in the presence of sympathetic neurons showing higher rates of proliferation as compared to myocytes cultured alone. This suggests that sympathetic signaling increases the proliferative capacity of cardiac myocytes before they withdraw from the cell cycle. The molecular mechanisms that underlie this proliferative regulation were examined in an *in vivo* lesion model in which sympathetic innervation to the heart was ablated. The expression of three genes involved in myocyte cell cycle regulation, c-myc, Meis1, and ALMS1, were compared between 6-OHDA lesioned hearts and control hearts at P2, P7, and 8 weeks. C-myc showed decreased expression in P2 lesioned hearts as compared to age-matched control hearts. Meis1 showed an increase in expression in both P2 and P7 lesioned hearts as compared to controls. These two results suggest that sympathetic signaling may interact with c-myc and Meis1 at different points in development to regulate cardiac myocyte proliferation. ALMS1, however, showed no difference in expression between control and lesion hearts at P2 and P7, suggesting that ALMS1 may not interact with sympathetic signaling to regulate myocyte proliferation. Further experiments will uncover the specific role of Meis1 in the complex signaling pathway that underlies sympathetic regulation.

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INTRODUCTION

I. Overview of Cardiac Myocyte Development

The cardiac muscle is made up of highly specialized and structured cells called cardiac myocytes. Through the course of development, the heart grows through two distinct mechanisms. In fetal life, heart growth is characterized by cardiac myocyte hyperplasia, or increase in cell number. But soon after birth cardiac myocytes withdraw from the cell cycle, and growth is achieved primarily through hypertrophy, or increase in individual cell size (Li et al., 1996; Ahuja et al., 2007). However, before terminal withdrawal, myocytes undergo an additional round of karyokinesis, resulting in binucleated cells (Ahuja et al., 2007). The timing of this key developmental transition from hyperplasia to hypertrophy sets the number of cardiac myocytes found in the adult heart, which in turn has implications for cardiac function.

There is mounting evidence that suggests the early regulation of cardiac myocyte number has consequences for cardiac function. For example, it has been shown that knocking down the apoptosis regulator, survivin, perturbs myocyte proliferation without inducing apoptosis (Levkau et al., 2008). This developmental change induces an adaptive mechanism of hypertrophy in myocytes (Levkau et al., 2008). However, this adaptive mechanism presents itself as pathological. The heart, with a fewer number of larger myocytes, has an increased hemodynamic load, which leads to progressive heart failure (Levkau et al., 2008). Conversely, mitogenic cardiomyopathy is a lethal neonatal cardiomyopathy that is characterized by increased myocyte hyperplasia (Chang et al., 2010). This evidence points towards an optimal number of cardiac myocytes that must be present in order to maintain a healthy fully functioning heart.

II. Overview of Sympathetic Regulation

During the prenatal and early postnatal period, sympathetic neurons mature and provide sympathetic drive to the heart (Dowell et al., 1985). This neuronal development occurs concomitantly with the hyperplasia-hypertrophy transition in cardiac myocytes. It has been shown that sympathetic signaling also regulates the proliferation of other cell types including cells that make up white adipose tissue and smooth muscle cells (Bevan, 1975; Bowers et al., 2004). This evidence suggests that sympathetic signaling may be a regulator of the hyperplasia-hypertrophy transition in cardiac myocytes. In addition, the sympathetic nervous system is involved in different facets of cardiac myocyte development, which will be further explained below.

The sympathetic nervous system is one of the two main branches of the autonomic nervous system. It is responsible for stimulating the body's "fight or flight" response when dealing with environmental stressors. It often works in coordination with the parasympathetic nervous system to maintain an internal state of homeostasis. Sympathetic neurons are primarily found in the paraverterbral ganaglia (Purves et al., 2001). These neurons receive input from the central nervous system via pre-ganglionic neurons from the spinal cord. Additionally, post-ganglionic sympathetic neurons project axons and innervate proximal and distal organs, providing sympathetic drive to these organs. The primary neurotransmitter released by post-ganglionic sympathetic cells is norepinephrine (Vance et al., 1975).

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Sympathetic regulation is particularly important in the cardiac system, where it governs many aspects of cardiac output. Sympathetic neurons that innervate the heart originate from the superior cervical ganglion. The heart expresses both α- and β- adrenoreceptors, which allows

the organ to respond to sympathetic signaling. Norepinephrine signaling mediates positive inotropic effects, causing an increase in heart rate, conduction velocity, and myocardial contraction and relaxation (Schäfers et al., 1994). In addition to these effects, sympathetic signaling has also been shown to play an important role in cardiac myocyte development. It has been shown that knocking out dopamine beta-hydroxylase, the enzyme responsible for synthesizing norepinephrine, in mice, results in death in utero (Thomas et al., 1995). Cardiac myocytes appeared to be disorganized and atrophied (Thomas et al., 1995). In addition, sympathetic signaling has been shown to regulate the development of electrophysiological properties in cardiac myocytes. Cardiac myocytes co-cultured with sympathetic neurons show the development of Na^+, L-type Ca^+, pacemaker, inward rectifier and transient outward K^+ currents (Qu & Robinson, 2004). Although the signaling pathway has not been definitively elucidated for each of these processes, the evidence suggests that the sympathetic nervous system plays an important role in the development of cardiac myocytes.

The purpose of this work is to elucidate the effect of sympathetic signaling on the hyperplasia-hypertrophy transition in cardiac myocytes. A more definitive role has been established for sympathetic regulation by previous work in the lab, which has shown that early postnatal chemical sympathectomy with 6-hydroxydopamine *in vivo* results in rats with smaller hearts (Kreipke & Birren, 2015). The model that has been used to explain this data is that sympathetic signaling in early postnatal development increases the proliferative capacity of cardiac myocytes. Therefore, in the absence of sympathetic signaling cardiac myocytes go through fewer rounds of proliferation, resulting in smaller hearts. However, further results from the lab show an interesting change in the regulatory role of sympathetic signaling later in development. While young postnatal cardiac myocytes respond to β-adrenergic signaling with

a decrease in cell size, it has been shown that mature cardiac myocytes show an increase in cell size in response to the signaling (Kreipke et al., 2015). These results summarize an interesting developmental change in the regulatory role of sympathetic signaling, and suggest that both hyperplasia and hypertrophy may be activated by the same underlying mechanism. Despite the evidence that points towards sympathetic signaling mediating the key developmental transition from hyperplasia to hypertrophy in cardiac myocytes, the underlying molecular mechanisms that interact with sympathetic signaling have yet to be understood.

III. Potential Underlying Mechanisms: C-myc

Transcription factors present themselves as attractive candidates for potential regulators, because they are molecules that can initiate genetic programs and alter downstream targets to modify developmental processes. One potential regulator of cardiac myocyte proliferation is the transcription factor c-myc, which shows a sharp reduction from fetal levels during the neonatal period (Komuro et al., 1988; Schneider et al., 1986). C-myc is a member of the basic helix-loop-helix-leucine zipper family of transcription factors that activate transcription when they are in a heteromeric complex with the Max protein (Ahuja et al., 2007). It is important to note that c-myc is a transcription factor that is involved in a broad array of processes during development.

In the cardiac system, c-myc is expressed in embryonic ventricular myocytes and is needed for proper cardiac development during the embryonic stage (Davis et al., 1993). In addition, the over-expression of c-myc in the murine fetal myocardium is associated with increased cellular proliferation, resulting in ventricular enlargement (Jackson et al., 1990). However, it has also been shown that c-myc is up regulated in response to hormonally induced cellular

hypertrophy (Starksen et al., 1986). In addition, induced c-myc activation in the adult myocardium provokes cardiac myocyte hypertrophy along with the reactivation of DNA synthesis, potentially through a cyclinD2 dependent signaling pathway (Xiao et al., 2001; Zhong et al., 2006). These results suggest that c-myc may switch roles through the course of cardiac myocyte development. In addition, this evidence again suggests cellular hyperplasia and hypertrophy may be alternatively activated by the same underlying mechanism. C-myc may serve as a potential regulator that mediates the growth of cardiac myocytes at different points in development in coordination with the sympathetic nervous system.

IV. Potential Underlying Mechanisms: Meis1

While c-myc is involved in a broad array of process, the homeodomain transcription factor Meis1 has been specifically linked to proliferation. Meis1 belongs to the three amino acid loop extension family of transcription factors, and plays a role in normal cardiac development and differentiation (Wamstad et al., 2012; Paige et al., 2012). It has been shown that *in vitro* knockdown of Meis1 in rat cardiac myocytes results in a three-fold increase in cardiac myocyte proliferation (Mahmoud et al., 2013). Results were recapitulated *in vivo* when conditional Meis1 knockout mice were analyzed for myocyte proliferation (Mahmoud et al., 2013). Conversely the over-expression of Meis1 leads to decreased cardiac myocyte proliferation in the neonatal period (Mahmoud et al., 2013). This evidence suggests that Meis1 acts to trigger cell cycle withdrawal.

Interestingly, Meis1 has also been shown to play a role in the sympathetic nervous system. Specifically, Meis1 inactivation in the peripheral nervous system results in compromised sympatho-vagal regulation of cardiac function, including cardiac conduction defects

(Bouilloux et al., 2016). A loss of Meis1 in sympathetic neurons impairs their distal target-field innervation abilities (Bouilloux et al., 2016). Furthermore, sympathetic neurons deficient in Meis1 progressively die via apoptosis from early embryonic stages to perinatal stages (Bouilloux et al., 2016). Meis1 plays a role in cardiac myocyte development and the development of sympathetic neurons, suggesting that it is an important component of normal development in this system. However, Meis1 regulation in response to sympathetic signaling has never been examined. Meis1 may work with sympathetic regulation to impact cardiac myocyte development.

V. Potential Underlying Mechanisms: ALMS 1

In addition to transcription factors, ALMS1 also presents itself as a potential regulator of cardiac myocyte proliferation. ALMS1 is a component of the non-motile primary cilium (Shenje et al., 2014). An ALMS1 variant allele was identified in two infant siblings with neonatal heart failure due to mitogenic cardiomyopathy (Shenje et al., 2014). The ALMS1 mutation resulted in frameshift and premature termination. Mutations in ALMS1 are known to cause a recessive disorder known as Alström syndrome, in which approximately two-thirds of patients suffer from dilated cardiomyopathies (Bond et al., 2005; Marshall et al., 2011). This suggests that the novel ALMS1 mutation may be responsible for the mitogenic cardiomyopathy seen in the infants. Further investigation showed that knocking down ALMS1 in neonatal mice cardiac myocytes resulted in increased myocytes in G2/M phase as compared to controls (Shenje et al., 2014). In addition, an $Alms1^{Gt/Gt}$ mouse model with truncated ALMS1 mRNA was analyzed for proliferation at 15 days, when cardiac myocytes are expected to be terminally differentiated. The results showed increased 5'ethynyl-2'-deoxyuridine (EdU), a marker of DNA synthesis, and increased expression of the proliferative

marker phospho-histone-H3 (PH3) (Shenje et al., 2014). These results suggest that cardiac myocytes have an extended proliferative window in the absence of ALMS1 and that ALMS1 is a potential regulator of cardiac myocyte cell cycle withdrawal.

As stated above ALMS1 is a protein that is associated with the non-motile primary cilium. However, there are many hypotheses that explain how ALMS1 may impact cardiac myocyte proliferation. Cardiac myocytes have cilia, and there seems to be a temporal association between the transformation of cardiac myocytes from mitotic to post-mitotic and the formation of cilia (Rash et al., 1969). Components of the primary cilium have been shown to restrain canonical Wnt/β-catenin signaling, which plays a key role in cardiomyogenesis (Kwon et al., 2007; Ajima et al., 2011). ALMS1 deficiency may promote Wnt/β-catenin signaling, which in turn may induce the transcription of genes that promote proliferation (Shenje et al., 2014). In addition it has been shown that ALMS1 localizes to the centrosome, which is an organelle that plays a key role in the regulation of the cell cycle (Hinchcliffe, 2003). This suggests that ALMS1 may regulate cell-cycle withdrawal through interactions with the centrosome.

ALMS1 has been shown to regulate the proliferation of cardiac myocytes; however, whether sympathetic signaling regulates ALMS1 expression has not been examined. ALMS1, along with Meis1 and c-myc, may interact with sympathetic signals to coordinate cardiac myocyte maturation.

VI. Experimental Models

In order to discern the role of sympathetic signaling in cardiac myocyte development, sympathetic signaling to cardiac tissue and individual cardiac myocytes needs to be

manipulated. In this work, tools have been used to modulate sympathetic signaling *in vivo* and *in vitro*. We can observe the mechanistic outcomes *in vivo* and then take those results to a more simplified system *in vitro* system. In the *in vitro* system we can then examine the effects of sympathetic signaling in isolation and modulate signaling pathways to uncover the role of specific molecules.

Modulation of Sympathetic Signaling in vivo – 6-hydroxydopamine

In order to uncover whether sympathetic signaling plays a role in regulating the expression of genes that have been linked to cardiac myocyte growth, an *in vivo* lesion model was used. In this model early sympathetic signaling to heart was abolished. In order to achieve specific degradation of sympathetic neurons, 6-hydroxydopamine (6-OHDA) was used. 6-OHDA is a toxin that specifically targets monoaminergic neurons (Kostrzewa & Jacobowitz, 1974). 6-OHDA is taken up by the monamine reuptake transporter, after which it disrupts sympathetic innervation and signaling in multiple ways. 6-OHDA replaces norepinephrine in the release vesicles, forms destructive free radicals, and inhibits complexes I and IV in the mitochondrial respiratory chain (Kostrzewa & Jacobowitz, 1974; Glinka et al., 2007).

6-OHDA has been used extensively in adult animals to achieve complete destruction of noradrenergic neurons. The toxin is used in the central nervous system to target noradrenergic and dopaminergic neurons, creating models that mimic neurodegenerative diseases, such as Parkinson's Disease (Deumens et al., 2002). In the peripheral nervous system, sympathetic neurons are the only neurons that express the monamine reuptake transporter. Therefore, if 6-OHDA is injected peripherally, sympathetic neurons will be specifically targeted.

<u>Modulation of Sympathetic Signaling in vitro – Cardiac myocyte and sympathetic neuron co-cultures</u>

Cardiac myocytes cultured *in vitro* exhibit many of the same developmental events they would *in vivo*, including regulating expression of ion currents, spontaneously beating, and undergoing the transition from proliferation to hypertrophy (Li et al., 1996). Likewise, sympathetic neurons cultured *in vitro* develop similarly to their *in vivo* counterparts. They are capable of developing processes and forming functional synapses both onto themselves and cardiac myocytes, where they can be stimulated to release norepinephrine (Furshpan et al., 1976; Luther & Birren, 2009; Luther et al., 2013).

By culturing cardiac myocytes in the presence of sympathetic neurons we can recapitulate the sympathetic signaling regulation that would occur in an *in vivo* system. We can then go further and manipulate β-adrenergic signaling and the expression of molecules like Meis1 in order to discern the signaling pathway that underlies sympathetic regulation of cardiac myocyte development.

<u>VII. Aims</u>

Cardiac myocytes in the fetal stage primarily grow via hyperplasia; however, shortly after birth cardiac myocytes withdraw from the cell cycle and go on to grow via hypertrophy. The timing of this key developmental transition from hyperplasia to hypertrophy establishes the number of cardiac myocytes present in the adult heart, which in impacts cardiac function and the heart's ability to recover from injury (Porrello et al. 2008). The sympathetic nervous system has been shown to regulate many aspects of cardiac myocyte development; however, the role of sympathetic signaling in regulating the hyperplasia-hypertrophy transition in cardiac

myocytes has not been elucidated. Furthermore, c-myc, Meis1, and ALMS1 are all molecules that have been implicated in cardiac myocyte proliferation and may serve as potential underlying mechanisms through which sympathetic signaling may regulate cardiac myocyte growth.

Our first aim is to discern the affect of early sympathetic signaling on cardiac myocyte development. Specifically, we will look at whether sympathetic signaling influences the hyperplasia-hypertrophy transition.

Our second aim is to look at the molecular mechanisms that underlie this regulation. We will begin by looking at whether sympathetic signaling affects the expression of genes like c-myc, ALMS1, and Meis1.

Our third aim is to start to uncover the specific role of each of these genes in the complex signaling cascade that underlies sympathetic regulation by manipulating the expression of these genes and seeing how cardiac myocyte development is affected.

Together, this work will demonstrate the importance of sympathetic signaling for the regulation of the hyperplasia-hypertrophy transition in cardiac myocytes.

MATERIALS AND METHODS

Animals

Isolation and Culture of Sympathetic Neurons and Cardiac Myocytes. Sympathetic neurons were isolated from the superior cervical ganglion (SCG) and cardiac myocytes were isolated

from the ventricles of P1-P3 rats. Cells were cultured as previously described (Lockhart et al., 1997; Luther & Birren, 2006).

Chemical Sympathectomy. Procedure was performed by Rebecca Kreipke. Chemical sympathectomy was carried out on neonatal rats (P0) via a single intraperitoneal injection of 100mg/kg 6-hydroxydopamine (6-OHDA Sigma, St. Louis, MO) diluted in .9% saline solution with 1 mg/ml of ascorbic acid. Control animals received a sham injection with the saline solution alone. Pups were sacrificed 48 hours after injection (P2), a week after injection (P7), or 8 weeks after injection (8 weeks). Previous work from the lab has shown successful destruction of sympathetic innervation in the heart following 6-OHDA lesion (Kreipke & Birren, 2015).

Immunocytochemistry

Fixing Cells. Cultures spanning 1 DIV to 10 DIV were fixed for 10min at room temperature with 4% paraformaldehyde. The cultures were then rinsed three times with phosphate buffering solution (PBS) and stored at 4 degrees in PBS.

Staining. A lysis buffer was made using 1% normal donkey serum, 0.1% NP-40, and PBS. 1ml of the buffer was administered to the cells for ten minutes in order to permeabilize them. 10% normal donkey serum was then used to block non-specific antibody binding. Primary antibody was then diluted in 1% donkey serum and administered to the cells for 1-2 hours at room temperature. The difference in timing made no significant difference in the outcome of the staining. The antibodies used were: α-actinin (Sigma, St. Louis, MO) at a concentration of 1:800 and Ki67 (Sigma, St. Louis, MO) at a concentration of 1:1500. Cultures were then

rinsed with PBS 3 times for ten minutes. Secondary antibody was diluted in 1% donkey serum and applied to cells for 45 minutes in the dark at room temperature. Both secondary antibodies were used at a concentration of 1:600. The secondary antibodies used were donkey anti-mouse Rhodamine and donkey anti-rabbit FITC (Jackson Immunoresearch). The cultures were then rinsed 2 times with PBS for 5 minutes each time. 4',6-diamidino-2-phenylindole (DAPI, Life Technologies (1ug/ml)) was diluted in PBS and added to plates for 10 minutes at room temperature in order to visualize the nuclei.

Proliferation Measure. Cells were imaged using Volocity software on an Olympus IX-81 microscope. 60 Random fields were captured. Rates of cellular proliferation were calculated as a percentage of cardiac myocytes that had nuclei that were double labeled with both DAPI and Ki67.

Gene Expression

RNA Extraction. Portions of P2, P7, and 2mo ventricles were flash frozen in liquid nitrogen and stored at -80 until used. RNA was extracted using a Direct-zol™RNA MiniPrep kit (Zymo Research).

Reverse Transcriptase PCR (RT-PCT). RT-PCR was performed as previously described (Moon & Birren, 2008).

Quantitative Real Time PCR. Pre-validated Taqman primers were used to assay levels of c-myc and ALMS1, using GAPDH as a reference gene (Life Technologies). The PCR assays

were run on a Rotorgene6000 real time PCR machine (Corbett Life Sciences). Data was quantified as previously described (Ramakers et al., 2003).

Transfections

Transfections were carried out *in vitro* on cultured rat neonatal cardiac myocytes. Cardiac myocytes were plated at a density of 50,000 cells per dish. Neurons were plated at a density of 10,000 cells per dish. Cells were transfected 24 hours after plating. 2mls of growth media was exchanged for 2mls of antibiotic free media (AB media). Lipofectamine (Invitrogen), Meis1 siRNA (Applied Biosystems, Ambion; siRNA ID: s8662), and negative control siRNA were diluted in OPTIMEM Reduced Serum Medium (Invitrogen). Lipofectamine and siRNA was comibined at a 1:1 ratio and incubated at room temperature for 20min. Complex was added to the dishes (25pmol of siRNA/dish and 7.5ul of lipofectamine/dish). The dishes were then incubated for another 24 hours before they were imaged.

Statistical Analysis

All data presented is the average of at least 3 independent experiments ($n \geq 3$) with a standard error indicated by error bars. Significance was determined using a student's t-test or ANOVA with the appropriate post-hoc test. Data was compiled and analyzed using Excel 2011 software (version 2011 for MacOS, Microsoft, Seattle, WA).

RESULTS

*The data in **Figure. 5** is shown courtesy of Rebecca Kreipke*

Sympathetic signaling increases the proliferative capacity of cardiac myoyctes in the early postnatal period.

In the prenatal and the early postnatal period, cardiac myocytes undergo hyperplasia; however, by about 10 days *in vitro* (10 DIV) cardiac myocytes withdraw from the cell cycle, and go on to grow via cellular hypertrophy (Li et al., 1996). In order to examine whether sympathetic signaling influences the proliferative capacity of cardiac myocytes during this postnatal period, we carried out a time course, in which cardiac myoyctes were cultured in the presence or absence of sympathetic neurons for 1,2,3,4,5,7, and 10 DIV. The percentage of Ki67-positive cardiac myocytes were quantified and levels of proliferation were compared at each time point.

Our results showed that cardiac myocytes in both the myocyte only and co-culture condition underwent a precipitous drop in proliferation after 5 DIV, and by 10 DIV cardiac myocytes were completely withdrawn from the cell cycle. This result recapitulates the known phenomenon of cardiac myoycte cell cycle withdrawal in the postnatal period observed *in vivo* (Li et al., 1996). These data demonstrate the validity of our culture system, specifically showing that cardiac myocytes follow a normal developmental course *in vitro*. Our results also showed a difference in the proliferative capacity of cardiac myoyctes cultured in the presence of sympathetic neurons as compared to cells cultured alone. At 2 DIV there was a strong trend where we saw about 10% more Ki67-positive nuclei in the co-culture condition as compared to the myocyte only condition (**Fig. 1**, 2 DIV = 53.72% ± 2.28 vs. 41.41% ± 3.73, n=5, trending p value for significance at 2 DIV p = .06 vs. age-matched co-culture). We saw the same trend at 3 DIV and 5 DIV. These data suggest that sympathetic signaling drives cardiac myocytes to proliferate at a higher rate before they withdraw from the cell cycle. These results are consistent with previous work in the lab, which shows that rats with chemical sympathectomy have smaller hearts. According to our data this could be because in

the absence of sympathetic signaling, cardiac myocytes have a decreased rate of proliferation before withdrawing from the cell cycle; thus, fewer cells make up the smaller heart.

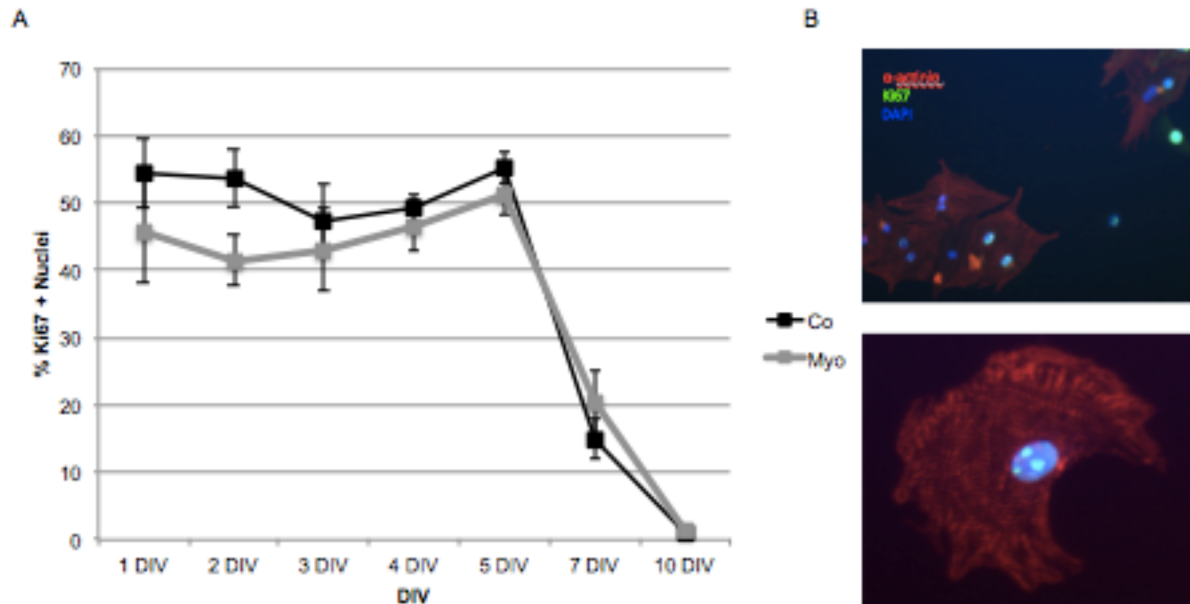

Fig. 1. Sympathetic signaling increases the proliferative capacity of cardiac myocytes. A) A plot of percent of proliferating cardiac myocytes cultured for 1, 2, 3, 4, 5, 7, and 10 DIV either in presence (black) or absence (gray) of sympathetic neurons. This plot shows that cardiac myocytes cultured in the presence of sympathetic neurons have an increased proliferative capacity ($n \geq 3$; trending p value for significance at 2 DIV $p = .06$ vs. age-matched co-culture). B) Representative images showing cultured myocytes. Proliferating cardiac myocytes are double labeled with α-actinin and Ki67.

Sympathetic signaling *in vivo* regulates the expression of the transcription factor c-myc.

C-myc is a transcription factor that is involved in a broad array of developmental processes. However, a significant body of work has also pinpointed c-myc as a molecule involved in regulating cardiac myocyte proliferation (Jackson et al., 1990; Machida et al., 1975). Thus, we hypothesized that c-myc could be involved in the underlying mechanistic pathway that coordinates sympathetic signaling and regulates the hyperplasia-hypertrophy transition in cardiac myocytes. In order to examine the effect of sympathetic signaling on c-myc, we

examined c-myc expression in sham injected (control) hearts and 6-OHDA lesioned hearts. In addition, in these set of experiments we switched to an *in vivo* lesion model because manipulations of c-myc expression *in vivo* have shown an effect on cardiac myocyte proliferation (Jackson et al., 1998; Machida et al., 1975).

We first assessed the expression of c-myc over time by comparing c-myc mRNA levels at P2, P7, and 8 weeks in control hearts. We extracted RNA from P2, P7, and 8 week old control hearts and assayed levels of c-myc using qPCR. We found that at P7 and 8 weeks c-myc expression significantly decreased as compared to P2 (**Fig. 2**, average fold change (P7) = .55 ± .13, n=4, p = .04; average fold change (8 weeks) = .095 ± .053, n=4, p =.0004). This result is consistent with what has already been shown by Komuro et al. This timeline is also consistent with the pattern of cell-cycle withdrawal in cardiac myocytes. At P7, when cardiac myocytes begin to withdraw from the cell cycle, there is a drop in c-myc. At 8 weeks, when cardiac myocytes are terminally withdrawn, we see even less c-myc. This data suggests that c-myc may be a good marker for cellular proliferation in cardiac myocytes.

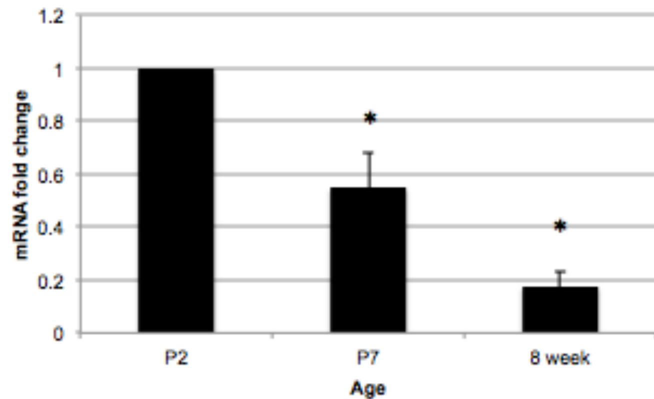

Fig. 2. C-myc expression decreases over time in sham injected hearts. Levels of c-myc expression were compared in P7 and 8 week rats that received a saline injection. There is a decrease in c-myc expression between P2 and P7 and P2 and 8 weeks (n=4, *p<.05 vs. P2).

In order to see if this expression pattern was affected by sympathetic signaling, we examined c-myc expression between control and 6-OHDA lesioned hearts. RNA was extracted from control and 6-OHDA lesioned rat hearts at P2, P7, and 8 weeks, and assayed for c-myc

expression. We saw a significant decrease in c-myc expression at P2 in lesioned animals as compared to control animals (**Fig. 3A**, average fold change = .49 ± .14, n=4, p = .036). This suggests that c-myc expression, early on in development, may be regulated by sympathetic signaling, and may serve as a genetic mechanism through which sympathetic signaling regulates cardiac myocyte proliferation. By P7, there was no significant difference in c-myc expression between the lesion and control hearts, which is consistent with the pattern of cell cycle withdrawal in cardiac myocytes. At eight weeks however, c-myc is up regulated in lesioned hearts as compared to control hearts (**Fig. 3B**, average fold change = 2.99 ± .68, n=6, p = .03). C-myc has been shown to be up regulated in pathological states (Wolfram et al., 2011); therefore, the increase in c-myc at 8 weeks could be an early indicator of future cardiac dysfunction.

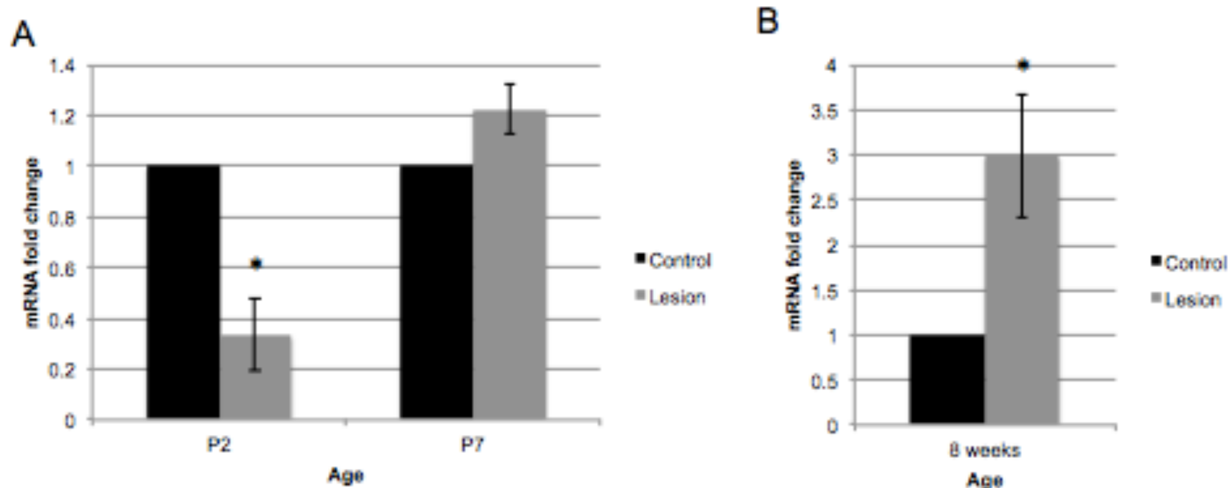

Fig. 3. Sympathetic signaling decreases the expression of the transcription factor c-myc early in postnatal development. A) Bar plot comparing levels of c-myc in rats given a sham (black) or 6-OHDA (gray) injection at P0. Chemical sympathectomy at P0 leads to a decrease in c-myc expression at P2 (n=4, *p<.05 vs sham). B) Bar plot comparing levels of c-myc in the same two conditions at 8 weeks. C-myc is up-regulated at 8 weeks (n=6, *p<.05 vs. sham).

In addition to looking at c-myc as a marker for cellular proliferation, we also looked at c-fos as

a marker for cellular hypertrophy. It has been shown that the expression of c-fos increases with cardiac myocyte age (Komuro et al., 1988), a pattern that is consistent with cardiac myocyte cell cycle withdrawal and the initiation of growth via hypertrophy. Thus, we hypothesized that c-fos expression may be regulated by sympathetic signaling. However, results from qPCR experiments were inconclusive.

Sympathetic signaling *in vivo* regulates the expression of the ciliary protein ALMS1. While c-myc is a gene that is associated with the proliferation and differentiation of many different cell-types, ALMS1 is a gene that has recently been identified as a specific regulator for cardiac myocyte proliferation (Shenje et al., 2014). Recent evidence has shown that knocking down ALMS1 in cultured neonatal mice cardiac myocytes results in increased cardiac myocyte proliferation (Shenje et al., 2014). This evidence led us to hypothesize that sympathetic signaling may regulate the expression of ALMS1.

We started off by looking at the expression of ALMS1 in control hearts at P2, P7, and 8 weeks. Our results showed no significant change in ALMS1 expression over this time course. This result demonstrates that ALMS1 levels stay relatively stable through the course of the developmental transition in cardiac myocytes (**Fig. 4**).

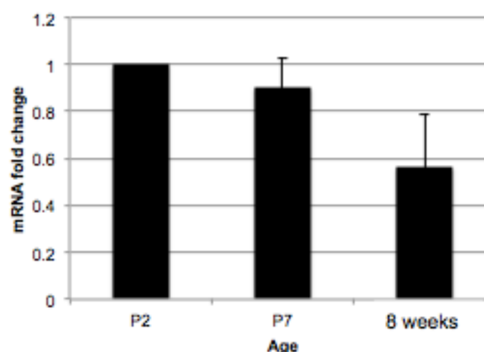

Fig. 4. ALMS1 expression remains relatively stable in control animals. ALMS1 expression was compared between P7 and 8 week old animals that received a saline injection. There was no significant change in the expression of ALMS1 between P2 and P7 and P2 and 8 weeks (n=4).

In order to see if expression was affected by sympathetic signaling we then examined ALMS1 expression between control and 6-OHDA lesioned animals. RNA was extracted from control

and 6-OHDA lesioned rat hearts at P2, P7, and 8 weeks. We then assayed levels of ALMS1 expression using qPCR. There was no significant difference in expression at P2 and P7 between lesion and control hearts; however, ALMS1 expression was significantly increased at 8 weeks in lesioned animals as compared to control animals (**Fig. 5**, average fold change = 2.41 ± .47, n=6, p =.03). This result is interesting because it suggests ALMS1 is not involved in regulating cardiac myocyte proliferation with sympathetic signaling in early postnatal development. But by 8 weeks sympathetic signaling interacts with ALMS1 to maintain stable low levels of the protein. Without this sympathetic signaling, ALMS1 is up regulated.

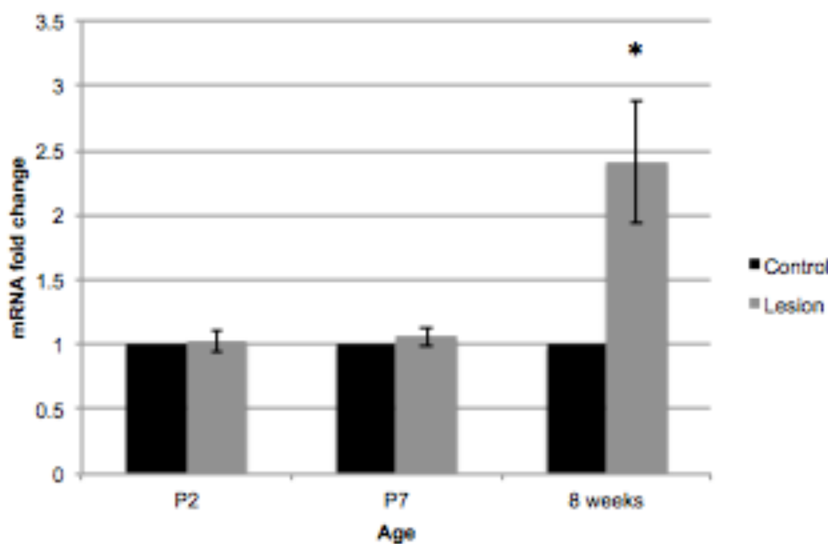

Fig. 5. ALMS1 expression increases later on in postnatal development. This bar plot compares the levels of ALMS1 in rats given a sham (black) or 6-OHDA (gray) injection at P0. Chemical sympathectomy at P0 leads to an increase in ALMS1 expression at 8 weeks (n=6, *p<.05 vs sham).

Sympathetic signaling *in vivo* regulates the expression of the transcription factor Meis1.

Like ALMS1, Meis1 has recently been pinpointed as a regulator of cardiac myocyte proliferation. It has been shown that the overexpression of Meis1 in mice cardiac myocytes drives cell cycle withdrawal (Mahmoud et al., 2013). Thus, we asked the question of whether the expression of Meis1 was affected by sympathetic signaling *in vivo*. In order to test our

hypothesis, the expression of Meis1 was compared between control and 6-OHDA lesioned hearts.

Rebecca Kreipke first compared the expression of Meis1 in control hearts at P2, P7, and 8 weeks. She found that there was a significant increase in Meis1 expression between P2 and P7 (**Fig. 6A**). This result is consistent with previous results in Mahmoud et al., and coincides with the pattern of cell cycle withdrawal in cardiac myocytes. Interestingly, Rebecca also found that Meis1 expression at 8 weeks returned to the levels seen at P2, which suggests that Meis1 levels are not maintained in the postmitotic period in mycoytes (**Fig. 6A**).

Next, Rebecca Kreipke looked at whether this expression pattern of Meis1 changed as a result of sympathetic signaling. She extracted RNA from control and 6-OHDA lesioned hearts at P2, P7, and 8 weeks and assayed levels of Meis1 using qPCR. She found that sympathetic lesion at P0 resulted in an increase in Meis1 mRNA in lesion animals at P2 and P7 as compared to the control animals (**Fig. 6B**). In addition, Meis1 levels between control and lesion animals did not significantly differ at 8 weeks. These results suggest that Meis1 may coordinate sympathetic signaling and regulate cardiac myocyte cell cycle withdrawal at a specific developmental time point.

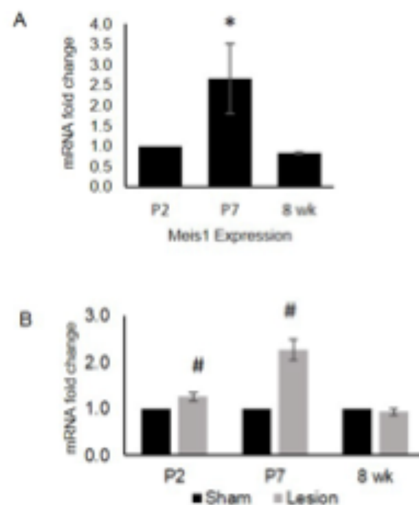

Fig. 6. Meis 1 is up-regulated following sympathetic lesion *in vivo*. A) Meis 1 increases in expression between P2 and P7, but returns to early developmental levels by 8 weeks. B) Lesioning the sympathetic nervous system at P0 leads to increased levels of Meis 1 mRNA at P2 and P7 as compared to sham animals. By 8 weeks, there is no difference in levels of Meis 1 expression (*p<.05 vs. P2; #p<.05 vs. sham). Data is taken from **Kreipke RE, Birren SJ. Innervating Sympathetic neurons regulate heart size and timing of cell cycle withdrawal. *J Physiol.* 2015; 593(23): 5057-73.**

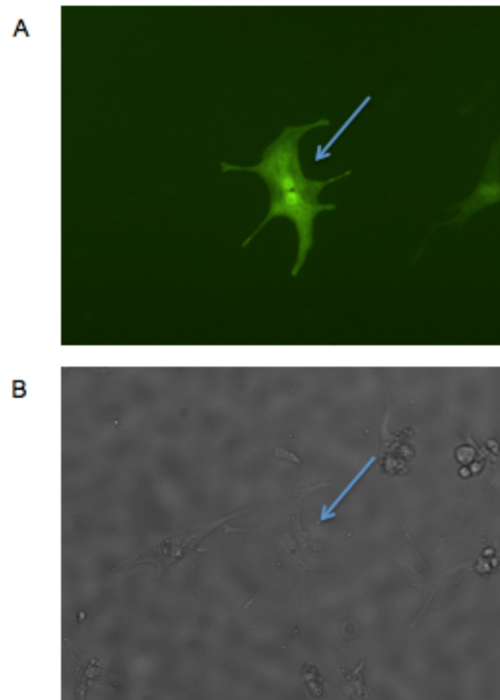

Fig. 7. Successful transfection of cardiac myocytes with GFP plasmid. A) The blue arrow points to a cardiac myocyte from a myocyte only culture at 3 DIV which is fluorescent due to the GFP transfection. B) The blue arrow points to the same cardiac myocyte seen in A and shows other myocytes in the vicinity that have not been transfected.

This result led us to investigate the mechanism of Meis1 and sympathetic regulation. For these experiments we returned to an *in vitro* co-culture system because of the feasibility of manipulating the expression of Meis1. Developing a co-transfection procedure proved to be challenging because of the sensitivity of the developing cardiac myocytes. Cardiac myocytes were sensitive to the reduced serum medium, the amount of GFP plasmid, and the amount of lipofectamine used. Through troubleshooting, a successful procedure was developed that resulted in GFP fluorescent myocytes (**Fig. 7**). In the future we hope to harness this procedure and conduct experiments where Meis1 expression is manipulated in the presence and absence of sympathetic neurons and the effect on myocyte proliferation is observed.

In these transfection experiments we will induce Meis1 knockdown or overexpression using siRNA or a Meis1 overexpression plasmid. All of theses experiments will be done at 2 DIV because that is the time point where we see the biggest effect of sympathetic signaling on cardiac myocyte proliferation. In the first set of experiments, Meis1 will be overexpressed in both myocyte only cultures and co-cultures. The proliferative capacity of these myocytes will be compared to the proliferative capacity of non-transfected myocytes cultured alone or in co-

culture conditions. We hypothesize that overexpressing Meis1 in myocyte only cultures will recapitulate the results seen in Mahmoud et al., where we will see decreased proliferation. Overexpressing Meis1 in co-cultures will give us some insight into whether Meis1 is involved in the same signaling pathway as sympathetic signaling or a different signaling pathway. If Meis1 is in the same signaling pathway as sympathetic signaling, then sympathetic signaling may act to decrease the level of Meis1, which in turn may result in increased cardiac myocyte proliferation. We hypothesize that if Meis1 is in the same signaling pathway as sympathetic signaling, then the proliferative capacity of transfected cardiac myocytes in co-cultures should be reduced to that of non-transfected myocytes cultured alone. This is because overexpressing Meis1 will essentially counteract the effect of sympathetic signaling on Meis1 expression. Meis1 levels will remain elevated, and cardiac myocyte proliferation will be decreased. However, if Meis1 impacts proliferation through an alternate signaling pathway, then we hypothesize that some intermediate level of proliferation will be observed. This is because overexpressing Meis1 may have a negative effect on myocyte proliferation, but that negative effect will be somewhat counteracted by the separate effect of sympathetic signaling, which has been shown to increase cardiac myocyte proliferation.

In the next set of transfection experiments we will knockdown Meis1 in myocyte only cultures and co-cultures and look at how the proliferative capacity of cardiac myocytes is affected. We hypothesize that knocking-down Meis1 in myocyte only cultures will recapitulate the results seen in Mahmoud et al., where an increase in proliferation will be observed. Sympathetic signaling in co-cultures has also been shown to increase the proliferative capacity of cardiac myocytes. Thus, it will be interesting to compare the level of proliferation in these transfected myocyte only cultures to proliferation levels in non-transfected co-cultures, to see whether

sympathetic signaling is enough to drive cardiac myocytes to maximum proliferative capacity. It will then be interesting to see the effect of Meis1 knockdown in cardiac myocytes cultured in the presence of sympathetic neurons. Together, all of these experiments will start to define the role of Meis1 and the pathway through which it acts to affect cardiac myocyte proliferation.

DISCUSSION

Early in postnatal development cardiac myocytes undergo a developmental transition in which they withdraw from the cell cycle and go on to grow via cellular hypertrophy. In this work we have shown preliminary data that is consistent with previous work in the lab, which shows that sympathetic signaling increases the proliferative capacity of cardiac myocytes in the early postnatal period (**Fig. 1**). We have also begun to uncover potential genetic mechanisms through which sympathetic signaling may regulate the proliferative capacity of cardiac myocytes at different points in development. C-myc has been identified as a potential mechanism through which sympathetic signaling may regulate cardiac myocyte proliferation early in postnatal development, around P2 (**Fig 3**). In contrast, Meis1 has been identified as a potential cell-cycle regulator that may act in coordination with sympathetic signaling around P7, when cardiac myocytes are about to withdraw from the cell cycle (**Fig. 6B**). Finally ALMS1 is a potential regulator that shows a unique pattern of expression and may not be involved in regulating cardiac myocyte proliferation in coordination with sympathetic signaling in early postnatal development (**Fig. 5**). All of this work together begins to define the regulatory role of the sympathetic nervous system in the development of the heart.

The final number of cardiac myocytes in the adult heart has a great impact on cardiac function (Porrello et al., 2008; Chang et al., 2010). It has been shown that having too many or too few myoyctes results in pathological consequences (Levkau et al., 2008; Chang et al., 2010). Our experiments *in vitro* have shown a strong trend at 2 days *in vitro* (DIV), where sympathetic signaling increases the proliferative capacity of cardiac myocytes (**Fig. 1**). In addition there is a trend seen at 3 DIV and 5 DIV, where cardiac myocytes that have been exposed to sympathetic signaling show an increased proliferative capacity as compared to cardiac myocytes cultured alone. These data suggest that sympathetic signaling ramps up the proliferative capacity of cardiac myocytes, allowing them to proliferate at a higher rate before they withdraw from the cell cycle. These data provide a potential explanation for a previous result seen in the lab, where denervated hearts are smaller than control hearts. In the absence of sympathetic signaling cardiac myocytes proliferate at a lower rate in the early postnatal period, resulting in fewer cells making up the smaller hearts. This data suggests an important regulatory role for the sympathetic nervous system that could have implications for cardiac function.

In order to further investigate the mechanisms that underlie this proliferative regulation, we looked at molecules that have been implicated in regulating the cell cycle in cardiac myocytes. The immediate early gene c-myc encodes a transcription factor that is involved in a broad array of developmental processes. However, c-myc has been identified as a regulator of cellular proliferation and differentiation in many systems, including the cardiac system (Gonda & Metcalf, 1984; Schneider et al., 1986; Witzgal et al., 1994). In normal development, c-myc expression decreases with cardiac myocyte age (Komuro et al., 1988). This pattern of expression corresponds to cardiac myocyte cell cycle withdrawal, and is

consistent with the results we have shown (**Fig. 2**). A significant body of work has pinpointed c-myc as a regulator of cardiac myocyte proliferation, where transgenic mice over-expressing c-myc show cardiac myoyctes with a greater proliferative capacity (Jackson et al., 1990). This evidence suggests that early in postnatal development c-myc is a good marker for cardiac myocyte proliferation. Thus, we asked whether c-myc expression was regulated by sympathetic signaling *in vivo*. Our results showed that early on in postnatal development c-myc is down regulated in denervated hearts (**Fig. 3A**). This fits the model that has been established, where cardiac myocytes that are not exposed to sympathetic signaling have a decreased proliferative capacity as compared to myocytes that have been exposed to sympathetic signaling. By P7, there is no significant difference in c-myc levels between control and lesioned hearts (**Fig. 3A**). This result is consistent with the pattern of cardiac myocyte cell cycle withdrawal seen in the *in vitro* data, where both myocytes that are exposed to sympathetic signaling and those that aren't start withdrawing from the cell cycle at P7 (**Fig. 1**). Our data suggests that in the early postnatal period c-myc may act in coordination with exogenous sympathetic signals to mediate cardiac myocyte proliferation.

C-myc's role later on in postnatal development may differ from its role in early postnatal development. Over expression of c-myc in the adult heart has been shown to promote hypertrophy in cardiac myocytes (Xiao et al., 2001; Robbins et al., 1992). In addition, C-myc has been shown to be up regulated in states of pathological hypertrophy (Wolfram et al., 2011). Our data shows a significant increase in c-myc expression in lesioned hearts at 8 weeks as compared to control hearts (**Fig. 3B**). This increase in c-myc could be an indication that c-myc switches roles in postnatal development, and may regulate hypertrophy in cardiac myocytes. This result coincides with the developmental switch seen in the role of

norepinephrine. Previous work in the lab has shown that cardiac myocytes in early postnatal development do not change size in response to isoproterenol (a norepinephrine agonist); however, by 24 DIV there is significant increase in cardiac myocyte size in response to isoproterenol (Kreipke & Birren, 2015). This result suggests that the role of sympathetic signaling is developmentally dependent, similar to c-myc. Furthermore, this increase in c-myc could also be an early indication of pathological dysfunction. Preliminary work from the lab shows that lesioned hearts at 8 weeks have an increased action potential duration and a decreased conduction velocity as measured by (Kreipke and Njon, unpublished). Both of these changes are thought to be signs of developing arrhythmias and are similar to changes seen in failing hearts (Tomaselli et al., 1994). Overall, c-myc may interact with sympathetic signaling at specific points in development to impact cardiac myocyte development differently and may even be involved in pathological developments in cardiac function.

While c-myc is a transcription factor that is involved in a broad array of developmental processes, Meis1 is a transcription factor that has been specifically linked to cardiac myocyte cell cycle withdrawal (Mahmoud et al., 2013). Cardiac myocytes that over-express Meis1 are prompted to withdraw from the cell cycle sooner (Mahmoud et al., 2013). Furthermore, in Meis1 knockout mice, cardiac myocytes continue to proliferate beyond their normal window (Mahmoud et al., 2013). Previous work by Rebecca Kreipke shows that denervated hearts have increased levels of Meis1 in the early postnatal period (Kreipke & Birren 2015; **Fig. 6B**). This is the same period in which sympathetic innervation extends the proliferative capacity of cardiac myocytes. This evidence suggests that Meis1 may interact with sympathetic signaling to regulate cardiac myocyte cell cycle withdrawal.

In order to place Meis1 within a pathway, *in vitro* transfection experiments were designed. By manipulating the expression of Meis1 in cardiac myocytes in the presence or absence of sympathetic neurons we can further discern the role of Meis1 and its interaction with sympathetic signaling. Through extensive troubleshooting, a successful co-transfection procedure has been developed that results in GFP fluorescent cardiac myocytes (**Fig. 7**). In the future we hope to harness this procedure and conduct experiments where Meis1 is knocked-down or overexpressed in cultures containing either myocytes alone or both myocytes and neurons. These experiments will hopefully recapitulate the results seen *in vivo* by Mahmoud et al. In addition, through these experiments we will be able to uncover if Meis1 is involved in the same pathway as sympathetic signaling.

The final cell-cycle regulatory protein that we identified was ALMS1. ALMS1 is a protein that has been implicated in cardiac myocyte cell cycle control and cardiomyopathies. Specifically, knockdown of ALMS1 in murine cardiac myocytes results in increased cardiac myocyte proliferation (Shenje et al., 2014). Thus, we asked whether ALMS1 expression was regulated by sympathetic signaling *in vivo*. Our data showed no significant difference in ALMS1 expression between lesion and control hearts at P2 and P7 (**Fig. 5**). This suggests that ALMS1 does not interact with sympathetic signaling to regulate cardiac myocyte proliferation in early postnatal development. However, at 8 weeks our data showed a significant increase in ALMS1 expression in lesioned hearts as compared to control hearts (**Fig. 5**). This result is interesting because at 8 weeks, sympathetic signaling is not necessarily involved in mediating cardiac myocyte proliferation since cardiac myocytes are terminally withdrawn from the cell cycle. It is possible that later on in postnatal development ALMS1 switches from its

role as a cell cycle regulator and is regulated by sympathetic signaling in some alternate functional pathway.

Together this work supports the model defined by previous work in the lab, which shows that sympathetic signaling increases the proliferative capacity of cardiac myocytes before they withdraw from the cell cycle. In addition, we have started to define molecules like c-myc and Meis1 that may be genetic mechanisms through which sympathetic signaling impacts cardiac myocyte development. All of the work presented here defines the effect of chemical sympathectomy within a limited postnatal period. In the future it will be interesting to explore the effect of early chemical sympathectomy on adult cardiac function.

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