The role of sympathetic innervation in the development of postnatal cardiomyocytes

Master’s Thesis

Presented to

The Faculty of Graduate School of Arts and Sciences

Brandeis University

Graduate Program in Molecular and Cell Biology

Susan Birren, Advisor

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in

Molecular and Cell Biology

by

Sagarika Utture

February 2015
I would sincerely like to thank Dr. Susan Birren for her support and guidance throughout my project. I am very grateful to Rebecca Kreipke for her valuable feedback, guidance and the training she provided. I would also like to thank Surbhi Sona for taking time to train and advise me on the Western Blot process. I extend my gratitude to all the other members of the Birren Lab for their support and discussions on my work.
ABSTRACT

The Role of sympathetic innervation in the development of postnatal cardiomyocytes

A thesis presented to the Graduate Program in Molecular and Cell Biology

Graduate School of Arts and Sciences
Brandeis University
Waltham, Massachusetts

By Sagarika Utture

Cardiomyocytes exhibit two distinctive modes of growth during development. Prenatal and early postnatal myocytes grow by the process of cell proliferation / hyperplasia and around the third day post birth; the cells exit the cell cycle irreversibly and grow via an increase in cell size/ hypertrophy (Ahuja et al., 2007). This critical transition establishes the number of cardiomyocytes and affects the ability of the heart to recover from injury. Yet, despite the importance of this developmental process the transition from hyperplasia to hypertrophy is not very well understood. One potential candidate for the regulation of this transition is the sympathetic nervous system. It is known that sympathetic innervation plays a vital role in the development of the heart and other systems; however the precise mechanism of the role is not well defined. To understand the mechanisms involved in the development of these myocytes, the role of sympathetic innervation in the development of postnatal cardiomyocytes was investigated.
I investigated the role of the sympathetic system in cardiomyocyte proliferation using both *in vivo* and *in vitro* systems. In the *in vitro* paradigm, myocytes were cultured with and without sympathetic neurons to study the effect of the neurons on the myocyte development. The sympathetic system exerts its effect via the release of noradrenaline (Dowell, 1985). I investigated whether the effects of sympathetic neurons were mediated by β-adrenergic signaling by using an agonist, isoproterenol and antagonist, propranolol to stimulate and block the noradrenergic neurotransmission. These experiments revealed that the sympathetic innervation promotes cell proliferation and delays myocyte hypertrophy via β-adrenergic signaling. It was found that the sympathetic innervation is necessary and sufficient to delay cell hypertrophy.

Hypertrophy is also characterized by change in levels of proteins in the cell. Proteins were extracted from control and experimental rats (sympathetic activity ablated) at different time points (*in vivo*). Quantification of vital protein expression levels, mainly cfos, was performed with the help of Western blot analysis. Preliminary results showed that there was an increase in the cfos protein level when 7 day lesion hearts were compared to the control. Additional experiments would need to be performed to establish these results.

However the ability of sympathetic innervation to alter cardiomyocyte growth indicated that the sympathetic nervous system has a direct causal role in the regulation of heart growth. The study of such mechanisms increases our knowledge about the underlining developmental processes which affect the ability of the heart in the adult stage. This may lead to potential therapeutic targets and provide an insight on the mechanism responsible for programing of adult cardiovascular diseases.
# TABLE OF CONTENTS

1. Abstract iii  
2. List of Tables vi  
3. List of Figures vii  
4. List of Abbreviations viii  
5. Introduction 1  
6. Material and Methods 6  
7. Results 12  
8. Discussion 23  
9. References 27
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Experimental conditions and controls used for 2D and 5D cultures</td>
<td>8</td>
</tr>
<tr>
<td>Table 2</td>
<td>Summary of conditions used for 4D cultures</td>
<td>8</td>
</tr>
<tr>
<td>Table 3</td>
<td>Primary and corresponding secondary antibodies used in Western Blot Analysis</td>
<td>11</td>
</tr>
</tbody>
</table>
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Timeline used to culture cardiomyocytes</td>
<td>8</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Microscopic analysis of immunostained myocytes in culture</td>
<td>13</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Quantification of cardiomyocyte cell proliferation for cultures fixed and stained after 2 days (2D) (N=5)</td>
<td>14</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Sympathetic innervation promotes cell proliferation in 5D (N=5) and 4D (N=3) cultures. Co-culture vs Myocytes only.</td>
<td>15</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Quantification of cardiomyocyte cell proliferation of 5D and 4D culture. Coculture control vs Co-culture + P</td>
<td>16</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Quantification of cardiomyocyte cell proliferation. Co-culture control vs Co-culture + IP</td>
<td>17</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Quantification of cardiomyocyte cell proliferation. Myocytes only control vs Myocytes only + IP</td>
<td>18</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Quantification of cardiomyocyte cell proliferation of 5D (N=5). Myocytes only control vs Myocytes only + P</td>
<td>18</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Sympathetic innervation delays hypertrophy. Average cell size analysis of 5D (N=5) and 4D (N=3). Co-culture vs Myocytes only.</td>
<td>19</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Average cell size analysis of Coculture control vs Co-culture + P</td>
<td>20</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Average cell size analysis of Myocytes only control vs Myocytes + IP</td>
<td>21</td>
</tr>
<tr>
<td>Figure 12</td>
<td>cfos protein expression in heart tissue extracts (Lesion hearts vs control).</td>
<td>22</td>
</tr>
</tbody>
</table>
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCG</td>
<td>Superior Cervical Ganglia</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Saline Buffer</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve Growth Factor</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>NDS</td>
<td>Normal Donkey Serum</td>
</tr>
<tr>
<td>NPG</td>
<td>N-propyl gallate</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris Buffered Saline Tween 20</td>
</tr>
<tr>
<td>IP</td>
<td>Isoproterenol</td>
</tr>
<tr>
<td>P</td>
<td>Propranolol</td>
</tr>
</tbody>
</table>
INTRODUCTION

Heart disease is the leading cause of death in the United States, claiming around one million lives annually. Adult development of cardiovascular disease is linked to events during prenatal and early postnatal phases (McMillen and Robinson, 2005). Increase in cell size / cardiac hypertrophy, is an essential predictor of cardiovascular morbidity and mortality (Levy et al., 1990). Yet the origin and developmental mechanisms that drive these diseases is not well understood. There is growing evidence that dysfunction of the neural formation in a developing organ might be instigating factor in the pathogenesis of that organ (Borden et al., 2013). Previous studies indicate that the number of cardiomyocytes in the heart play an important role in responding to demands of increased workload (Porrello et al., 2008), suggesting that an understanding of cardiomyocyte development could shed light on important underlying factors in heart disease.

Cardiomyocytes exhibit two distinctive developmentally regulated growth processes. Fetal and early postnatal hearts grow by active division of cardiomyocytes, or hyperplasia. Around the third or the fourth day after birth, the cardiomyocytes withdraw from cell cycle and are arrested in the G1 phase, irreversibly blocking mitosis (McGill and Brooks, 1995). Thereafter, heart growth takes place via increases in individual cardiomyocyte cell size, or hypertrophy (Ahuja et al, 2007). According to Li et al., 1996, a rapid switch from hyperplasia to hypertrophy occurs during the 3rd or the 4th day after parturition in rat pups. The molecular mechanisms underlining the switch from hyperplasia to physiological hypertrophy in the
developing heart is not fully understood, yet the final number of cardiac myocytes in the adult heart is dependent upon the timing of this transition.

Sympathetic innervation is known to contribute to morphogenesis of the target organ / tissue (Glebora and Ginity, 2004). Hence investigation of the role of sympathetic innervation in postnatal cardiomyocyte development may shed light on the mechanisms underlying the transition from hyperplasia to hypertrophy. Since this critical transition establishes the number of cardiomyocytes in the heart, which affects its ability to recover from injury, study of this regulatory mechanism will provide insight to develop potential therapeutic approaches.

Norepinephrine is a neurotransmitter released by the sympathetic neurons and plays a key role in regulating cardiac development (Dowell, 1985; Olson and Schneider, 2003). Sympathetic activity is mediated via norepinephrine binding to the adrenergic receptors and activating β-adrenergic signaling. Isoproterenol (IP) is a β-adrenergic agonist which is used to stimulate the sympathetic activity in cultured cells (Akuzawa-Tateyama et al., 2006). Conversely, propranolol (P) is a β-adrenergic antagonist which is known to block sympathetic activity (Tseng et al., 2001). Both of these drugs are used in this study to investigate the role of β-adrenergic signaling in cardiac development.

The switch from hyperplasia to hypertrophy is characterized by the change in DNA levels and protein content. Schneider et al., 1986, have shown that mRNA levels of cmyc were abundant in the embryo stage of the rat pups and consequently decrease by the first week after birth. Conversely the cfos gene is said to be induced during hypertrophy (Izumo et al., 1988). Therefore, one would expect the levels of cmyc protein to decline and cfos protein level to increase post birth.
Previous work- Birren Laboratory:

Work in the Birren laboratory has shown that eliminating the sympathetic activity via chemical sympathectomy achieved by 6-hydroxydopamine (6-OHDA) treatment of newborn rat pups, significantly reduced heart size as compared to sham injected pups (Kreipke et al., unpublished). This indicates that sympathetic innervation plays a role in the development of the heart. The decrease in heart size in rat pups with no sympathetic input could be due to the absence of sympathetic input affecting the modes of heart tissue growth at early development times. If so, sympathetic innervation could play a role in either increasing cardiomyocyte cell proliferation, or by affecting hypertrophy, or by a combination of both mechanisms.

Previous studies (Birren at al., unpublished) indicate that in vitro experiments at day 3 and 4 post culturing the myocytes grown with sympathetic neurons were significantly smaller in size and had a higher proliferating percentage of myocytes when compared to the myocytes only cultures. This suggests that sympathetic neurons may play a role in promoting cardiomyocyte proliferation and delaying their switch to hypertrophy. These experiments were performed using phosphor-histone H3 protein as a marker for cell proliferation. However, this marker labels only myocytes in a particular cell cycle phase and may not label other proliferating myocytes which happen to be in a those stages of cell cycle thus marking less than 20% of the myocytes in cultures.

As molecular changes take place during the transition from proliferation to hypertrophy, the m-RNA levels of key molecular markers such as transcription factors (cmyc, cfos), have been shown to vary (Birren et al., unpublished). The next step here would be to evaluate the change in expression levels of the proteins in presence and absence of sympathetic innervation.
Therefore, in this study I will be investigating the role of sympathetic innervation in the regulation of postnatal cardiomyocyte development. For my in vitro studies, cardiomyocytes were isolated from rat hearts on day 0 (birth), and cultured to study the effect of sympathetic innervation on cardiomyocyte hypertrophy at 2 day, 4 day and 5 days post culturing. Myocytes were cultured either in presence (Co-culture) or absence (myocytes only) of sympathetic neurons and percentage of proliferating myocyte cells as well as average size of myocytes was compared. This would tell us whether the sympathetic innervation plays a role in the development of heart.

Due to the phosphor-histone H3 protein labeling limitation, a new approach was designed using an alternative proliferating marker, Ki-67 to verify these results. Ki-67 is a protein expressed during the different stages of the cell cycle except at the G0/rest phase. Thus, only mitotically proliferating cells get labeled with this marker, leading to a more comprehensive result for comparison.

To further narrow down whether the effects observed in the cultures are specifically due to sympathetic activity and no other factor and also to confirm if the effect is mediated via β-adrenergic signaling, the isoproterenol and propranolol drugs were used to evaluate whether the same results could be mimicked. Two main parameters were used to evaluate the effect: percentage of cells proliferating and average cell size. Co-cultures were compared to myocyte only cultures. Isoproterenol drug was used to mimic the sympathetic activity and propranolol drug was used to block the activity. The results from these experiments help demonstrate the role of sympathetic innervations and the mechanism by which it has its effect as well as exclude any additional factors playing a role.

Since hypertrophy is characterized by molecular changes in the cell, western blot analysis was used to determine if there is any significant change in the protein expression, particularly of
cfos transcription factor during the early stages of heart development. Sympathetic innervation was shown to promote cell proliferation and delay hypertrophy, and since hypertrophy induces cfos, we would expect the levels of cfos protein to increase in absence of the innervation. Thus the protein expression levels would vary in presence and absence of the sympathetic activity. The levels of proteins from hearts of experimental and control rat pups were compared. The experimental rat pups were injected with 6-OHDA to ablate sympathetic activity, which would allow us to assume that cardiomyocytes enter hypertrophy at a faster rate and induce cfos as compared to control rat hearts, injected with saline. Proteins were extracted from 2 day and 7 day experimental and control hearts and subjected to western blot analysis.
MATERIAL AND METHODS

Animals
Sprague-Dawley neonatal rats were used to obtain cardiomyocytes and superior cervical ganglion for the immunocytochemistry studies. Chemical sympathectomy was performed by administrating 6-hydroxydopamine (6-OHDA, 100mg/kg) (Sigma) to neonatal rat pups 24 hours post birth and hearts were extracted at day 2 and day 7 to quantify loss of sympathetic innervation of the heart. Control animals were injected with saline.

Cell Culture
Cells were obtained through the dissection of newborn rat pups (dissection and cell plating were performed by PhD candidate Rebecca Kreipke and methods used were as previously described in Luther and Birren, 2009). Cardiomyocytes were obtained from the lower left ventricle and sympathetic neurons from the superior cervical ganglia (SCG). Prior to plating the cells, glass bottomed dishes (MatTek Corporation) were coated with collagen (50μg/mL) for 20 minutes and rinsed 3 times with phosphate buffer solution (PBS 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4 ). 2mL MAH food (25mL Fetal Bovine Serum, 10mL 1:1:2 (0.3% Dextrose, 0.5% glutamine and 0.5% Penn/Strep (GIBCO) in water); and 5 mL FVM (10 μg/mL DMPH4 (Calbiochem), (50 μg/mL glutathione and 1 mg/mL ascorbic acid in distilled water; set pH between 5-6 using 0.5 N KOH) in 250 mL L15CO2)) supplemented with 5μg/mL
of Nerve Growth Factor (NGF) (BD Biosciences) was then added to the dishes containing the cells. Cultures were grown in presence (Co-culture) and absence (Myocytes only) of sympathetic neurons. Density was set at 50,000 myocytes per dish and supplied with 10,000 neurons for co-culture dishes. Cardiomyocytes were grown with and without sympathetic neurons and fixed at different time points. Cultures were treated with β-adrenergic agonist, isoproterenol (2μM, Sigma) and β-adrenergic antagonist, propranolol (10μM, Sigma). Cultures were fixed with 4% paraformaldehyde for 10 minutes at different time points post culturing (2D-2days, 4D-4 days and 5D-5days). The dishes were then rinsed with PBS and stored at 4°C for immunostaining.

**Experimental Design (Cell culture)**

Cardiomyocytes were grown in the presence (Co-culture) and absence (Myocyte only) of sympathetic neurons. The myocyte cell cultures were subjected to six different conditions run in duplicates; Co-culture controls (without drugs)- CoC1, Co C2, Co-culture with isoproterenol drug- Co IP1, Co IP2, Co-culture with propranolol drug- Co P1, Co P2, Myocytes only control cultures- Myo C1, Myo C2, Myocyte only with isoproterenol drug- Myo IP1, Myo IP2 and myocyte only with propranolol drug- Myo P1, Myo P2. Each condition was tested in duplicates. Post plating, the cultures were analyzed after 2 days (2D), 4days (4D) and 5 days (5D). Table 1 summarizes the condition details used in case of 2D and 5D cultures. Table 2 illustrates the conditions used to culture 4D cells. Figure 1 illustrates a timeline used for the cell culture experiments. The dishes were incubated at 37° C. The same experimental procedure was used to calculate the percentage of proliferating myocytes for the 5D (N=5) and the 4D time point (N=3). For the 5D and 4D cultures, the media was changed after 2 days, fresh MAH food and
NGF was added. The isoproterenol and propranolol drugs were added to the respective plates and incubated at 37°C.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myocyte only</td>
<td>Myo C1</td>
<td>Myo C2</td>
<td>Myo IP1</td>
<td>Myo IP2</td>
<td>Myo P1</td>
<td>Myo P2</td>
</tr>
<tr>
<td>Coculture</td>
<td>Co C1</td>
<td>Co C2</td>
<td>Co IP1</td>
<td>Co IP2</td>
<td>Co P1</td>
<td>Co P2</td>
</tr>
</tbody>
</table>

Table 1: Experimental conditions and controls used for the 2D and 5D cultures. Conditions: Myocytes only culture controls, Myocytes with drug IP, myocytes with drug P, Coculture (Myocytes + sympathetic neurons) controls, and co-culture with drug IP and co culture with drug P. All conditions were tested in duplicates.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myocyte only</td>
<td>Myo C1</td>
<td>Myo C2</td>
<td>Myo IP1</td>
<td>Myo IP2</td>
</tr>
<tr>
<td>Coculture</td>
<td>Co C1</td>
<td>Co C2</td>
<td>Co P1</td>
<td>Co P2</td>
</tr>
</tbody>
</table>

Table 2: Summary of conditions used for the 4D cultures. Myocytes only controls, myocytes with drug IP, co-culture controls and co-culture with drug P. Each condition was performed in duplicate.

Figure 1: Timeline used to culture cardiomyocytes. The dishes were incubated at 37°C. Cardiomyocytes were extracted from rat pups at day 0, incubated with MAH food, NGF and respective drugs. 2D cultures were fixed and stained after 2 days. 4D and 5D cultures were replenished with fresh media and drugs on day 2. 4D were fixed and stained on day 4, 5D on day 5.
Immunocytochemistry

Fixing and staining

Cell cultures were plated at a density of 10,000 neurons and 50,000 myocytes per dish. Cells were fixed at 2D, 4D and 5D time points with 4% paraformaldehyde (PFA). The cells were then rinsed with PBS and permeabilized by incubating it for 10 minutes in permeabilizing solution (0.1% NP40 and 1% Donkey serum (Gibco) in PBS). Followed by blocking for 30 minutes with 10% Donkey Serum and rinsed with PBS. The dishes were then incubated in primary antibody 1% NDS containing rb-α-Ki67 (Abcam); 1:1500 and ms-α-Actinin (Sigma); 1:800 for an hour at room temperature. Excess antibody was washed off using PBS. Secondary fluorescent antibody 1% NDS containing dk-anti-rb-FITC (Abcam); 1:600 and dk-anti-ms-Rhodopsin (Abcam); 1:600 incubation was carried out for 45 minutes at room temperature. Post incubation dishes were rinsed with PBS and incubated for 10 minutes in 0.1% DAPI (Invitrogen Life Technologies) solution at room temperature to stain the nuclei. A final PBS wash was performed and the cells were submerged in 1mL N-propyl gallate (NPG) to protect from photobleaching and stored at -20°C.

Image Acquisition and statistical analysis

Fixed and stained cells were imaged using the 20X objective on an Olympus 1x81 fluorescence microscope. Volocity software was used to capture images. 60 random points were selected per dish and photographed through Rhodamine, FITC, and DAPI filters. Image J software was used to measure cell size and count proliferating myocytes. Myocytes were identified by the α-Actinin staining (red), proliferating myocytes were identified by positively double stained nuclei for Ki-67 (green) and DAPI stain (blue). Total number of Ki-67 positive myocyte nuclei was divided by
the total number of myocyte nuclei to determine percentage of proliferating myocytes. Cell size was calculated using area measurement on Image J. Excel was used to perform Students T-test (one tailed) to compare results and significance level. A probability of p<0.05 was considered to represent a significant difference.

Proliferation %= \frac{\text{total number of double labeled DAPI+ Ki-67 positive myocytes}}{\text{total number of DAPI myocytes}}\times 100

**Western Blot Analysis**

Hearts of normal and treated rats were harvested 2 days and 7 days post injection and frozen in liquid nitrogen. Heart tissue was homogenized in 500-600µL of ice cold RIPA lysis buffer (50mM Tris pH 7.5, 150 mM NaCl, 1% NP40, 0.5% DOC, 0.1% SDS, 2mM EDTA and Complete Mini Protease inhibitor cocktail (Roche Diagnostics, Germany, #1836170001). The tubes containing the homogenized tissue were incubated at 4°C in a rotating device for 30 minutes. The sample was then passed through 22-gauge needle and centrifuged (13,000rpm for 10 minutes at 4°C) to collect the supernatant. Protein concentrations were determined by Bradford Assay.

Samples were subjected to SDS-PAGE and transferred to either Nitrocellulose membrane (0.2 µM, Biorad) or PVDF membrane (0.2, µM, Biorad). The membranes were blocked using either 5% nonfat dry milk in PBS or 5% BSA in 1% TBST for an hour at room temperature. The membranes were incubated in primary antibody solutions for 1 hour at room temperature or overnight at 4°C. The membranes were then washed 4 times for 10 minutes each with TBS with 1% Tween 20 (1% TBST) and incubated in appropriate horseradish peroxidase conjugated secondary antibody for an hour at room temperature (Table 3). 1% TBST was used to wash off
secondary antibody. Blots were developed using LumiGLO Chemiluminescent Substrate (KPL# 546100) on Blue Devil X-ray Films (Genesee Scientific #30-100). Primary antibody was diluted using 5% BSA in TBST and secondary was diluted using 5% nonfat milk powder in TBST.

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Corresponding secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>rb-actin (1:5000; Odyssey # 92642210)</td>
<td>gt anti-rb HRP (1:7500; Jackson ImmunoResearch #111035144)</td>
</tr>
<tr>
<td>rb-cfos (1:1000; CST #4348)</td>
<td>gt anti-rb HRP (1:7500; Jackson ImmunoResearch #111035144)</td>
</tr>
<tr>
<td>ms-cmyc (1:1000; Santa Cruz #sc-40)</td>
<td>gt anti-ms HRP (1:7500; Jackson ImmunoResearch #111035144)</td>
</tr>
</tbody>
</table>

Table 3: Primary and corresponding secondary antibodies used in Western blot analysis.
RESULTS

To demonstrate the effect of sympathetic innervation, cell cultures were used to calculate percent of proliferation of the myocytes and measure average myocyte cell size. I investigated if sympathetic innervation regulates cardiomyocyte proliferation by examining the expression of the presence and absence of the proliferation marker, Ki-67 in the myocytes grown in presence and absence of co-cultured neurons. Each dissection/extraction of myocytes and SCG was considered as individual experiments. 5 such experiments were conducted (N=5) for 2D and 5D cultures and 3 experiments were conducted for the 4D cultures. Each experiment consisted of 3 different conditions in duplicates for myocyte only cultures and myocyte with sympathetic neuron cultures (co-cultures). The drug isoproterenol being an agonist was expected to mimic the condition of myocytes with sympathetic neurons when added to the myocyte only cultures and enhance the effect of sympathetic activity in co-cultures. The reciprocal results were expected when the antagonist drug propranolol was added ie to block the sympathetic activity. Percentage of proliferating myocytes was calculate and averaged to give the overall results. Average cell size of myocytes in each culture condition was compared to the control. The data for these experiments have been summarized in graphs in the following sections.

To determine if the sympathetic innervation has an effect on cardiomyocyte development, the co-cultures in presence of the sympathetic innervation was compared to the myocytes only culture, in absence of the innervation. Further to evaluate whether this effect is mediated via β-adrenergic signaling, the agonist isoproterenol and antagonist propranolol were added to the cultures. Figure 2 is a representative immunostaining image. As seen in the image, the α-Actinin
antibody specifically binds to the cardiac α-actinin, which forms a structural component of the contractile sarcomere. This staining results in a characteristic ladder-like pattern indicating the structure of the cardiomyocyte. The DAPI positive nuclei are stained in blue and the proliferating marker Ki-67 stained within the nucleus as green.

Figure 2: Microscopic analysis of immunostained myocytes in cultures. The cultures were stained for myocytes-specific marker α-Actinin (red), cell proliferation marker, Ki-67 (green). The cultures were also stained with DAPI (blue) to determine the total nuclei count.

No significant effect of sympathetic innervation on proliferation of myocytes was observed in the 2D cultures:

Cardiomyocyte cultures were grown in presence and absence of sympathetic neurons. Previous experiments showed that myocytes proliferated at a higher rate when grown in presence of sympathetic neurons at 2 and 5 days in vitro. However there was no difference in the rate of proliferation in presence or absence of sympathetic innervation in the experiment I performed.
The agonistic drug isoproterenol, did not exhibit any significant change when added to both culture conditions. Sympathetic blocking drug propranolol, which was expected to decrease percentage of proliferating myocytes when added to co-culture showed a limited effect which was not statistically significantly. Overall the 2D cultures showed a lot of variation between each experiment rendering them inconsistent to derive any statistically significant results (Figure 3). At the 2 day time point, my results were unable to confirm the previous results of the laboratory, either directly in co-cultures, or by perturbing β-adrenergic signaling. I therefore went on to examine the later time points.

Figure 3: Quantification of cardiomyocyte cell proliferation (N=5) for cultures fixed and stained after 2 days (2D). Percent of proliferating myocytes was calculated across 60 fields. Presence of sympathetic neurons showed no significant effect on percentage of cardiomyocyte in 2D cultures.
(a). The graph (b) shows the percentage of proliferating myocytes when supplied with sympathetic neurons. Control dishes are compared to dishes with the IP and P drug (c) similar graph shows results in absence of sympathetic neurons. Drug P seemed to reduce the percentage of proliferation but was not statistically significant when compared to the controls.

**Sympathetic innervation promotes cell proliferation in 4D and 5D cultures:**

**Co-culture vs Myocytes only**

To specifically determine the effect of sympathetic innervation on rate of myocyte proliferation at later times in development, the level of Ki67 staining was assessed in myocyte only cultures and compared to myocytes co-cultured with sympathetic neurons. At these time points, I found that the percentage of proliferating myocytes was higher in co-cultures compared to myocytes only. The difference was statistically significant (Students T test was used to calculate significance level) for both 4D and 5D cultures (5D p=0.0001; 4D p=0.008). This result is consistent with earlier work in the laboratory and indicates that sympathetic innervation promotes cell proliferation of cardiomyocyte.
Figure 4: **Sympathetic innervation promotes cell proliferation in 4D and 5D cultures.**
Quantification of cardiomyocyte cell proliferation of 5 day (N=5) and 4 day (N=3) cultures. Shows Myocytes supplied with sympathetic neurons have a higher rate of proliferation when compared to myocytes only culture. (a) 5D cultures (p=0.0001). (b) 4D cultures (p=0.008).

**Sympathetic activity is mediated through β-adrenergic signaling:**

Co-culture controls vs Co-culture + propranolol drug

To confirm a role for β-adrenergic signaling in the difference in rate of proliferation seen above, and to exclude the possibility of a role for other factors, the sympathetic antagonist drug propranolol was used. Here we would expect that co-cultures when supplied with propranolol would reduce the percentage of proliferating myocytes, mimicking an absence of sympathetic neurons. As seen in the graph, the expected result was confirmed. Co-cultures with the propranolol showed significant reduction in cell proliferation when compared to controls (5D p<0.005; 4D p=0.004; students T test).

![Graph](image)

Figure 5: Quantification of cardiomyocyte cell proliferation of 5D (N=5) and 4D (N=3). Drug P blocks the sympathetic activity and significantly reduces the percentage of proliferating myocytes in cocultures. (a) 5D cultures (p<0.05) (b) 4D cultures (p=0.004).
Co-culture controls vs Co-culture + isoproterenol drug

When co-cultures were grown with isoproterenol (agonist), an increase in cell proliferation was expected, since they mimic sympathetic activity. However this was not observed. The drug isoproterenol did not seem to have this effect on 5D cultures. (4D cultures were not subjected to IP condition). There was no significant difference between the control and isoproterenol condition.

![Graph showing Co culture Control vs Co culture + IP (5D)]

Figure 6: Quantification of cardiomyocyte cell proliferation. 5D cultures (N=5), comparing coculture control and coculture with drug IP. No significant difference in percentage of proliferating myocytes was obtained.

Myocyte only control vs Myocyte only + isoproterenol drug

We hypothesized that myocytes, when treated with isoproterenol, would show an increase in cell proliferation. Surprisingly, the opposite result was observed. The addition of the isoproterenol drug seemed to reduce percentage of proliferating cells in myocyte only cultures. This suggests that sympathetic innervation exerts its effect by other factors in addition to β-adrenergic signaling.
Figure 7: Quantification of cardiomyocyte cell proliferation of 5D (N=5) and 4D (N=3) cultures. Comparison of myocyte only culture controls with myocytes only when IP drug is added. Contrary to what was expected, drug IP seemed to reduce the percentage of proliferation. (a) 5D cultures (b) 4D cultures

Myocyte only control vs Myocyte only + propranolol drug

When myocytes were cultured in absence of sympathetic neurons, the propranolol drug would be expected to show no effect in this condition. As predicted, the graph below shows that there was no difference in the control and propranolol condition, since the propranolol drug did not have sympathetic activity to block. (4D cultures were not subjected to this condition.)

Figure 8: Quantification of cardiomyocyte cell proliferation of 5D (N=5). No difference in percentage of proliferation was observed when Myocytes only cultures were supplied with drug P.

Hence we can conclude that β-adrenergic signaling is necessary, but may not be sufficient, for sympathetic regulation of cardiomyocyte proliferation.
**Sympathetic innervation delays hypertrophy:**

Hypertrophy is characterized by increase in cell size. When cardiomyocytes irreversibly switch from the proliferative phase to hypertrophy, the cell size is expected to increase.

**Co-culture vs Myocyte**

As concluded in the previous result section, if sympathetic innervation promotes cell proliferation it would indicate that it may also delay hypertrophy. Myocytes co-cultured with sympathetic neurons, then, would be smaller in size as compared to myocytes grown alone. As predicted, the average cell size of co-cultured myocytes was significantly smaller than myocytes grown in absence of sympathetic innervation (5D p= 0.003; 4D p=0.0003; Students T test).

![Graph](image)

(a) (b)

**Figure 9:** Sympathetic innervation delays hypertrophy. Average cell size analysis. Myocytes only cultures were significantly larger in average cell size when compared to cocultures in both 5 day old cultures (a) (N=5) (p=0.003) and 4 day old cultures (b) (N=3) (p=0.0003).
Sympathetic innervation delays hypertrophy via β-adrenergic signaling:

Co-culture control vs Co culture + propranolol

To determine if β-adrenergic signaling underlies sympathetic regulation plays a role in the regulation of cardiomyocyte size, the co-cultures were supplied with the drug propranolol (blocks sympathetic activity). Here, we would expect co-cultures with propranolol to enter into hypertrophy earlier and have a larger average cell size as compared to controls. From the graph we can see that the myocytes in the propranolol condition are trending towards a larger cell size (5D \( p = 0.02 \); 4D \( p = 0.07 \)). Though the difference in cell size was not statistically significant, myocytes in the propranolol condition were consistently larger through all experiments performed for the 4D as well as 5D cultures.

Figure 10: Average cell size analysis of cocultures and cocultures with drug P. Blocking of sympathetic activity, in condition Co P, lead to increase in average cell size of myocytes when compared to the controls (Co C) in both 5 day old cultures (a) \((N=5)\) and 4 day old cultures (b) \((N=3)\).
Myocytes only control vs Myocytes only + isoproterenol

Isoproterenol stimulates the sympathetic activity and would mimic the co-culture condition when supplied to myocyte only cultures. Average cell size in case of myocytes only was larger when compared to the isoproterenol condition, as expected. Although the difference was not statistically significant the difference was uniform and maintained across individual experiments (5D p=0.06; 4D=0.13).

Figure 11: Average cell size analysis for myocyte only cultures. Myocytes only cultures when supplied with drug IP decreased the average cell size when compared to the controls.

**Hence, we can state that sympathetic innervation is necessary and sufficient to cause a delay in hypertrophy via β-adrenergic signaling.**

**Ablation of sympathetic activity had an effect on the expression of cfos protein:**

Cfos protein is said to be induced when the cells transition into hypertrophy. I therefore asked whether there were changes in the cfos expression in hearts in which sympathetic innervation had been disrupted. These experiments were performed *in vivo*. To ablate the sympathetic activity, rat pups were injected with 6-OHDA, and control rat pups were injected with saline 24 hours after birth. Whole hearts from these pups were extracted and proteins were
analyzed by Western blot analysis to determine if the levels of cfos protein expression varied in presence of sympathetic activity.

As established earlier sympathetic activity tends to delay hypertrophy. Thus we can assume that when the activity is ablated (lesion hearts), cells would enter hypertrophy earlier, thus causing the cfos protein levels to increase as compared to controls. The analysis revealed that the levels of cfos in P7 lesion hearts were greater as compared to the control P7 hearts (Figure 12). However the cfos levels did not seem to be evidently different when compared on the basis of timeframe (2 day old hearts vs 7 day old hearts) as well as 2 day lesion hearts vs control. Additional experiments would need to be performed since these are preliminary results. Unfortunately conditions to determine cmyc protein levels could not be standardized and would require further adjustments to obtain conclusive results.

Figure 12: cfos protein expression in heart tissue extracts. Hearts were extracted 2 days and 7 days after treatment, from rat pups. Total protein content was extracted from whole heart. Equal amounts of proteins were loaded in each well (20µg). Lane 1 contains proteins extracted form 7 day old hearts, control condition, injected with saline; lane 2 contains lesion hearts from 7 day old hearts, injected with 6-OHDA. Lane 3 contains sample from 2 day normal hearts, injected with saline and lane 4, lesion hearts from 2 day old pups injected with 6-OHDA. Sympathetic ablation by 6-OHDA showed to increase the level of cfos protein expressed when compared to control.
DISCUSSION

Cardiomyocytes, in response to sympathetic innervation, are shown to exhibit changes during development (Hansson et al, 2003). Besides signals from heart tissues also alter the development of sympathetic neurons (Qu and Robinson 2004). Nerve growth factors released by the heart tissue are required for survival, growth and differentiation of the sympathetic neurons (Chun and Patterson, 1977). In this study, we have characterized the role of sympathetic innervation in developing postnatal cardiomyocytes and demonstrated that the effect is mediated via β-adrenergic signaling.

I found that the 2D cultures showed a lot of variation when results were analyzed. Previous work in the Birren laboratory had shown that sympathetic innervation did have an effect on 2D cultures. I could not reproduce the same results; this could be because the cells in 2D cultures may still be in the phase of acclimatizing to the in vitro conditions. I therefore proceeded to evaluate later time points.

In vitro studies of 4D and 5D experiments demonstrate a consistent effect of the sympathetic innervation on the development of cardiomyocytes. First, myocytes in presence of sympathetic innervation were shown to proliferate more as compared to myocytes grown in absence of the innervation. These results imply that sympathetic innervation does play a role in the development of cardiomyocytes. To investigate whether sympathetic innervation mediates its effect via β-adrenergic signaling mechanism and to exclude other factors which may cause an effect, the activity was blocked using an antagonist drug in co-cultures. In this condition, the myocytes seemed to reduce the percent of proliferating myocytes in co-culture, due to the
sympathetic blocking effect. However, when myocyte only cultures were supplied with agonist drug isoproterenol there was no increase in the proliferation percent. This suggests that sympathetic activity mediated via β-adrenergic signaling is vital but may not be sufficient to cause the increase in proliferation. These results matched previous results from the Birren laboratory. Other factors, such as cardiomyocyte derived factors like neurotrophins, NGF or signals from surrounding cells may play a role in combination with the sympathetic activity.

Co-cultures when supplied with the isoproterenol drug did not show any difference in proliferation when compared to controls. There are few scenarios why this condition did not give the expected result. Absence of effect could be contributed to the fact that alternative signaling mechanisms aside from β-adrenergic signaling may regulate sympathetic control of cellular proliferation. Or it could also mean that sympathetic neurons have a limited effect, which cannot be enhanced by supplying the isoproterenol drug.

As sympathetic neurons have been shown to promote cell proliferation, it could be assumed that they delay cells from entering hypertrophy. So, the absence of the neurons would, in turn, lead to myocytes exiting the cell cycle earlier and shifting into the hypertrophic mode. This was tested by measuring the cell size of myocytes in presence and absence of sympathetic neurons. Similar to the pervious experiment, to confirm that the activity is mediated through β-adrenergic signaling and to exclude the possibility of other factors that may have a similar effect; sympathetic activity was blocked by drug propranolol in co-cultures and stimulated by isoproterenol in myocytes only cultures to confirm the effect. Co-cultured myocytes had smaller average cell size as compared to myocytes only culture, indicating that myocytes enter hypertrophy and increase in size earlier in absence of sympathetic innervation. This result was further established when an increase in average cell size was observed in case of cocultures when
supplied with drug propranolol, blocking the activity of the neurons. To further make the effect concrete, myocyte only cultures were grown in presence of the isoproterenol (stimulates sympathetic activity), which lead to a decrease in average cell size as compared to controls. Hence, sympathetic activity mediated via β-adrenergic signaling is necessary and sufficient to delay hypertrophy in cardiomyocytes.

Results from previous studies with Histone H3 as the proliferative marker showed similar results and establish the role of sympathetic innervation in the development of postnatal cardiomyocytes. Further experiments with different sympathetic activating mechanisms in addition to β-adrenergic signaling pathway would be useful to further characterize the role. Investigating other factors like signals from the cardiomyocyte to the sympathetic innervation may help us learn the complementary mechanism since each of them has an effect on the other.

The transition from hyperplasia to hypertrophy is also characterized by the change in protein expression. In vivo experiments were performed wherein the newborn rat pups were injected with 6-OHDA to ablate the sympathetic activity. To determine the effect of this, protein expression from whole hearts of experimental and control rat pups were compared. Western blot analysis was used to quantify the levels of proteins such as cfos and cmyc. The analysis for cfos protein levels revealed that lesion hearts when extracted at 7 days post birth shows an increase in the protein level when compared to control hearts, which was consistent with other literature. However since this is a preliminary result, additional experiments to confirm the same would have to be performed. But the protein expression results verify mRNA expression obtained from PCR studies in Birren Laboratory. It would be interesting to see if other proteins such as cmyc and meis fluctuate as their corresponding mRNA has been shown to vary during development (Birren et al. unpublished).
The overall results are consistent with the model in which sympathetic innervation plays a role in cardiac development. This study gives a possible explanation as to how the total number of cardiomyocytes within the heart is determined, uncovering the mechanism which is vital for stability of cardiac functioning and cardiac pathology. Studies such as these would increase our knowledge about the mechanism underlining the development of heart and the role that the nervous system plays. These studies may reveal potential targets for therapeutic approaches and provide insight into the mechanisms responsible for the programming of adult cardiovascular diseases.
REFERENCES


Olson, E. N. & Schneider, M. D. Sizing up the heart: development redux in disease.*Genes Dev.* 17, 1937–1956 (2003).


