CARP Plays a Key Role in Cellular Growth Under Hypertrophic Stimuli in Neonatal Rat Ventricular Myocytes

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Cardiac hypertrophy is a growth process characterized by an increase in individual myocyte size and overall cardiac muscle mass. Although such growth can occur through physiological means such as exercise, this study examined molecular components of the pathological process that occurs in response to long-term stress, injury, or disease states. Such hypertrophic stimuli induce unique cardiac muscle growth, increasing the risk of ischemia and congestive heart failure. During the pathological process, the fundamental unit of cardiac muscle—the sarcomere—is disrupted, both as a consequence of changes in gene expression and local sarcomeric proteins. Cardiac-restricted ankyrin repeat protein (CARP) is a crucial protein that functions both in the cardiac sarcomere and at the nuclear transcriptional level. We postulated that due to this dual nature, CARP plays a key role in maintaining the cardiac sarcomere and inducing hypertrophic growth pathways. GATA4 is a transcription factor with known importance during cardiac hypertrophy. Our studies uniquely suggest that CARP and GATA4 interact, and affirm that GATA4 is activated by hypertrophic stimuli. In order to partly delineate the complex process of cardiac hypertrophy, we applied a novel approach by testing cardiomyocytes for changes in CARP levels and GATA4 activation after hypertrophic stimuli over time. Neonatal Rat Ventricular Myocytes (NRVMs) were used in this study, and induced by the stimuli agents of phenylephrine (PE) and fetal bovine serum (FBS). Aggregate results of changes in GATA4 activation over time after both stimuli did not generate a consistent trend over time and did not gain statistically significance. CARP levels over time after such stimuli, however, were documented for the first known time, and generated a relative trend of increasing then decreasing levels over time after both stimuli with relatively more significance after PE stimuli. The functional role of CARP during hypertrophic growth was clearly and significantly demonstrated in this study through immunofluorescence. Results show that hypertrophic cardiomyocyte growth was significantly blunted when NRVMs were co-treated with CARP small-interfering RNA (siRNA), which disrupts CARP function. These data suggest that CARP plays a crucial role in the hypertrophic response in cardiomyocytes, and catalyzes further study.

INTRODUCTION

Cardiac hypertrophy is a growth process characterized by an increase in each cardiomyocyte size, without cellular division (Haung et al., 2013). One characteristic consequence of such growth is thus an overall increase in heart muscle mass and size, as shown by the schematic illustration in Figure 1 (Nucleus Medical Art, 2009). Although the heart can also increase in size and muscle mass as a result of exercise, this occurs through a physiological process in which the muscle increases to directly compensate for increased work conditions, blood vessel growth occurs simultaneously, and molecular components of each muscle are maintained (McMullen and Jennings, 2007; Bernardo et al., 2010; Anversa et al., 2003). The pathological process of cardiac hypertrophy occurs in response to hypertrophic stimuli of long-term stress, injury, or disease states such as hypertension (ibid; Dorn et al., 2005). These induce unique cardiac muscle growth that causes irreversible changes in gene expression of molecular components, apoptosis (cardiomyocyte-self-induced death), and fibrosis of cardiac muscle (ibid). Due to these pathways, pathological cardiac hypertrophy increases the risk of ischemia and heart failure (Bernardo et al., 2010). This study examines some of the molecular changes that occur during the pathological cardiac hypertrophy growth process.

As illustrated in Figure 2, the sarcomere is the basic functional component of all cardiac muscle cells, and regulation of its structure through local sarcomeric proteins and appropriate gene expression is crucial for overall proper contraction of the heart (University College London, 2011). Under cardiac
Hypertrophy pathological conditions, sarcomere structure and function is disrupted, and the overall cardiac muscle’s ability to contract efficiently is limited. Hypertrophic stimuli increase growth requirements in each individual cell and transmit increased loading conditions for all sarcomeres within. These conditions not only stretch sarcomeres from their optimal contractile positions, but also induce influential pathways that lead to altered gene expression of important sarcomeric proteins within each myocyte (Van Berlo et al., 2011). For example, it has been widely shown that such conditions cause activation of mechano-sensitive proteins such as, but not limited to, calcineurin, FHL1, and MLP within the sarcomere (Butandelger et al., 2011; Miller et al., 2003; Inoue et al., 2009). Such proteins translocate to the nucleus, relay conditions, and can initiate specific muscle gene transcription to compensate for increased workload through growth (Linke et al., 2008).

Cardiac-restricted ankyrin repeat protein (CARP/ANKRD1) is an important protein found only in the heart. It serves as a cofactor in myocytes, regulating several nuclear transcription factors. Additionally, CARP is detected in the N2A region of cardiac protein titin, which is in the I-band of the sarcomere, as illustrated in Figure 1 (Link, Wolfgang et al., 2007; Bang ML et al., 2001). Due to this dual localization in the nucleus and sarcomeres, it has been widely proposed that CARP mechanistically acts as a mechanosensory unit to control stretch-based signaling in the sarcomere, regulating cardiomyocyte hypertrophic growth, as shown in Figure 3 (Chen et al., 2012; Miller MK et al., 2003; Alhara Y et al., 2000). This is supported by studies showing that CARP is translocated to the nucleus under hypertrophic stimuli, affecting the presence of CARP in the sarcomere, and thus the maintenance of the cardiomyocyte sarcomere structure, as well as, gene expression in the nucleus (Singal et al., 2000; Zou et al., 2012; Miller MK et al., 2012). Such dual localization of CARP is shown through our studies in Figure 3, in which CARP is illustrated by green staining. Each striation in this Figure is an individual sarcomere, and the dense round-shaped structures are nuclei (Chen et al., 2012).

Three models of cardiac hypertrophy have elevated CARP levels, suggesting a potential link between CARP and the regulation of hypertrophy. Knockdown of CARP with small-interfering RNA (siRNA) has been shown to disrupt the normal
tions in cardiomyocyte sarcomere structure. This effect demonstrates the importance of CARP’s role in the sarcomere (Chen et al., 2012). Recently, the importance of CARP in overall cardiac function has also been highlighted by several studies that link CARP mutations with cardiomyopathies (Mever et al., 2012; Arimura T et al., 2009).

GATA4 is a transcription factor that regulates the expression of cardiac specific genes, including CARP, during cardiac development and in response to stress stimuli. (Schlesinger J et al., 2011; Broderick TL et al., 2012). GATA4 has been shown as up-regulated during cardiac hypertrophy, and similarly, an overexpression of GATA4 in cardiomyocytes has been shown to induce hypertrophy, causing accumulation of cardiac specific proteins, enhanced sarcomeric organization, an increase cell size, and progressive heart failure in animal models (Liang et al., 2011; Han et al., 2012). In cells treated with hypertrophic stimuli, GATA4 is activated by phosphorylation at serine 105 (van Berlo et al., 2001). This activation has been shown to result in enhanced DNA binding activity and enhanced cardiac gene expression (Liang et al., 2001). These data underscore the importance of precisely understanding and regulating GATA4 protein levels and activity. GATA4 is a known cardiomyocyte hypertrophic inducer, and studies indicate that CARP is also involved; however, this is a novel idea. A recent study found that when CARP levels decrease, activated GATA4 levels also decrease, suggesting a correlation (Sawyer et al., 2012). Previous data published by our lab further validates this connection. We showed that GATA4 siRNA knockdown in cardiomyocytes inhibited CARP expression and myofilament gene transcription. A scheme of these findings is illustrated below in Figure 3. Furthermore, co-transfection experiments showed that GATA4 operates upstream of CARP by activating the proximal CARP promoter to induce increased expression (Chen et al., 2012). These results and our previous studies suggest that CARP and GATA4 interact. Most importantly for this study, they also strongly suggest a role for CARP in mediating cardiomyocyte hypertrophy via regulation of GATA4 activation; however, this has not previously been investigated.

In order to better understand the process of cardiac hypertrophic growth, we examined how CARP and GATA4 are regulated under such conditions. The mechanisms by which this growth process occurs are of particular importance in studying the maintenance of the cardiac sarcomere under hypertrophic stimuli considering CARP’s functional role in the I-band of the sarcomere, and demonstrated interactions with GATA4. Sarcomeric proteins such as CARP and titin contribute to this maintenance by serving as mechano-sensors, reacting to stress conditions and inducing changes in sarcomeric structure and gene expression pathways. We

![Figure 3: CARP localization shown as green in the sarcomere (striated) and nucleus. (Chen et al., 2012).](image)

![Figure 4: Cardiac sarcomere structure is maintained by a balance between sarcomere protein synthesis and degradation. As seen on the left, GATA4 regulates CARP expression and induces sarcomere gene transcription. CARP expression is subject to a negative feedback regulatory mechanism. As seen on the right, GATA4 siRNA and CARP siRNA each inhibit CARP signaling and sarcomere gene expression, resulting in an imbalance of protein turnover and eventual sarcomere disruption. (Chen et al., 2012)](image)
hypothesized that hypertrophy induces GATA4 activation and CARP levels, and that these specific proteins play a key role in mediating cardiomyocyte hypertrophic growth. In this study, our first aim was to test the correlation between CARP protein levels and GATA4 activation over time in cardiomyocytes treated with hypertrophic stimuli phenylephrine (PE) and high serum media (7% FBS) (Prasad AM et al., 2007; Rokosh et al., 1996). Our second aim was to determine whether in vivo knockdown of CARP by siRNA impairs hypertrophy-induced cardiomyocyte growth. The overall goal of this project is to elucidate the effective role of CARP during both normal and pathologic cardiac hypertrophy. Examination of such regulation, and potential differences between the two conditions, allows for further focused research on the pathological process of cardiac hypertrophy to understand potential preventative measures and therapeutics.

**METHODS AND MATERIALS**

**Ethics Statement**
This study was carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols for all experiments using vertebrate animals were approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center.

**Aim One**
**Cell Culture and Treatment Procedures**
Neonatal Rat Ventricular myocytes (NRVMs) were harvested from freshly dissected ventricles of 1 to 2 day-old Wistar rats. Cells were plated and cultured in Dulbecco’s Modified Eagle Media (DMEM), supplemented with 7% fetal bovine serum (FBS, Invitrogen), containing 2 mg/mL albumin (Sigma), 5 mM creatine (Sigma), 2 mM L-carnitine (Sigma), 5 mM taurine (Sigma), 1% 100 units/mL penicillin/streptomycin (Invitrogen), and 100 µM bromodeoxyuridine (BrdU, Sigma). Culture media was changed 1 hr after plating. NRVMs were grown for three days to ~80% confluence in separate petri dishes and then serum-starved with 1% FBS for 24 hrs. Hypertrophic stimuli treatments of 100mM phenylephrine (PE) in 1% FBS or high (7%) FBS were then administered to induce cellular hypertrophy. NRVMS within each biological set were either un-stimulated for 0hr controls, or stimulated with one of the respective hypertrophic-inducing agents at the 0hr time point and subsequently harvested. NRVMs were harvested at 0, 1, 6, 24, and 48hr time points to detect changes in levels of GATA4 activation and CARP over time. Analysis of GATA4 activation at each of these time points was determined through densitometry quantification of NRVM lysates on western blots (WBs). A total of eleven western blots were obtained within this study, each of which represents a different biological sample of NRVMs.

**Gel Electrophoresis and Immunoblotting**
Gel electrophoresis and immunoblotting were carried out using standard western blot (WB) procedures, as described by Burnette et al. Alterations in materials and technique are as follows: Primary antibodies to total GATA4 (Santa Cruz Biotech, clone G4, sc-25310) and the phosphorylated form of GATA4 (abcam, ab5245 phospho S105) were incubated with membranes overnight at 4°C. Membranes were washed three times with PBST while shaking, and incubated with appropriate secondary antibodies of Odyssey-detectible anti-rabbit or anti-mouse for 1hr at 37°C. Membranes were washed again for 45-minutes and stored at 4°C to dry.

**IRDye Imaging**
Dry membranes were imaged to detect reactive banding by an Odyssey scan and LiCore imaging software. Detected proteins were compared to the detected IRDye (680/800) protein marker (LiCore, 2012). Densitometry analysis of scanned blots was performed using Odyssey 3.0 software.

**Membrane Stripping**
Membranes were soaked in PBST for 10 minutes, then immersed in Invitron stripping buffer for 30 minutes and washed three times with PBST. Membranes were re-blocked using 5% BSA in PBST for 1hr at 37°C. Primary antibodies to CARP (Santa Cruz Biotech, sc-30181, H-120) and α-tubulin (Santa Cruz Biotech, sc-5286, clone B-7) were incubated with the membranes overnight at 4°C. Membranes were washed three times with PBST while shaking. Appropriate secondary antibodies were incubated with the membranes, as previously described.

**Aim Two**
**Immunofluorescence**
Neonatal Rat Ventricular myocytes (NRVMs) were harvested and cultured in petri dishes as described in Cell Culture Procedures. For this experiment, some NRVMs were co-treated, along with hy-
pertrophic stimuli, with either small interfering RNA (siRNA) or non-silencing (NS) siRNA against CARP. Sequences for these previously designed molecules are shown in Figure 5. Lipofectamine 2000 (Invitrogen) was added to NRVMs to allow for transfection of siRNAs, which were diluted with Opti-MEM (Invitrogen). Nonsilencing (NS) or CARP-silencing siRNAs were then added to the cells, and allowed to incubate for 24hrs. Cardiomyocytes were then fixed with 4% paraformaldehyde (PFA) in PBS, washed with cold PBS three times, permeabilized with 0.5% Triton-X/ PBS in the dark for 30 minutes, and washed again.

Our experiments then used a monoclonal antibody to myomesin (B4, University of Iowa Hybridoma Bank), which stains the center M-line of the sarcomere. This was followed by incubation with goat anti-mouse FITC-conjugated secondary antibody (Alexa dyes, Invitrogen) and Texas-Red phalloidin (for staining filamentous actin). The cells were mounted on a cover slip using VectaShield (Vector Labs), containing DAPI (to stain nuclei), and mounting media. Sarcomere morphology was analyzed using an Olympus IX81 inverted fluorescent microscope (LSM 510, Zeiss).

Images of cells incubated with NS siRNA and CARP siRNA were compared. To quantify the size of imaged cardiomyocytes, the space between each striation (illustrated by the distance between myomesin bands stained green and actin bands stained red) was accepted as 2um as previously described (Wang X et al., 2012; Klabunde R, 2007). Striations were counted and the area of representative cells was calculated for comparison analysis.

**Statistical Analysis**

Data are reported as mean±SD. Where appropriate, results were either analyzed by Student’s t-test or ANOVA with a Bonferroni’s multiple comparison post-hoc test. P<0.05 was considered statistically significant.

### Results

**Aim One**

**Part One: Hypertrophy-induced activation of GATA4 over time:**

Previous studies have shown that GATA4 activation by phosphorylation of serine 105 occurs during hypertrophic-induced pathways (van Berlo et al., 2001). In order to study our first aim in understanding the roles of GATA4 and CARP within such hypertrophic pathways, NRVMs were stimulated with PE or 7% FBS hypertrophic-inducing agents. Activation of GATA4 was evaluated by measuring an increase in GATA4 phosphorylation of serine 105 levels (pGATA4), which was detected by immunoblotting with a specific antibody to separate its level from total GATA4 (tGATA4). Because GATA4 activation is linked with hypertrophy, this was used as our positive control to detect and confirm an effect induced by hypertrophic stimulation. The extent of changes in GATA4 activation over time was determined through analysis of several western blots comparing the pGATA4 levels with the tGATA4 levels. Below, sets of data are illustrated in Figures 6 and 7.

Figure 6 shows results from representative western blots (WBs) from different NRVM biological samples induced under PE (Parts A and B). Each WB contains data from one biological sample. In Part A, detected levels of pGATA4 are shown as green, while tGATA4 levels are shown as red. These channels are overlaid in the third row, creating a clear visual trend that GATA4 activation decreased over time. In Part B, results from another blot from NRVMs under hypertrophic PE stimulation are shown, with less apparent changes in GATA4 activation than results from Part A. Densitometry results from Odyssey 3.0 software provided quantitative measurement of GATA4 activation at each time point, relative to the 0hr-control time point, and accounting for changes in the A-Tubulin loading control. Averages of biological samples for each time point are plotted as a bar graph in Figure 6C, as a function of pGATA levels divided by tGATA4 levels. While results show a transient increase and subsequent decrease in GATA4 phosphorylation over time.

<table>
<thead>
<tr>
<th>CARP siRNA</th>
<th>Non-silencing (NS) CARP siRNA</th>
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<tr>
<td>Against exon 4 of the rat CARP sequence</td>
<td>5′-GAACCGGAGCCTGAAATTATT-3′</td>
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<tr>
<td>5′-AATTCTCCGAACGTGTCACGT-3′</td>
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**Figure 5:** siRNA molecules used for Aim Two studies.
under PE stimulation, statistical significance was not reached. This conclusion is demonstrated in Figure 6C by the overlapping error bars, which represent standard deviations within each time point data set. Due to differences in values of GATA4 activation within each of these sets, error bars are large. Each of the time points demonstrates this variance, and thus error bars overlap. Such overlap illustrates that a P value of < 0.05 was not achieved, and this significantly reduces evidence that actual differences exist between the time point data sets.(Cummings et al., 2007).

Figure 7 incorporates results from WBs on two other different NRVM biological samples induced under 7% FBS (Parts A and B). In Part A, results determined that the highest degree of activation occurred one hour after NRVMs were stimulated. In Part B, though, the highest degree of GATA4 activation was detected twenty-four hours after stimulation. Data from all blots illustrating changes in GATA4 activation under hypertrophic 7% FBS stimulation are plotted as a bar graph in Figure 7C. Results also show a slight transient increase and subsequent decrease in GATA4 phos-

![GATA4 activation over time under hypertrophic PE stimulation](image-url)
phorylation over time; however, statistical significance was not achieved under this condition either. As shown in Figure 7C, statistical significance of P< 0.05 is not achieved. This is visualized by the clearly overlapping error bars, which represent standard deviations within each time point data set. (Cummings et al., 2007).

**Part Two: Hypertrophy-induced changes in total CARP levels over time:**

The second part of our first aim seeks to test if cardiomyocyte hypertrophy induces CARP levels. NRVMs were treated with PE or 7% FBS to induce hypertrophy. Membranes were previously confirmed to show a degree of GATA4 activation to control for hypertrophic induction, and thus were re-probed for CARP, as well as, A-Tubulin for a loading control. These WBs were analyzed to determine CARP levels, and CARP is detected as green in the representative WBs (A) of Figures 8 and 9 below. Figure 8 shows results from NRVM’s under hypertrophic PE stimuli and Figure 9 shows results from NRVMs under hypertrophic 7% FBS stimuli. Densitometry results from Odyssey 3.0 software provided quantitative measurement of CARP levels at each time point, relative to the 0hr-control time point, and accounting for changes in the A-Tubulin loading control. These results were plotted
Changes in CARP levels over time under hypertrophic PE stimulation:

A. 

Figure 8: A: WB. Detected levels of CARP are shown in green. A-Tubulin served as the loading control. B: Densitometry data from five biological samples (N=5) were gathered to calculate average levels of CARP for each time point across the sample sets. Standard deviations within each set are shown as error bars, which overlap between time points, making differences no more significant that by chance.

B. Changes in CARP Levels Over Time Under Hypertrophic PE Stimuli (N=5)

Figure 9, changes in CARP levels for each of the six biological samples (N=6) under hypertrophic 7% FBS stimuli were analyzed from WBs. In this WB, changes in CARP levels over time were undistinguishable by simple visualization, but densitometry analysis allowed for quantitative measurements. Aggregate results were plotted as a bar graph (Figure 9B). After an insignificant decrease between the initial time point and 1hr, a more noticeable trend of increasing CARP levels emerges, with a decrease after the 24hr time point.

In Figure 9, changes in CARP levels for each of the six biological samples (N=6) under hypertrophic 7% FBS stimuli were analyzed from WBs, one of which is shown as Figure 9A. In this WB, changes in CARP levels over time were undistinguishable by simple visualization. Figure 9B, though, shows a clear increase in CARP levels twenty-four hours after hypertrophic 7% FBS stimulation, but overall data from all WB under these conditions does not match with this WB result.

Aim Two

Detection of CARP's Role in Cardiomyocyte Growth Under Hypertrophic Stimuli:

To determine whether CARP regulates hypertrophy, NRVMs were treated with PE or 7% FBS to in...
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Changes in CARP levels over time with 7% FBS stimulation:

**Figure 9:**

A: WB. Images are blurry due to imaging limitations, however densitometry data was still effectively analyzed. Detected levels of CARP are shown in green. A-Tubulin served as the loading control. B: Densitometry data from five biological samples (N=6) were gathered to calculate average levels of CARP for each time point across the sample sets. Standard deviations within each set are shown as error bars, which overlap between time points, making differences no more significant that by chance.

Several previous studies confirm that hypertrophic growth in cardiomyocytes is caused by intracellular responses to development, stimuli, and stress (Harvey et al., 2011; Schlesinger et al., 2011; Linke et al., 2008). The mechanism by which this occurs is of particular importance in studying the maintenance of the cardiac sarcomere, and thus overall heart function. Sarcomeric proteins such as titin and CARP contribute to this maintenance by serving as mechano-sensors, reacting to such conditions by inducing changes in sarcomeric structure, as well as, gene expression pathways (Van Berlo et al., 2011). In order to better understand...
the process of cardiac hypertrophic growth, we tested how important proteins, CARP and GATA4, are affected and involved. We hypothesized that hypertrophic agents induce GATA4 activation and CARP levels, and that CARP plays a key role in mediating cardiomyocyte hypertrophic growth.

**Aim One**

In this study, our first aim was to test the correlation between GATA4 activation and CARP levels over time in NRVMs treated with hypertrophic stimuli phenylephrine (PE) and high serum media (7% FBS) (Prasad AM et al., 2007; Rokosh et al., 1996). Simulation under PE occurs through a well-recognized pathway that activates adrenergic receptors (Bogoyevitch MA et al., 1996). 7% FBS High serum media stimulates hypertrophic growth through a different pathway by use of growth factors and glucose contained within the media (Ren et al., 2003). Our study did not seek to test the differences in GATA4 activation or CARP levels between the two hypertrophic stimuli, however several previous studies have shown that PE is a relatively stronger hypertrophic-inducing agent (Vara et al., 2003; Lu et al., 2009).

Within the GATA4 activation data that was analyzed, an interesting trend emerged. While results showed a transient increase and subsequent decrease in GATA4 phosphorylation over time under both PE and 7% FBS stimulation, statistical significance was not reached in either case. As described by Cummings et
al., standard deviations within each time point data set can be plotted as inferential error bars to show how the data is spread, and provides a graphical representation of how much uncertainty exists. Long inferential bars indicate large error, and the larger the amount of overlap in bars among the time point data sets, the larger the P value. Since a significant portion of overlap occurred in error bars among the GATA4 activation graphs for both PE and 7% FBS stimulation, there is not strong evidence for a true difference in GATA4 activation levels over time. Differences in GATA4 activation within the time point data sets was mostly likely due to inconsistent protocols between experiments, including NRVM harvest procedures, ineffective administration of hypertrophic stimuli, and human error. Innate biological differences in the NRVMs also could have affects the variability in results. Additionally, low sample numbers likely had an effect, as several more biological samples would have provided more data, and thus increased the statistical power of our experiment. Smaller sample sizes of only three data sets for PE-stimulated NRVMS (Figure 6) and five data sets for 7% FBS-stimulated NRVMS (Figure 7) were available due to loss of early data in the Odyssey 3.0 software system. Files from western blot Licore-scans were moved from the original database to an external hard drive, and in the transfer, files were converted to a version that opens as read-only. Since data must be analyzed via Odyssey 3.0 after transfer from the scan device, some early files were unable to be analyzed. At the time that this problem surfaced, several western blots were already stripped of GATA4 antibodies for the next step of CARP level analysis, and thus re-scanning to analyze for GATA4 activation was not possible.
In the second part of Aim One, our study aimed to test if CARP levels changed over time as a result of hypertrophic stimulation. Such changes are important to better understand how CARP acts as both a sarcomeric protein and co-factor for gene expression pathways. Results under hypertrophic 7% FBS stimulation showed a transient increase and subsequent decrease in CARP levels over time. Similarly, results under PE hypertrophic stimulation showed a delayed, yet transient increase and subsequent decrease in CARP levels over time. Yet, induction of CARP levels due specifically to cardiac hypertrophy pathways could not be interpreted as conclusive in this study for a few reasons. GATA4 activation with hypertrophic stimuli phenylephrine (PE) and high serum (7% FBS) were crucial to interpreting further results because this activation would have confirmed effects of the hypertrophic agents used. For example, PE-stimulation has been shown to specifically activate GATA4 in several studies (Broderick et al., 2012; Schlesinger et al., 2011; Zhou et al., 2012). However, GATA4 activation over time in this study was inconsistent through the biological samples, and resulted in non-significant changes over time as a whole. Thus, hypertrophic pathways cannot be confirmed.

Aim Two

Our second aim was to determine whether in vitro knockdown of CARP by siRNA impairs hypertrophy-induced cardiomyocyte growth. The overall goal of this project is to elucidate the role of CARP in key molecular events that occur during normal and pathologic cardiac hypertrophy. Immunofluorescence results from experiments in this study support the role of CARP in mediating hypertrophy. Interpretation of these Immunofluorescence images confirms that CARP is an important protein involved in cardiac hypertrophy. Staining for myomesin (green) first confirmed that the imaged cells were in fact cardiomyocytes, because this is a cardiac-specific protein. Although other types of cells, such as fibroblasts, were detected by isolated actin (red) staining, this does not cause a contributory effect, and was likely due to imperfect isolation of NRVMs. During the Aim Two studies, hypertrophic stimulation was confirmed by comparison of stimulated and un-stimulated cells, and thus further interpretation of the effect of CARP knockdown in relation to hypertrophy can be confirmed through analysis of siRNA CARP knockdown experiments. Our study found that CARP knockdown clearly impedes hypertrophic-induced growth for both PE and 7% FBS agents. CARP siRNA specifically had a stronger effect when acting on cardiomyocytes that were stimulated with hypertrophic agents when compared to those not stimulated by PE or 7% FBS. This suggests that CARP, or its functional role, is altered during cardiac hypertrophy. Quantification of such data has not been completed at this time, yet it is important to note that images shown are representative of a larger set of analyzed cardiomyocytes. Additionally important is the fact that CARP knockdown with CARP siRNA also caused sarcomeric disarray, as illustrated by disruption.
of normal striations stained green and red in the immunofluorescence images in this study. This suggests the importance of CARP in maintaining the cardiac sarcomere.

In order to control for the effect that transfection could have on cardiomyocytes, non-treated siRNA cells were also imaged and compared to both NS siRNA and CARP siRNA-treat cardiomyocytes. Although elongation of cells might be a side-effect of NRVMs treated with CARP siRNA (compare Figure 13 with Figures 10a and 10b), this is not a confirmed effect, nor is it contributory to the study of cardiomyocyte growth based on cellular size comparisons.

**CONCLUSION**

Results of changes in GATA4 activation over time were substantial, and generated a trend, yet did not gain statistically significant differences. Measurements of CARP levels over time also did not gain a statistically significant trend, but were documented for the first known time. The role of CARP in hypertrophic cellular growth, however, was clearly and significantly demonstrated through immunofluorescence in this study. Our results show that cardiomyocyte growth was significantly blunted when NRVMs under hypertrophic stimuli were co-treated with CARP small-interfering RNA (siRNA), which disrupts CARP function. These data suggest that CARP plays a crucial role in the hypertrophic response in cardiomyocytes.

**Further Studies**

Cardiac hypertrophy is a complex growth process. Events, such as those tested in this study, are not isolated in hypertrophic pathways and several other important proteins are involved. In order to better delineate how this process occurs, our studies have focused on the role of GATA4 and CARP. Further studies will repeat GATA4 activation over time experiments under both PE and 7% FBS hypertrophic stimulation. These studies will compose of much larger sample sets, and be carried out according to a specific protocol for all biological samples. In addition, experiments that examine direct GATA4 binding to specific DNA sequences, such as a CARP promoter, can be carried out through EMSA assays in order to understand the effects of GATA4 activation and relationship to induced CARP levels. Once GATA4 activation over time is confirmed, CARP levels over time will be studied. Co-immunoprecipitation experiments could be performed to confirm direct interaction of GATA4 and CARP. Future studies could also support the hypothesis that CARP, in part, mediates a hypertrophic-response pathway via regulation of GATA4 activation through replication and alternate knockdown experiments. These studies, and more, could contribute to elucidating the role of CARP in key molecular events that occur during normal and pathologic cardiac hypertrophy. This has not previously been investigated, and thus this study catalyzes further research in order to better understand the complex growth process of cardiac hypertrophy.

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**REFERENCES**


Zou Y, Evans S, Chen J, Kuo HC, Harvey RP, Chien KR. CARP, a cardiac ankyrin repeat protein, is downstream in the Nkx2-5 homeobox gene pathway.