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Nuclear Targeted CREB Regulated Transcriptional Co-activator 2 (ntCRTC2) in the Setting of Chronic Heart Failure

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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Nuclear Targeted CREB Regulated Transcriptional Co-activator 2 (ntCRTC2) in  
the Setting of Chronic Heart Failure

A Thesis submitted in partial satisfaction of the requirements for the degree of

Master of Science

in

Biology

by

May Ling Tay

Committee in Charge:

Wilbur Lew, Chair  
Brenda Bloodgood, Co-Chair  
Ngai Chin Lai  
James Posakony

2016



The Thesis of May Ling Tay is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

Chair

University of California, San Diego

2016

## **DEDICATION**

I would like to dedicate this to my family and all my friends, for their constant words of encouragement, and for their belief in my abilities to achieve.

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## **ABSTRACT OF THE THESIS**

Nuclear Targeted CREB Regulated Transcriptional Co-activator 2 (ntCRTC2) in  
the Setting of Chronic Heart Failure

by

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Master of Science in Biology

University of California, San Diego, 2016

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As the number one cause of death in the world, and the leading cause of death in the United States, heart disease claims more lives than all forms of cancer combined (Go et al., 2013). Though several therapies are available to

alleviate its symptoms and delay the progression of cardiac deterioration, there is currently no cure for heart failure. The discovery of new proteins and pathways, and understanding their mechanisms, create targets for potential therapeutic approaches.

Calcium and  $\beta$ -adrenergic receptor ( $\beta$ -AR) signaling are both important factors in cardiac function. Here, we examined CREB Regulated Transcriptional Co-activator 2 (CRTC2), a known converging point between  $\beta$ -adrenergic receptor and calcium in glucose metabolism. Little is known about the function of CRTC2 in cardiomyocytes. Upon comparison of survival and cardiac function of wildtype and transgenic nuclear targeted CRTC2 (ntCRTC2) mice in the setting of chronic heart failure (CHF), we provide evidence of trends favoring mice with upregulated ntCRTC2. Understanding of a  $\beta$ -AR and calcium point of convergence will enable development of de novo treatments that modulate both systems, both of which are fundamental to cardiac function.

## **INTRODUCTION**

### **1.1 Heart Failure and Disease**

Although death rates from cardiovascular disease as a whole are decreasing, incidences of heart failure related deaths are still on the rise (Cook et al., 2014). As the number one cause of death in the world and the leading cause of death in the United States, heart failure affects an estimate of 5.1 million people in the country. Those that are diagnosed have poor long term prognosis, nearly half dying within 5 years of diagnosis (Go et al., 2013). Aside from imposing restrictive lifestyles on its patients, heart failure carries many direct and indirect economic and social costs in the form of health care expenditures on medical services, loss of productivity from morbidity and mortality, and welfare benefits and support (Cook et al., 2014). In 2012, the estimated direct cost resulting from heart failure totaled \$20.9 billion whereas the indirect cost totaled \$9.8 billion (Go et al., 2013). With such high economic and social burdens, continued research and development of new treatments and preventative methods can help significantly reduce both social and economic costs.

### **1.2 Animal Models and Therapeutic Potential**

The study of heart failure necessitates the ability to study chronic structural and functional changes in myocardium and the ability to quantify the progression of left ventricular remodeling. Small animal models enable these pathophysiological studies, necessary in the screening and understanding of new

molecules of interest. Mice and rats, having lower housing and maintenance costs than larger animals, are ideal for increasing statistical power through study replicability. Thus, they are commonly used in preliminary proof of concept studies. In addition, cell type specific and inducible knock out and transgenic methods in mice have allowed study of overexpression or deletion of genes in cardiomyocytes.

Studies utilizing mice and rat models help identify molecular targets. Potential therapies can then be created and tested on larger animals that carry greater physiological similarities to humans. Dependent on the etiology, heart failure models in different species have been established, the most common being dogs, swine, and sheep. Swine possess similar cardiac physiologies to that of humans and contain human relevant cardiac and coronary anatomies, making them a popular model in cardiac gene transfer therapies (Ishikawa et al., 2012). Successful large animal studies may then advance to human clinical trials that test the efficacy of therapies.

A successful example of identifying and testing molecular targets in preclinical studies and its clinical translation into humans is seen in recent gene transfer therapies conducted by Hammond and colleagues. In their study, the safety and efficacy of cardiac intracoronary administration of adenovirus 5 encoding adenylyl cyclase 6 (Ad<sub>5</sub>.hAC6) was tested. The completion of phase 2 clinical trials with promising results indicate the potential of AC6 gene transfer to safely benefit left ventricular function, carrying prospects of larger trials (Hammond et al., 2016).

### 1.3 Modeling Heart Failure

Transverse aortic constriction (TAC) and myocardial infarction (MI) are two common surgical techniques performed to model chronic heart failure (CHF). In the TAC model, as the name suggests, the transverse aorta is sutured and constricted to create cardiac hypertrophy and failure by pressure overload (deAlmeida et al., 2010). In response to the damage, cardiac hypertrophy develops as a compensatory mechanism to maintain cardiac function. This change, though initially beneficial, is maladaptive in a chronic setting and causes cardiac dilation and failure.

Alternatively, myocardial infarction is performed via occlusion of the left coronary artery. In comparison to TAC, cardiac remodeling resulting from the MI procedure is more complicated than simple compensatory hypertrophy. Following initial injury, there is an initial inflammatory phase to remove necrotic tissue, followed by a healing phase where fibroblasts proliferate and collagen deposits form to create scar tissue around the infarcted area (Westman et al., 2016). The duration of this process can range from weeks to months depending on the species. In order to preserve stroke volume, increased shortening and heart rate occur as a result of sympathetic stimulation of non-infarcted myocardium. In late remodeling, hypertrophy occurs in response to increased wall stress (Sutton and Sharpe, 2000). If the compensatory responses are inadequate, heart failure ensues (Westman et al., 2016).

## 1.4 Available Therapies

Heart failure (HF) is classified into two distinct classes, each with differing cardiac physiologies resulting from functional or structural deficits of ventricular filling or blood ejection. These two categories include HF with reduced ejection fraction (EF) (HFrEF), and HF with preserved EF (HFpEF), characterized by LV enlargement and decreased EF ( $\leq 40\%$ ), and preserved or normal EF, respectively (Writing Committee et al., 2013). Efficacious therapies have only been demonstrated in patients with HFrEF though similar guidelines defining medical therapy are used for HFpF patients (Writing Committee et al., 2013).

Several treatments are currently prescribed and have been shown to improve the long term survival in patients with heart failure. These therapies, including  $\beta$ - blockers, angiotensin converting enzyme (ACE) inhibitors, and spironolactone (aldosterone antagonist), target maladaptive LV remodeling pathways (Davies et al., 2000).

In response to LV abnormalities, the body initiates compensatory mechanisms in which renin-angiotensin and sympathetic nervous system activities increase in the body.

Increased activation of the sympathetic nervous system and adrenergic activity improves contractility. Although this is initially beneficial, chronic stimulation causes desensitization and selective reduction of  $\beta$ - adrenergic receptor density, resulting in cardiac apoptosis and contractile dysfunction (Lympelopoulous et al., 2013). Preventing chronic stimulation with  $\beta$ -adrenergic

receptor blockers can improve heart function and survival (Writing Committee et al., 2013).

The renin-angiotensin-aldosterone pathway is involved with vascular tone regulation and water and salt metabolism (Abramov and Carson, 2012).

Activation of this system results in arterial blood pressure and myocardial contractility, as well as sodium and water retention and vasoconstriction. In this pathway, renin cleaves angiotensinogen to form angiotensin I, which is then cleaved by angiotensin-converting enzyme (ACE) to form angiotensin II.

Angiotensin II causes aldosterone release from the adrenal cortex (Ma et al., 2010). ACE inhibitors and aldosterone blockers (spironolactone) suppress the activity of this pathway and have been shown to improve survival in heart failure with reduced ejection fraction (Writing Committee et al., 2013).

### **1.5 The $\beta$ -adrenergic Pathway, Calcium signaling, and CRT2**

Increased activation of both  $\beta_1$  and  $\beta_2$  adrenergic subtypes are known to increase cardiac contractility and heart rate.  $\beta_1$  adrenergic receptors ( $\beta_1$ -AR) are expressed at a 70:30 ratio in comparison to  $\beta_2$  (Triposkiadis et al., 2009).

Sympathetic epinephrine and norepinephrine ligand induced stimulation of cardiomyocyte  $\beta_1$ -ARs activate  $G_s$  protein cascades, a subtype of G-protein cascades that activate a cyclic AMP (cAMP) dependent pathway. The  $G_{\alpha s}$  subunit of the G protein complex, activated by ATP when the G protein coupled receptor is activated, binds to, and activates adenylyl cyclase (Madamanchi, 2007). Activated adenylyl cyclase goes on to upregulate the conversion of ATP

to cyclic- AMP (cAMP). cAMP activates protein kinase A (PKA), that then phosphorylates and activates troponin, L- type calcium channels, and phospholamban (PLB), all molecules responsible for cardiomyocyte contraction, enabling increased contractility (Madamanchi, 2007).

Another fundamental molecule in cardiac contractions, and found to be dysregulated in hearts with systolic failure, is calcium. In a healthy cardiomyocyte, excitation contraction coupling begins with membrane depolarization induced voltage gated L- type calcium channel opening. The increase in intracellular calcium via these channels triggers a calcium induced calcium response in which ryanodine receptors (RyR2s), located on the sarcoplasmic reticulum (SR), open to release calcium to further increase intracellular calcium concentrations. Cytosolic calcium then associates with troponin C, a complex that activates cardiac myofilaments to promote contraction. Cytosolic removal of calcium through sarcolemmal sodium calcium exchangers, and reuptake into the sarcoplasmic reticulum via calcium ATPase pumps (SERCA2a), allow for cardiac relaxation (Luo and Anderson, 2013).

In heart failure, excitation contraction coupling is defective, resulting in a decreased concentration of calcium transients, increased SR calcium leak during diastole, and decreased SR calcium sequestration (Luo and Anderson, 2013). These abnormalities impair cardiac contractility and relaxation.

It is unclear as to whether calcium dependent signaling and  $\beta_1$ -AR activity are linked in affecting cardiac remodeling and function. However, the relation between the two is clearly understood in glucose metabolism, providing insight

on possible mediating proteins and factors that may be reflected in cardiomyocyte. If a similar pathway occurs in cardiac cells, understanding its components can pioneer new grounds for drug targeting.

In studies done on insulin secreting pancreatic tumor cells, increases in cyclic- AMP (cAMP) and  $Ca^{2+}$  signaling allowed the shuttling of the transcriptional modulator, CREB regulated transcriptional coactivator 2 (CRTC2), previously known as TORC2, to the nucleus (Screaton et al., 2004). CREB is a transcriptional factor that binds to cAMP response element (CRE) sequences on DNA. CRTC2 promotes the transcription of genes targeted by CREB. At rest, CRTC2s remain in a phosphorylated state in which they associate with 14-3-3 proteins and reside in the cytoplasm (Altarejos and Montminy, 2011). The introduction of cAMP, followed by calcium, dephosphorylates and translocates CRTC2 to the nucleus. There, CRTC2 binds to cyclic- AMP responsive element binding protein (CREB) to affect gene transcription. These processes are confirmed by studies in HeLa cells that used  $\beta_1$ -AR agonist, which are, as previously mentioned, also responsible in cardiac dysfunction, and adenylyl cyclase agonist, a protein in the  $\beta_1$ -AR activation cascade, and resulted in accumulation of CRTC2 in the nucleus (Bittinger et al., 2004). Since CRTC2 serves as a converging point in calcium signaling and  $\beta_1$ -AR in glucose metabolism, it is possible that CRTC2 may too be a mediating factor in cardiac function gene transcriptional control, thus a protein of interest in cardiac research.

## 1.6 ntCRTC2 and Transverse- Aortic Constriction (TAC)

Preliminary studies by Lew and colleagues studied the effects of cardiac CRTC2 on LV function in TAC induced pressure overload (Lai et al., 2014). Transgenic mice with tetracycline-regulated cardiac myocyte-specific, nuclear targeted CREB regulated transcriptional co-activator 2 (ntCRTC2) expression were used. Three weeks post TAC procedure, mice with activated ntCRTC2 expression had attenuated pressure overload- induced LV hypertrophy and dilation. In addition, mice with activated ntCRTC2 expression measured higher LV developed pressures, increased rates of pressure development, and more rapid rates of LV relaxation, as compared to ntCRTC2- off mice.

The therapeutic potential of ntCRTC2 for treating established heart failure was then tested in wild type mice via intracoronary gene therapy. Adenoviral ntCRTC2 (Ad5.ntCRTC2) was administered to mice three weeks post TAC procedure. Increased LV function and decreased LV hypertrophy was seen in ntCRTC2 treated mice as compared to those treated with intracoronary saline, three weeks post administration.

Based on these promising results, it was hypothesized that CRTC2 may play a role in other models of heart failure, including post myocardial infarction (MI). Therefore, this study examined if upregulation of nuclear targeted CRTC2 may have beneficial effects to improve survival and cardiac function in heart failure following MI.

## MATERIALS AND METHODS

### 2.1 Animals

Animal use and care were approved by the Institutional Animal Use and Care Committee of the VA San Diego Healthcare System in accordance with NIH guidelines. Male and female nuclear targeted CREB regulated transcriptional co-activator 2 (ntCRTC2) transgenic mice ( $6.3 \pm 0.3$  months old, n=64) and their transgene negative littermates ( $5.0 \pm 0.2$  months old, n=64) were used. Genomic DNA purified from tail tips was used to confirm gene presence (**Figure 1**). Mice were housed with free access to food and water and exposed to 12h light/dark cycles. Normal ntCRTC2 mice were characterized against negative siblings, and subsequently a separate group was induced with heart failure (CHF- ntCRTC2) to determine the efficacy of ntCRTC2.

### 2.2 Genetics and DNA Preparation

Genomic DNA was extracted and purified from mice tail using the Qiagen® DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. DNA was amplified using the MJ Research PtC-200 Dual 48 Well Thermocycler and PCR primers TT20 (MHCP1) and TT681 (CRTC2R).

TT20 (MHCP1): 5' – CACATAGAAGCCTAGCCCACACC – 3'

TT681 (CRTC2R): 5' – TAGCAGGCTGGTCAGGAGAT – 3'

### **2.3 Myocardial Infarction**

Myocardial Infarction (MI) was induced by occluding the left coronary artery. Mice were anesthetized with isoflurane (1-2% in 1L/min oxygen) and a small incision was made at the neck to expose the trachea. The mice were intubated and mechanically ventilated with a pressure controlled rodent ventilator (100 breaths per minute, 0.3 mL tidal volume, 12-14 cm H<sub>2</sub>O). The mice were placed in a left lateral decubitus position and the fourth intercostal space was opened to expose the heart. The proximal left coronary artery was tied with 6-0 silk suture. The chest and skin were closed separately with 7-0 prolene, buprenorphine (0.05-0.1 mg/kg, sc) was given, and the mice were recovered before returning them to their quarter.

### **2.4 Survival Study**

A five week randomized, blinded survival study following MI was performed on 40 ntCRTC2 and 44 transgene negative siblings. Mice that died in one day post procedure were not recruited and mortality was checked daily. Only mice with large MI at the end of five weeks confirmed by echocardiography and terminal visual assessment of the LV were used in the Kaplan Meier analysis.

### **2.5 Echocardiography**

Animals were anesthetized (1-1.5% isoflurane) and echocardiography was performed with a Vevo 2100 System (Visualsonic, Toronto, Canada) with a 9-18

MHz transducer. With mice in supine position, long axis of the LV and Doppler signals were obtained. Data were analyzed without knowledge of group identity.

## **2.6 *In Vivo* Hemodynamics**

Five weeks post MI, left ventricular (LV) pressure was measured in 11 ntCRTC2 and 9 control mice. In addition, LV pressure was also measured in 6 ntCRTC2 and 4 control normal, non-MI mice. Mice were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and mechanically ventilated. A 1.4F conductance-micromanometer catheter (SPR 839, Millar Instruments, Houston, Texas) was advanced via the right carotid artery across the aortic valve and into the LV cavity. Left ventricular pressures was recorded and stored digitally for processing (IOX1.8 Emka Technologies, Christchurch, VA). Peak rates of LV pressure development (LV +dP/dt) and decay (LV -dP/dt) were calculated. Subsequently, tissue samples were obtained for future biochemistry and molecular experiments. Data were acquired and analyzed without knowledge of group identity.

## **2.7 Cardiac Cell Isolation and Calcium Studies**

Cardiac myocytes from 3 ntCRTC2 positive and 3 ntCRTC2 negative controls were isolated. Hearts from anesthetized mice were excised and placed in chilled KRH solutions (125 mM NaCl, 5mM KCl, 11 mM glucose, 1.2 mM MgCl<sub>2</sub>, 15 mM HEPES, pH 7.38-7.40). The aorta was cannulated and the heart was perfused with oxygenized Langendorff buffer (1.2 ml taurine (0.5M stock) and 1.5 ml BDM

(0.5M stock) in 100 ml KRH solution) for 3-5 minutes via perfusion apparatus. The hearts were then perfused with digestion enzyme solution (15 mg collagenase, 2 mg protease, 3.75 CaCl<sub>2</sub> (0.1M stock) in 15 ml Langendorff perfusion buffer) for 13-15 minutes. Ventricular myocytes were separated by gentle mechanical agitation and placed in a 37°C water bath for 7-8 minutes, during which it was intermittently shaken. The cells were filtered into a 50 ml tube using a cell strainer and centrifuged at 0.7 – 0.8 x 1000 rcf for 1 – 2 minutes. The supernatant was discarded and increased concentrations, 50 µM, 100 µM, 250 µM, 500 µM, 1 mM, of calcium solution (250 mg BSA powder in 40 ml Langendorff perfusion buffer, CaCl<sub>2</sub>) was added to the precipitate in 10 minute intervals. 250µl of the cardiac myocyte solution, along with 1µl Fluo-4 loading buffer was added to laminin topped coverslips. Calcium transients were measured with the FelixGX 4.1.2 PTI Fluorescence Master. Data were analyzed with the Clampfit 10.5 system.

## **2.8 Statistics**

Data are reported as mean±sem; group differences were tested for statistical significance with using Student's *t*-test (unpaired, 2-tailed). The null hypothesis was rejected when  $p < 0.05$ . Group differences in mortality were assessed using Kaplan-Meier analysis and the log-rank test.

## RESULTS

### 3.1 Characterization

DNA was extracted from mice tails, amplified, and run on gels. Figure 1 shows a typical PCR result for ntCRTC2 as indicated by the 1 kb band. Mice positive for ntCRTC2 are shown on lanes 1 - 4, 6, 8, and 9, while the negative siblings are indicated on lanes 5 and 7.

### 3.2 Mice with no Intervention (no Myocardial Infarction)

#### 3.2.1 Echocardiography

Echocardiography provides information on cardiac function and dimensions in ntCRTC2 and the negative mice (**Table 1**). Diastolic and systolic areas, heart rates, and aortic ejection times were measured in lightly sedated normal mice. The diastolic and systolic areas, ejection fractions, and heart rates between ntCRTC2 and the negative mice were not significant. However, there was a significant increase in aortic ejection time for ntCRTC2 mice. The ejection fraction values of both control and ntCRTC2 mice were around 48%.

#### 3.2.2 *in Vivo* Hemodynamics

Left ventricular pressure obtained from intact hearts of control and ntCRTC2 mice showed that both +dP/dt and -dP/dt values were similar (**Fig. 2A & 2B**). In normal mice, the p values for developed pressure (**Fig. 2C**) and heart rate (**Fig.**

**2D)** were not significant in both the ntCRTC2 and its negative siblings. Thus, *in vivo* hemodynamics did not differ between control and ntCRTC2 mice at baseline (with no intervention).

### **3.2.3 Calcium Handling**

Calcium transients were measured to determine peak cytoplasmic calcium content (**Fig. 3A**), time to reach the peak (**Fig. 3B**), and tau (**Fig. 3C**), the rate of calcium transient decay. P values for peak, tau, and time to peak values were not significant between normal and transgenic mice (**Fig. 3**).

### **3.2.4 Morphometrics**

Morphometric assessments of body weight (BW), left ventricle and intraventricular septum (LV + IVS), lung, and liver mass were performed to determine for organ abnormalities in ntCRTC2 mice. Mass-specific LV weight was calculated to determine proportionality between heart size and body weight. Table 2 shows no differences in these parameters.

## **3.3 Mice with Myocardial Infarction (CHF-control and CHF-ntCRTC2)**

### **3.3.1 Survival**

A Kaplan-Meier analysis was performed to test if overexpression of ntCRTC2 prolonged survival in MI afflicted mice. After 35 days, no significant difference was seen in survival rates of CHF-ntCRTC2 mice when compared to their CHF-

control littermates. However, CHF-ntCRTC2 mice trended towards better survival between 8 and 28 days (**Fig. 4**).

### ***3.3.2 Echocardiography***

Echocardiography were performed 5 weeks post MI in lightly sedated mice. The long axis view of diastolic and systolic areas, heart rates, and aortic ejection times were acquired for MI mice. Differences between CHF-control mice and their CHF-ntCRTC2 siblings were not significant (**Table 3**). The ejection fraction values of both groups 5 weeks post MI were about 7%, which is indicative of severe heart failure (**Table 3**).

### ***3.3.3 in Vivo Hemodynamics***

LV pressure measurements were obtained from intact MI afflicted hearts. Figure 5 compared CHF-control mice with their CHF-ntCRTC2 siblings for rates of pressure development and decay, developed pressure, and heart rate. Although statistically not significant, mice with the ntCRTC2 transgene showed greater rates of pressure development, decay, and contractility as compared to CHF-control siblings.

### ***3.3.4 Morphometrics***

Left ventricular, lung, and liver weights were similar in both CHF-control and CHF-ntCRTC2 mice (**Table 4**). When this data were compared to the non-CHF mice, differences in LV and lung mass were higher in the CHF groups, showing

the severity of MI. Surprisingly, liver mass were lower in the CHF groups (Tables 4 & 2).

## DISCUSSION

The CREB regulated transcriptional coactivator 2 (CRTC2) may represent a novel target to treat heart failure. Although CRTC2 exists in the heart, little is known about its function. Preliminary studies conducted by Lew and colleagues (Lai et al. 2014) utilized the TAC pressure overload model in mice with tetracycline-regulated cardiac myocyte specific ntCRTC2. Their findings showed attenuation of LV dilation and hypertrophy along with elevated LV contractile function and relaxation in ntCRTC2 expressing mice 3 weeks post TAC. The goal of this study was to determine if increased cardiac expression of ntCRTC2 may similarly have a beneficial effect in another setting of heart failure, the volume overload model via myocardial infarction (MI).

No significant difference was seen between normal and ntCRTC2 mice in left ventricular (LV) contractility and decay, and developed pressure (**Fig. 2**). In addition, organ masses and mass specific LV weight between control and ntCRTC2 mice were similar (**Table 2**). Diastolic and systolic areas, ejection fraction, and heart rate were comparable between control and ntCRTC2 mice. There was a small, but significant increase in aortic ejection time in ntCRTC2 mice (**Table 1**), but not enough to influence overall LV function as measured by other echocardiographic indices or *in vivo* hemodynamics. Calcium transients were similar in control and ntCRTC2 mice (**Fig. 3**). Collectively, these results indicate no significant difference in cardiac function between ntCRTC2 and control mice under baseline conditions.

A high mortality that resulted from MI was seen in mice recruited into the study. 18 of 44 (41%) of control mice and 14 of 40 (35%) of ntCRTC2 mice died from MI within the 35-day study period. Although there was no significant difference in survival between CHF-ntCRTC2 mice and their CHF-control siblings near the completion of the 35-day study (**Fig. 4**), the ntCRTC2 mice appeared to have better survival between 8 to 28 days, before the two curves converge after 32 days. A post hoc analysis of the data was done with a 20 day end point and yielded a trend that favored ntCRTC2 mice with a p value of 0.13. Thus, ntCRTC2 may have cardio-protective effects that prolonged survival during the earlier period of 1-4 weeks following MI, although this advantage was not sustained after 5 weeks, in large due to the severity of this model.

The severity of heart failure induced in these mice are revealed in the ejection fractions obtained from echocardiography. Post MI, both control and ntCRTC2 mice had ejection fractions of approximately 7% (**Table 3**), compared to the ejection fractions of about 48% (**Table 1**) seen in mice with no myocardial infarction. There was no difference in LV size, as measured by systolic and diastolic areas, heart rates, or aortic ejection time between the two groups (**Table 3**).

CHF-ntCRTC2 mice that survived up to 35 days appeared to have a higher LV +dP/dt and -dP/dt (**Fig. 5**). The developed pressure were also higher. However, these data were not statistically significant because the power calculated sample size of n=15 per group was not met.

Several other reasons may have attributed to the results of the study. As previously mentioned, it is possible that CRT2 exerted its effects at an earlier time point and was unable to sustain a statistically significant population at 5 weeks. If cardiac function had been measured at an earlier time point, there may be significant differences between CHF-control and CHF-ntCRT2 mice. The MI induced on these CHF mice was so severe that ntCRT2 overexpression could not improve the heart function. A less severe MI model or perhaps a higher expression level of ntCRT2 may elevate heart function.

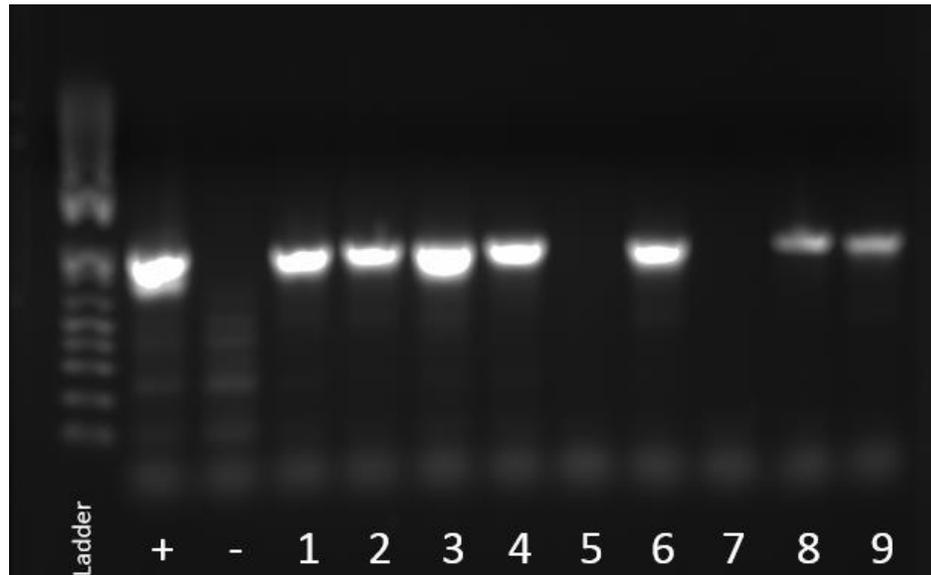
As preliminary results indicated, ntCRT2 was efficacious in reducing heart failure in the TAC model. These benefits were not reflected in the MI model, possibly due to the complexity of the remodeling when compared to the compensatory hypertrophy seen in TAC. The ejection fractions seen in the MI model averaged around 7% in both control and ntCRT2 groups. Post TAC procedure, control mice averaged an ejection fraction of 33%, compared to values of approximately 58% seen in mice with increased ntCRT2 expression. It is plausible that increased ntCRT2 in the current study was able to compensate (between 1-4 weeks) after MI but was unable to maintain its effects to 35 days with such severe heart failure. This hypothesis can be tested by further increasing ntCRT2 with gene therapy, as was done successfully to improve heart failure in the TAC model.

The compensatory responses seen in the MI model is more complex than that of the TAC model. In the TAC model, the heart responds with compensatory

cardiac hypertrophy. In the MI setting, acute inflammatory responses are first activated. A few days later, mobilization of inflammatory cells occur to remove necrotic tissues and initiate fibrosis, as well as other repair processes (Westman et al., 2016). Over the course of weeks to months, cardiac remodeling, including strengthening of infarcted areas via scar formation, and compensatory hypertrophy of non-infarcted areas occur. The upregulation of ntCRTC2 may play a role at one or more of these stages. The results of its intervention, whether beneficial or deleterious, are currently unknown.

In conclusion, while this study did not demonstrate a significant effect of ntCRTC2 on survival or cardiac function 35 days after MI, it does show a trend leading to better LV function and survival. The latter is observed between 8 and 28 days post infarction. Future studies may be structured to determine if preservation of LV function by ntCRTC2 occurs during this period, if ntCRTC2 could be beneficial with less severe heart failure following MI, or if additional gene therapy with ntCRTC2 may be beneficial.

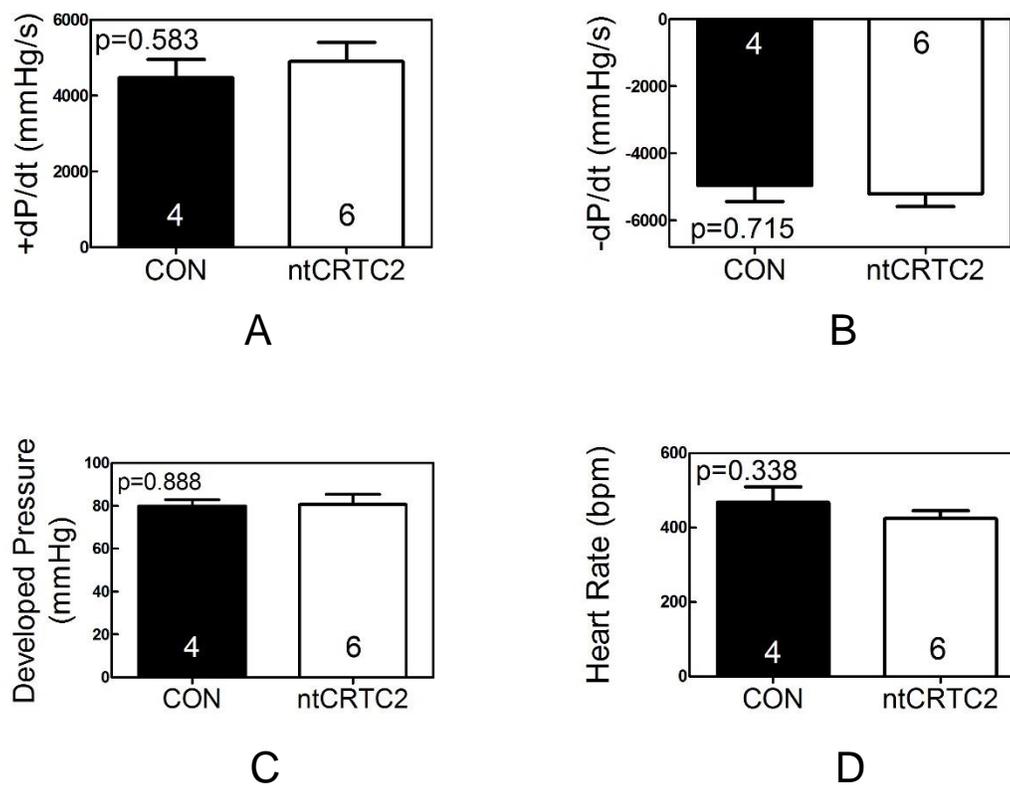
## TABLES AND FIGURES



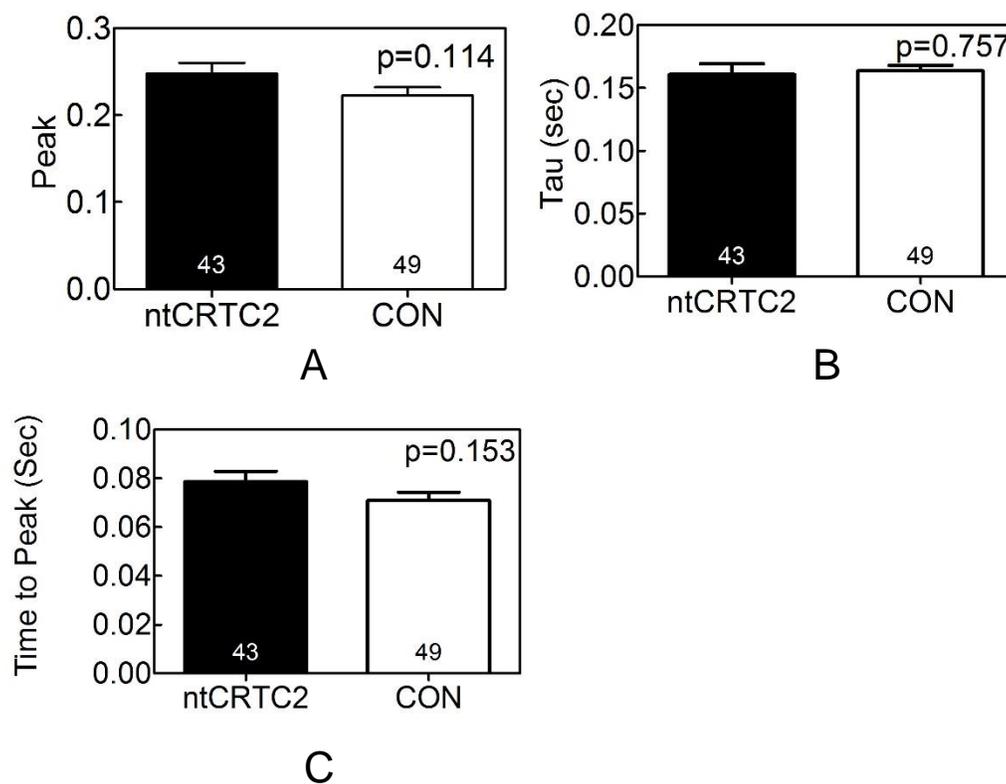
**Figure 1.** *Characterization.* Gel image showing transgene expression in the ntCRTC2 line of mice. Positive and negative controls are denoted + and -, respectively.

**Table 1.** *Echocardiography of Mice with No Intervention/ No Myocardial Infarction.* Diastolic area, systolic area, ejection fraction (EF%), heart rate, for ntCRTC2 transgenic mice and their wild type littermates. Data are reported as mean $\pm$ sem; group differences were tested for statistical significance with using Student's *t*-test (unpaired, 2-tailed).

<b>Measurement</b>	<b>CON (<i>n</i>=4)</b>	<b>ntCRTC2 (<i>n</i>=4)</b>	<b>P</b>
<b>Area Diastole (mm<sup>2</sup>)</b>	22.7 $\pm$ 0.9	20.0 $\pm$ 1.6	0.338
<b>Area Systole (mm<sup>2</sup>)</b>	15.5 $\pm$ 1.0	13.7 $\pm$ 1.2	0.297
<b>EF (%)</b>	48.9 $\pm$ 0.8	47.5 $\pm$ 1.4	0.419
<b>Heart Rate (bpm)</b>	567 $\pm$ 18.8	529 $\pm$ 18.4	0.203
<b>Aortic Ejection Time (ms)</b>	40.0 $\pm$ 0.8	44.7 $\pm$ 0.7	0.004



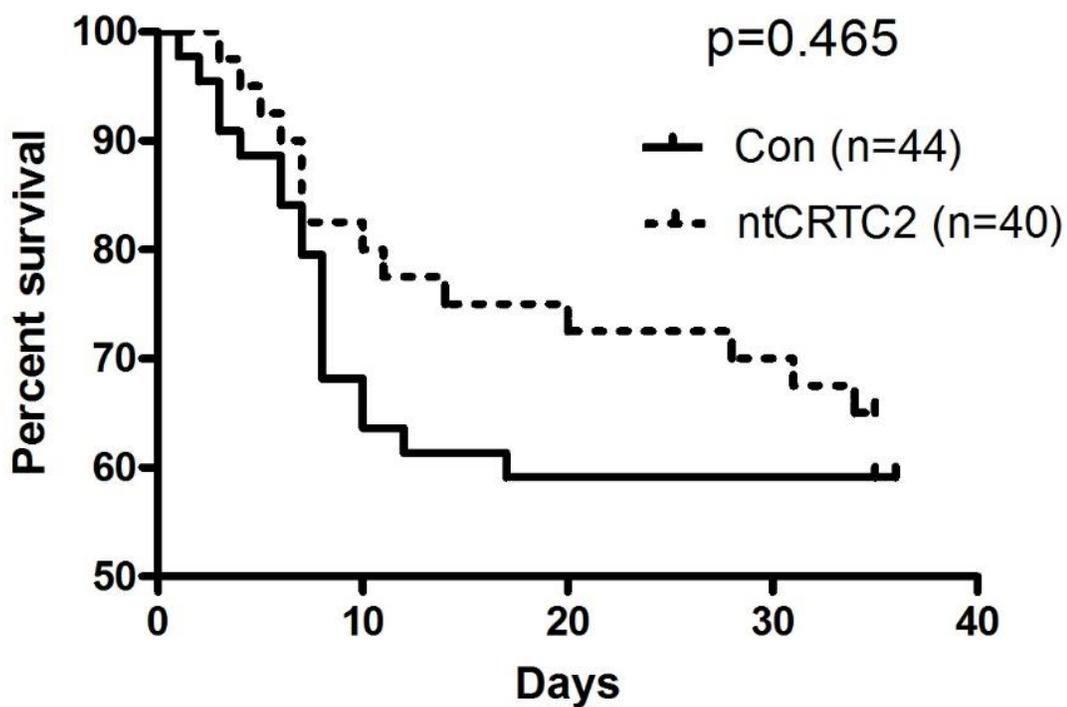
**Figure 2.** Hemodynamics of Mice with No Intervention/ No Myocardial Infarction. Rate of LV pressure development +dP/dt (A), Rate of LV Pressure Decay -dP/dt (B), Developed Pressure (C), and Heart Rate differences (D) between CRTC2 mice (ntCRTC2) and their wildtype littermates (CON).



**Figure 3.** Calcium Studies of Mice with No Intervention/ No Myocardial Infarction. Peak (A), tau (B), and time to peak (C) measurements of calcium transients between CRTC2 mice (ntCRTC2) and their wildtype littermates (CON). Group sizes are reported as individual cells.

**Table 2.** *Morphometrics of Mice with No Intervention/ No Myocardial Infarction.* Body weight (BW); left ventricle + interventricular septum (LV + IVS); Lungs; Liver in ntCRTC2 mice. Data are reported as mean $\pm$ sem; group differences were tested for statistical significance with using Student's *t*-test (unpaired, 2-tailed).

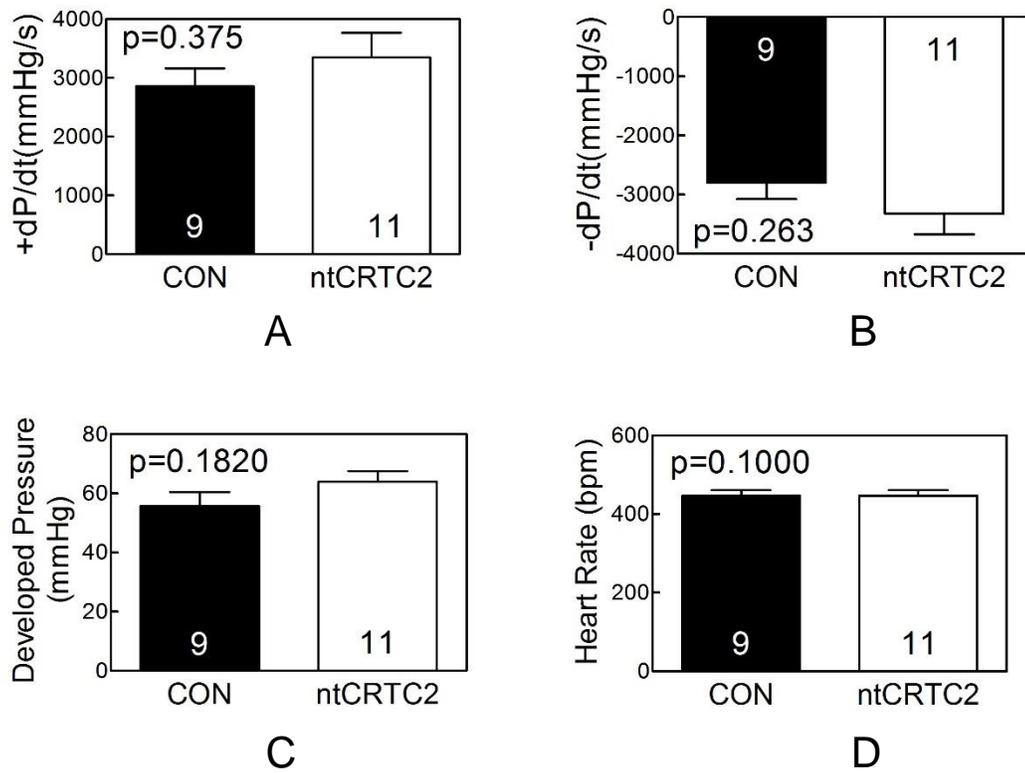
	<b>CON</b> ( <i>n</i> =3)	<b>ntCRTC2</b> ( <i>n</i> =7)	<b>P</b>
<b>BW (g)</b>	27.7 $\pm$ 1.5	28.3 $\pm$ 0.8	0.717
<b>LV + IVS (mg)</b>	95.0 $\pm$ 6.0	95.3 $\pm$ 1.9	0.953
<b>Lungs (mg)</b>	160.7 $\pm$ 14.7	158.3 $\pm$ 3.2	0.821
<b>Liver (mg)</b>	1393.0 $\pm$ 139.7	1355.0 $\pm$ 76.4	0.804
<b>(LV + IVS)/BW</b>	3.4 $\pm$ 0.03	3.4 $\pm$ 0.07	0.657



**Figure 4.** *Kaplan- Meier analysis* showing survival after myocardial infarction (MI) in ntCRTC2 transgenic mice and their wildtype littermates (CON). Subjects that survive 1 day post procedure are recruited into the study. Subjects alive 35 days post procedure are considered to have survived to completion of the study.

**Table 3.** *Echocardiography 5 weeks after Myocardial Infarction.* Diastolic area, systolic area, ejection fraction (EF%), heart rate, for CHF-control and CHF-ntCRTC2 mice. Data are reported as mean $\pm$ sem; group differences were tested for statistical significance with using Student's *t*-test (unpaired, 2-tailed).

<b>Measurement</b>	<b>CHF-Control (n=10)</b>	<b>CHF-ntCRTC2 (n=21)</b>	<b>P</b>
<b>Area Diastole (mm<sup>2</sup>)</b>	41.1 $\pm$ 3.2	44.2 $\pm$ 1.5	0.333
<b>Area Systole (mm<sup>2</sup>)</b>	39.3 $\pm$ 3.3	42.2 $\pm$ 1.5	0.364
<b>EF (%)</b>	7.0 $\pm$ 1.5	6.7 $\pm$ 0.9	0.813
<b>Heart Rate (bpm)</b>	542 $\pm$ 36.0	546 $\pm$ 19.1	0.915
<b>Aortic Ejection Time (ms)</b>	37.2 $\pm$ 1.3	35.8 $\pm$ 0.6	0.287



**Figure 5.** Hemodynamics 5 weeks after Myocardial Infarction. Rate of LV pressure development +dP/dt (A), Rate of LV Pressure Decay -dP/dt (B), Developed Pressure (C), and Heart Rate differences (D) between CRTC2 mice (ntCRTC2) and their wildtype littermates (CON) in the setting of myocardial infarction.

**Table 4.** *Morphometrics 5 weeks after Myocardial Infarction.* BW, body weight; LV + IVS, left ventricle + interventricular septum; Lungs; Liver in CHF mice. Data are reported as mean $\pm$ sem; group differences were tested for statistical significance with using Student's *t*-test (unpaired, 2-tailed).

	<b>CHF-CON</b> ( <i>n</i> =9)	<b>CHF-ntCRTG2</b> ( <i>n</i> =13)	<b>P</b>
<b>BW (g)</b>	24.9 $\pm$ 1.1	25.4 $\pm$ 0.8	0.728
<b>LV + IVS (mg)</b>	119.2 $\pm$ 6.6	127.7 $\pm$ 12.2	0.595
<b>Lungs (mg)</b>	326.1 $\pm$ 12.9	274.8 $\pm$ 28.4	0.170
<b>Liver (mg)</b>	1128.4 $\pm$ 67.0	1133.7 $\pm$ 41.4	0.945
<b>(LV + IVS)/BW</b>	4.8 $\pm$ 0.2	5.2 $\pm$ 0.7	0.645

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