Regulation by a developmental transcription factor

of signaling enzymes related to atrial fibrillation

by

Christina Law

A PROJECT

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in BioHealth Sciences (Honors Scholar)

Presented May 20th, 2015
Commencement June 2015

ABSTRACT OF THE THESIS OF

Christina M. Law for the degree of Honors Baccalaureate of Science in BioHealth Sciences presented on May 20th, 2015. Title: Regulation by a developmental transcription factor of signaling enzymes related to atrial fibrillation

Abstract Approved: _____

Theresa M. Filtz

Atrial fibrillation is the most common cardiac arrhythmia, and the mechanisms that lead to this heart defect are not fully understood. Consequently, treatments for this defect do not currently reverse structural or molecular variations that lead to cardiac arrhythmia. Loss of the gene coding for the transcription factor Pitx2 leads to abnormal cardiogenesis, and as a result mice with Pitx2 mutations are models susceptible to atrial fibrillation. In both humans and mice, increased expression of the enzyme Phospholipase C beta (PLC-β) correlates with atrial remodeling, hypertrophy, and apoptosis. Pitx2 was hypothesized to suppress the expression of PLC-β, and the reduction of Pitx2 was hypothesized to up-regulate hypertrophic and apoptotic signaling pathways that typically accompany atrial fibrillation. To test these hypotheses PLC-β expression was measured in mice mutated to have no Pitx2 gene, and compared to PLC-β expression in normal mice. Expression of PLC-β was measured by isolating mRNA from the mouse atria, and performing quantitative real-time polymerase chain reaction (qRT-PCR) assays for PLC-β. Western blot assays were utilized to determine relative PLC-β protein levels after elevation of Pitx2 in cultured cardiomyoblast cells. The consequences of this research will be followed in adult mice to indicate mechanisms for congenital heart defects, prospectively identifying factors in increased susceptibility to atrial fibrillation, and impart to potential drug development.

Key Words: atrial fibrillation, abnormal cardiogenesis, PLC-β, Pitx-2

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<u>Honors Baccalaureate of Science in BioHealth Sciences</u> project of <u>Christina M. Law</u> presented on <u>May 20th, 2015</u>.

APPROVED:

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

_____

Christina M. Law, Author

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

INTRODUCTION

Atrial Fibrillation

Congenital heart defects are among the most common birth defects. Atrial septal and

atrioventricular canal defects are closely correlated with atrial fibrillation, the most common

heart arrhythmia (Gatzoulis 1999, Berger 1999). Atrial fibrillation includes abnormal, irregular

beating of the upper heart chambers, and is associated with chronic dilation of the atria (Liu,

2005). Septal or valvular malformations increase atrial pressure, which can lead to dilation, and

eventually cardiomyocyte hypertrophy and apoptosis as compensatory mechanisms (Nattel

2008). The mechanisms for these adverse transformations are not well understood; current

pharmacotherapies for atrial fibrillation specifically treat the symptoms related to susceptibility

to blood clots and to suppression of arrhythmias without treating the underlying atrial

remodeling.

Pitx2

Pitx2 is a homeodomain transcription factor, controlling the rate of sequence-specific

transcription of genetic information from DNA to messenger RNA. Homeodomain transcription

factors are involved in the regulation of anatomical development; Pitx2 is responsible for

specifying the "left-sidedness" of the atria in cardiogenesis (Kioussi 2002, Kitamura 1999,

Kirchof 2011). During embryonic development in vertebrates, the signaling pathway which

establishes left-right asymmetry is completed by Pitx2 (Kirchof 2011). Mice embryos

heterozygous for Pitx2 show an increase in mesodermal cells in the left lateral body wall,

resulting in failure of embryonic ventral body wall closure (Kitamura 1999). Right pulmonary

isomerism, swelling of atrioventricular canals, and atrial juxtapositioning have also been

detected in Pitx2 heterozygous embryos (Kitamura 1999). Cardiac malformations from total loss of Pitx2 in mice are embryonically lethal; these malformations include atrial isomerism, atrioventricular, atrial, and ventricular septal defects, and outflow tract defects (Kirchof 2011).

Axenfeld-Reiger Syndrome (ARS) is an autosomal dominant disorder that is defined by mutations and deletions of Pitx2 in humans. ARS is characterized by a wide range of defects, including malformation and underdevelopment of teeth and eyes, hearing loss, and abnormal cardiac myogenesis (Grosso 2002). Because atrial septal defects and sensorineural hearing loss have been shown to mutually occur in patients with this syndrome, ARS may be a formative disorder of the neural crest, which is responsible for the development of cardiac structures such as the outflow tract and the aortic arch system (Grosso 2002). Both humans and mice with Pitx2 deletions and alterations have isomeric and abnormal atria, specifically septal ventricular and valvular defects (Grosso 2002).

Phospholipase C beta
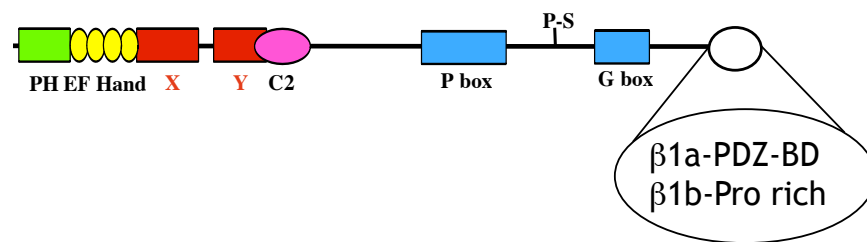
Figure 1: Linear schematic of Phospholipase C beta family enzymes. Shown are the known structural domains of Phospholipase C beta based on sequence homology and crystal structures. PH, EF Hand, and C2 are common lipid and/or calcium-binding domains. The X and Y refer to the two subdomains that comprise the catalytic core. The P box and G box are subdomains that are relevant for membrane association and G-protein stimulation, respectively. For PLC-β1 splice variants a and b, the difference in the C-terminal domains is indicated. The PLC-β1a splice variant contains a PDZ domain known to be involved in protein-protein interactions. The proline-rich domain in the PLC-β1b variant has been shown to be involved in interactions with scaffolding proteins at the plasma membrane (Gresset 2012).

Phospholipase C beta (PLC-β) is a signaling protein that hydrolyzes the membrane lipid PIP2 into two signaling molecules, diacylglycerol and inositol triphosphate. There are four isoforms of PLC-β, 1,2,3 and 4, with PLC- β1 having two prominent splice variants, a and b (for review, see Figure 1). PLC-β isoforms 1 and 3 are known to be present in human and mice hearts and are stimulated by G-protein coupled receptors in a wide array of physiological processes, including cardiomyocyte hypertrophy (Arthur 2001). PLC-β1 specifically has been found in high concentration in the brain and high PLC-β1 activity has been measured in heart tissues (Filtz 2009). PLC-β both mobilizes intracellular calcium and activates protein kinase C, generating normal cellular responses such as smooth muscle contraction, neurotransmitter release, and stimulation of cell growth. However, overexpression of the splice variant PLC-β1b in neonatal rat cardiomyocytes has been shown to cause increased cell size (Figure 2), apoptosis, elevated protein to DNA ratio, and up-regulation of atrial natriuretic peptide, a gene related to cardiac hypertrophy (Filtz 2009). These results from rat neonates suggest that PLC-β has a role in cardiac remodeling (Woodcock 2009).
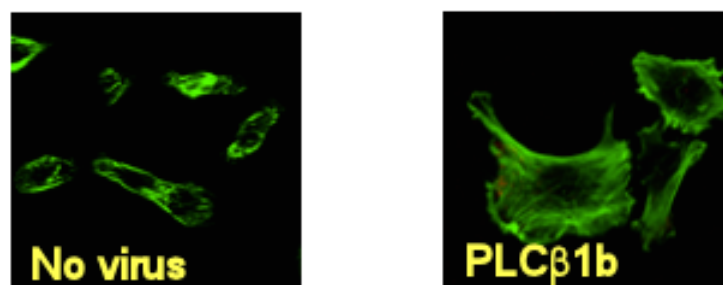
Figure 2: Overexpression of PLC-β1 in rat neonatal ventricular cardiomyocytes causes enlargement. Shown left is normal rat neonatal ventricular cardiomyocytes: no evidence of hypertrophy. Shown right is rat neonatal ventricular tissue with high levels of PLC-β1 with obvious hypertrophy. (Filtz 2009)

PLC-β1 has been shown to be associated with heart cell enlargement and is increased in diseased atrial tissue. PLC-β1b expression is selectively elevated in the left atria of valvular heart disease

patients who are undergoing surgery for coronary artery bypass when compared to normal atrial tissue and the diseased heart cells are clearly hypertrophic (Woodcock 2009). Overexpression of PLC-β due to gene rearrangement occurs in a variant of Wolf-Parkinson-White Syndrome (WPWS), a pre-excitation disorder which causes premature ventricular contraction. WPWS is the second most common cause of paroxysmal supraventricular tachycardia, and is clinically characterized by presyncope, syncope, shortness of breath, and sudden death (Gollob 2001). Conduction anomalies in the heart associated with WPWS also increase susceptibility to atrial fibrillation (Mills 2013).

Hypothesis

Both Axenfeld-Reiger Syndrome and Wolf-Parkinson-White Syndrome are associated with atrial cardiac arrhythmias; the former is defined by a decrease in Pitx2 and the latter is associated with high levels of PLC-β.

This project aimed to further define the relationship between Pitx2 and PLC-β in regards to the development of atrial fibrillation in mouse hearts. We hypothesized that alterations in signaling pathways in myogenesis due to Pitx2 mutations and deletions increase susceptibility to malformation and atrial fibrillation. Reduction of Pitx2 was hypothesized to increase hypertrophic and apoptotic signaling pathways, including the PLC-β signaling pathway in mouse hearts. In studying the Pitx2 mutant mouse model of cardiac malformation, we focused on the PLC-β signaling pathway, which is associated with atrial hypertrophy. Previously published microarray data suggested that the deletion of Pitx2 up-regulates PLC-β1 gene expression in embryonic mice at day 12.5 (Kirchof 2011).

QRT-PCR
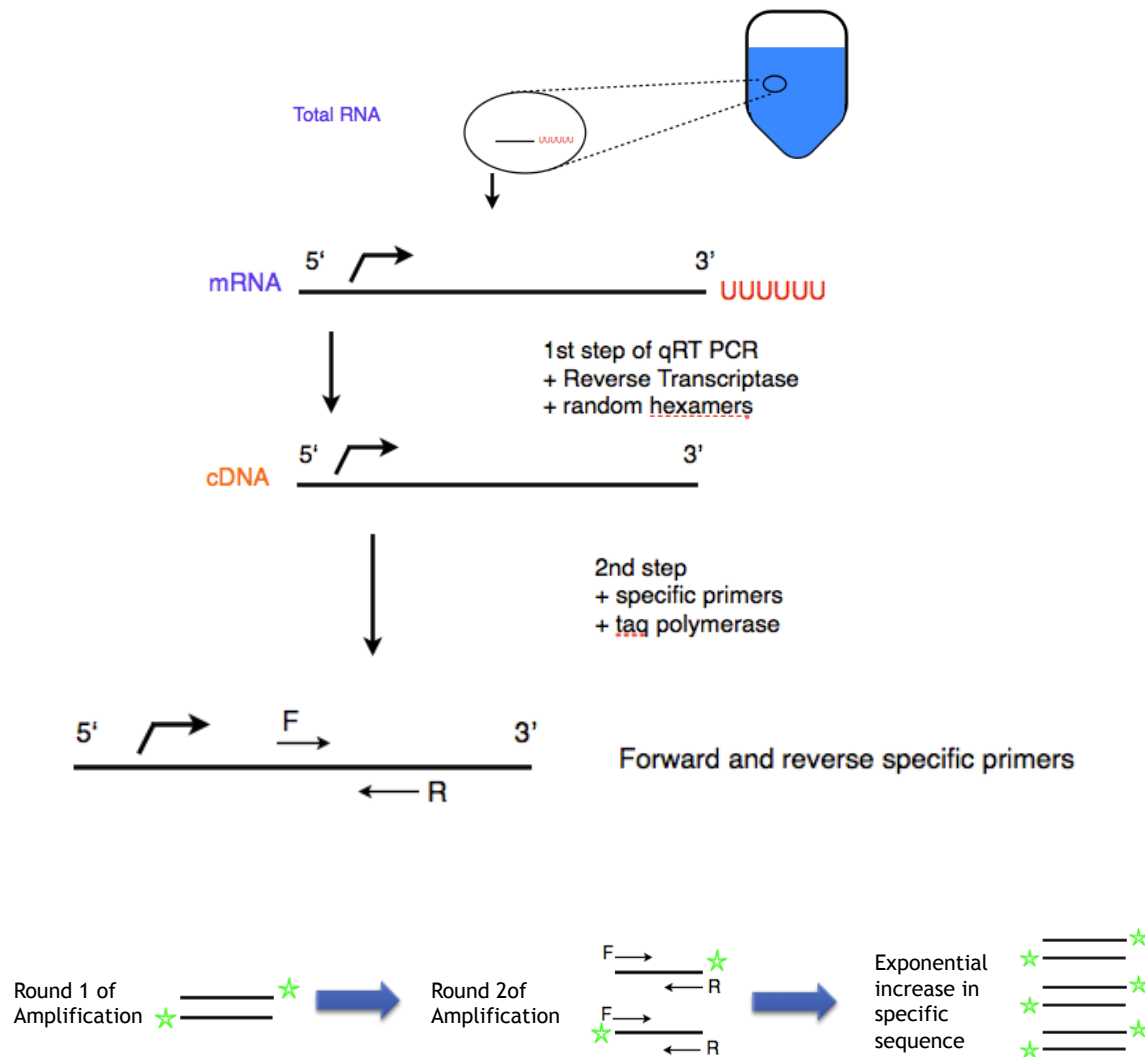
Figure 3: Linear schematic of qRT-PCR from total RNA from isolated tissues to amplification of specific sequences in cDNA. Specific sequences are detected with SYBR Green fluorescent dye.

Our first approach in identifying, quantitating, and validating changes in cardiac gene expression due to changing levels of Pitx2 was to use quantitative real-time polymerase chain reaction assays (qRT-PCR). Using mice in which one or both alleles for Pitx2 have been deleted, we quantitated expression of PLC-β isoforms by isolating RNA from heart tissue and performing qRT-PCR. In qRT-PCR reactions, reverse transcription of isolated mRNA to cDNA in samples is followed by incubation with specifically designed primers and *Taq* polymerase, thus

facilitating selective amplification of a sequence of interest (Figure 3). An exponential increase in this specific sequence is detected with SYBR Green fluorescent dye, and can be expressed as specific mRNA levels normalized to an invariant transcript, generally a housekeeping gene; HPRT was our chosen control gene. Fluorescence intensity data from the exponential increase in transcript amplification is interpolated at a set threshold where the increase in fluorescence is most linear to yield a threshold cycle or C_T value. The natural log of the C_T value for the transcript of interest is divided by the value for the housekeeping gene to yield a ΔC_T for comparative purposes.

H9c2

Our second hypothesis was that if Pitx2 regulated PLC-β levels in heart cells, then over-expression of Pitx2 would cause a decrease in PLC-β levels. To test this hypothesis, we attempted to overexpress Pitx2 by transfection into a cardiomyocyte-like cell line in culture and quantify subsequent PLC-β levels (Figure 4). The cardiac cells used were H9c2 cells, a cell line of rat ventricular myoblasts that has been validated as a working model for studies in heart development and disease. When stimulated with hypertrophic agents, H9c2 cells showed nearly identical hypertrophic responses and signaling pathways to cardiomyocytes in culture (Watkins 2010). H9c2 cells don't beat like heart cells, but they do respond to stress with hypertrophic mechanisms.
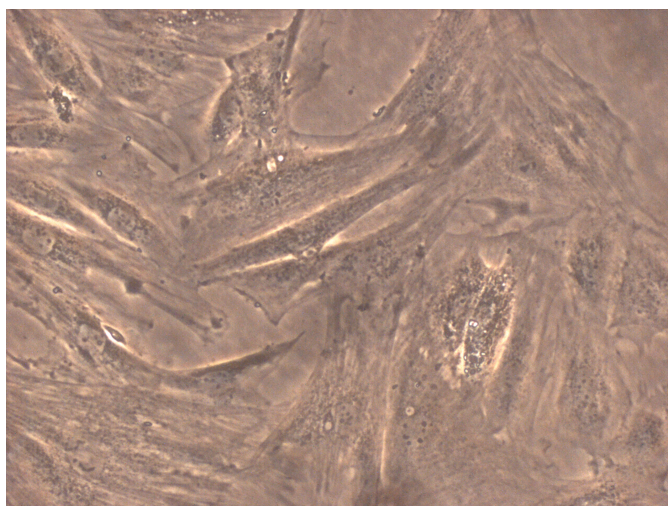
Figure 4: H9c2 rat ventricular myoblasts at 40X magnification.

METHODS

Purification of Total RNA

Hearts were excised from mice at embryonic at day 16.5 (E16.5), and atria were separated from ventricles under a stereomicroscope prior to freezing in liquid nitrogen. Embryos were identified as Wild Type (WT) or Pitx2$^{+/Z}$ (heterozygous knock out, HET) by X-gal staining for β-galactosidase expression in the heads of heterozygous embryos at the time of dissection.

Total RNA was extracted and purified from atria and ventricles with the RNEasy Qiagen Protocol following tissue disruption for 5 min in RNALater buffer (Qiagen®) by 0.2 mm glass beads. Purified RNA was quantitated on a microliter scale by UV spectrometer (Nanodrop®) and was stored at -80° C.

QRT-PCR

PLC-β isoenzymes were quantitated by qRT-PCR using the primers listed in the Table below and designed using NCBI/Primer Blast software. Primers were designed for specificity across the transcriptome, to cross intron-exon boundaries, and to minimize GC content and primer-dimer formation within a 22 to 23 bp size (Table 1).

The qRT-PCR reactions were conducted using the protocol in the Superscript III qRT-PCR kit (Life Technologies). The reactions included 200 μM forward and reverse primers, Superscript III high fidelity *Taq* polymerase, and SYBR Green dye. HPRT was quantitated as a invariant control transcript for each sample.

Table 1: List of PCR primers for PLC-β isoenzyme quantitation

Variant	Sequence 5' to 3'	Forward / Reverse	Tm (°C)	Size (base pairs)	%GC
PLCB1a	CGGAGCTGGAGCAAGAATAC	F	55.6	20	55
PLCB1a	TCACCTTTGCAGCATCTGAG	R	55.3	20	50
PLCB1b	GAAAAACTCGTGGAGAAACACA	F	53.5	22	40.9
PLCB1b	CTTGAGAGCTTGAGGGTTGG	R	55.6	20	55
PLCB2	TGCTGATCGAAAACGGGTGG	F	58	20	55
PLCB2	AGCTTTAGAGTGGTAGGAAGTGA	R	55.1	23	43.5
PLCB3	GGAGCGTGTGGAGAGAGCAG	F	60.1	20	65
PLCB3	GTCCACCTCCATGTTGGGTC	R	58	20	60
PLCB4	CAAGGGAGGCCGAGTTGATT	F	57.4	20	55
PLCB4	TTTCCGTATGGTGTCGGTGG	R	57.3	20	55
HPRT	GTTAAGCAGTACAGCCCCAAA	F	55.3	21	47.6
HPRT	AGGGCATATCCAACAACAAACTT	R	55.1	23	39.1

Cell Lines

H9c2, rat ventricular cardiomyoblast-like, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% Fetal Bovine Serum (FBS) and antibiotics (penicillin and streptomycin). The cells were grown in humidified 5% CO_2 / room air at 37° C. The myoblast phenotype was maintained throughout culture by visual inspection. Cells were routinely subcultured at 80% confluency and transfected at 50% confluency.

Brain from adult mice was isolated and used as positive control for PLC-β isoenzymes and for Pitx2 expression. Following brain tissue disruption by 10 sec homogenization with a Polytron tissuemizer, tissue was agitated for 2 hours in RIPA Lysis buffer (20 mM Hepes, 150 mM NaCl, 2 mM EDTA, 1% NP40, 0.5% Sodium deoxycholate, 0.1% SDS) with protease inhibitors (E64, leupeptin, pepstatin A, and PMSF) to liberate nuclear and membrane-bound proteins. Following

agitation, the tissue was sonicated on ice at an amplification of 25% for 5x5 seconds, then centrifuged at 4° C , 12 kxg for 20 minutes. The supernatant was divided into 20 aliquots of 200 µl each and stored at -80° C.

Transfection

Four lipid reagents were tested to optimize transfection efficiency (Figure 7). Cultured H9c2 cells were transfected with Metafectene® (Invitrogen), Cellfectin (Life Technologies), Lipofectamine 2000 (Life Technologies), and Lipofectin (Life Technologies) in 10 cm tissue culture plates in DMEM + 10% FBS medium without antibiotics as recommended by the manufacturer. Plasmid DNA (14 ug per transfection) and reagent (30 ul per transfection) were mixed in serum-free OPTI-MEM medium (Life Technologies) prior to addition to H9C2 cells. After 48 hours, the cells were examined under a fluorescent microscope to detect green fluorescent protein (GFP) for quantitation of transfection efficiency, and then harvested for PLC-β detection.

Following a wash with 1X PBS, cells were harvested in RIPA Lysis buffer plus protease inhibitors (listed above). After intermittent vortexing on ice for 30 minutes, the cells were sonicated at 25% amplitude for 5 x 5 seconds. The sonicated samples were then centrifuged at 2000 rpm for 10 minutes at room temperature, and the supernatant (whole cell lysate) was stored at -80° C in preparation for immunoblotting protocols.

SDS-PAGE and Immunoblotting

Samples were run on NuPage Bis-Tris 4-12% gradient gels (Life Technologies) by standard protocols for SDS-Polyacrylamide gel electrophoresis (SDS-PAGE). Each sample was loaded with 16.5 µl NuPage sample buffer plus 50 mM DTT. After electrophoresis at 125-160 V and adequate size separation, proteins were transferred from gel to Nitrocellulose by horizontal

electrophoresis in Transblot buffer (0.10 M Tris, 0.79 M Glycine, 10% MeOH). Blots were separated horizontally at the 100 kDa band to create high molecular weight blots for detection of PLC-β and low molecular weight blots for detection of Pitx2 or GFP. Blots were blocked in 10 ml of Odyssey blocking buffer (Licor®) for 30 min. The blots were then incubated overnight at 4°C with swirling in the following primary antibodies in Odyssey blocking buffer plus 0.2% Tween-20 detergent: PLC-β1 G12 anti-Rabbit (1:500, Santa Cruz Biotechnology), PLC-β2 anti-Rabbit (1:250, Santa Cruz Biotechnology), PLC-β3 C20 anti-Rabbit (1:500, Santa Cruz Biotechnology), PLC-β4 anti-Rabbit (1:250, Santa Cruz Biotechnology), Pitx2 anti-Goat antibody (1:200, Santa Cruz Biotechnology), and GFP anti-Guinea Pig (1:500; courtesy of Dr. Chrissa Kioussi; Kioussi 2002). After 24 hours, the blots were washed with deionized water and Tris-buffered saline plus 0.1% Tween-20 (TBS-T), and then incubated with swirling at room temperature in anti-rabbit (1:5,000) or anti-goat (1:20,000) IgG secondary antibody coupled to IR dye with emission at 680 or 800 nm for detection by the Odyssey Imager (Licor®). Licor ImageStudio® software was used to quantitate the fluorescent intensity of the PLC- β-specific bands at 135 to 150 kDa.

Gentle Stripping of Blots

For detection of a second set of proteins, some blots were stripped using three rounds of 15 minutes in warm 0.1 M glycine, 1% SDS stripping buffer with a pH of 2.0. The blots were then rinsed in TBS-T wash buffer to bring the pH back to neutral, then re-blocked as described above. The blots were then incubated overnight at 4° C with swirling in anti-tubulin antibody (1:700, Cell Signaling Technology) and anti-Mouse (1:5000, Li-Cor Biosciences) IgG secondary antibody coupled to IR dye with emission at 800 nm for detection by the Odyssey Imager.

RESULTS AND DISCUSSION

Axenfeld-Reiger Syndrome is defined by decreased Pitx2, and Wolf-Parkinson White Syndrome is associated with increased PLC-β1 (Grosso 2002, Gollob 2001). Axenfeld-Reiger Syndrome and Wolf-Parkinson-White Syndrome are both associated with susceptibility to atrial cardiac arrhythmia or atrial fibrillation. Reduction of the transcription factor Pitx2 was hypothesized to increase hypertrophic and apoptotic signaling pathways, including the PLC-β signaling pathway, in mouse hearts. We wished to investigate this hypothesis by quantitating levels of PLC-β isoforms in mouse hearts from Pitx2$^{+/+}$ (Wild Type) and Pitx2$^{+/Z}$ (heterozygous) mice.
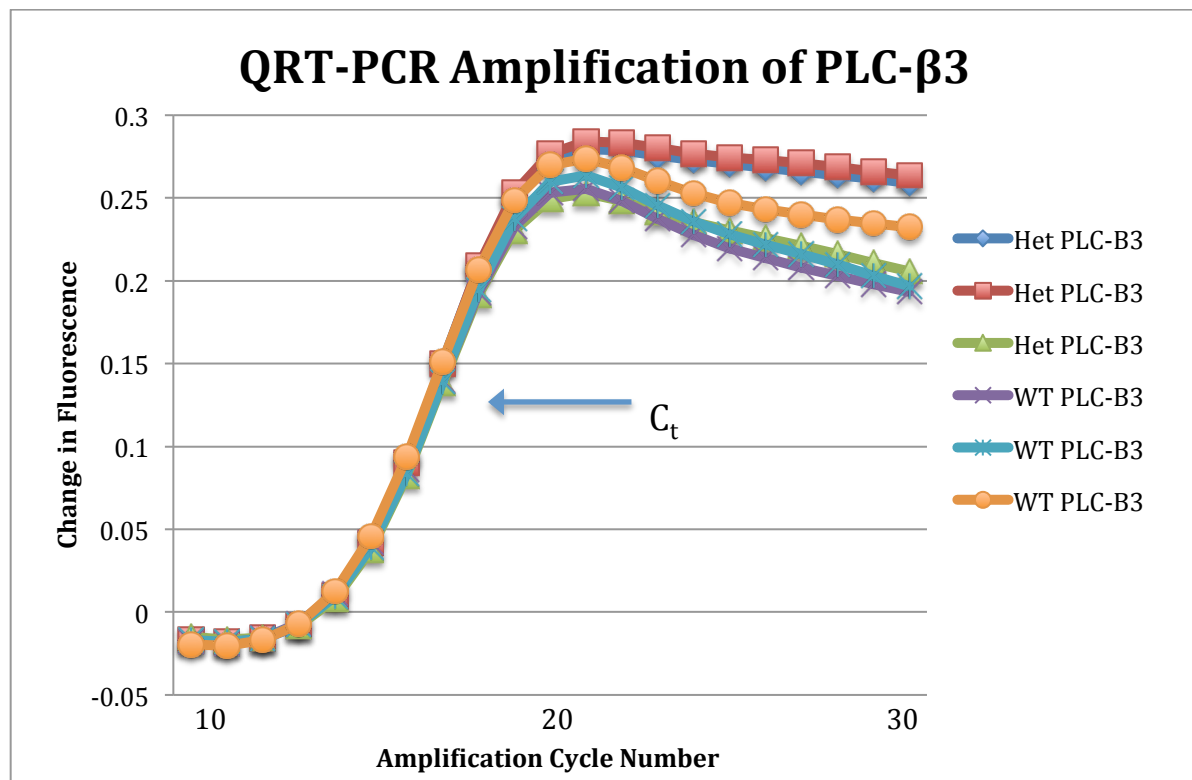
Primer Optimization

Figure 5: Example of qRT-PCR amplification data showing quantitation of fluorescence in a qRT-PCR assay over 30 amplification cycles using primers selected for mouse PLC-β3. C_t indicates the threshold cycle used for quantitation of mRNA levels.

Quantitative real-time PCR (qRT-PCR) was used to quantitate and compare expression of PLC-β isoforms (quantitation of mRNA) between Wild Type and heterozygous mice.

Primers were designed and constructed to specifically amplify our sequences of interest. In qRT-PCR, an exponential increase in a specific sequence is expected over many rounds of amplification. We tested a total of 14 primer sets for all isoforms of PLC-β, and analyzed both the fluorescence dissociation curves and the PCR products on a DNA gel . In the process, we determined that most of the primers were not completely selective and amplified non-specific DNA sequences or perhaps formed primer dimers. Nevertheless, we found a primer pair for each isoform of PLC-β that functioned well for selective amplification (Table 1). We used these validated primer sets for all further qRT-PCR experiments in this thesis.

Quantitation of PLC-β with Pitx2 Deletions

We quantitated PLC-β levels in Wild Type mice, with normal levels of Pitx2, and mice with one allele of Pitx2 knocked out, heterozygous mice, to investigate a correlation between reduction of the transcription factor Pitx2 and up-regulation of PLC-β isoforms. Expression of all isoforms was normalized to HPRT, a housekeeping gene. Heterozygous mice with only one Pitx2 allele showed a higher expression of PLC-β isoforms than Wild Type mice (Figure 6). Wild Type adult mice atria expressed low levels of all isoforms, except for PLC-β3, which showed moderate expression relative to HPRT. This relatively high expression of the PLC-β3 isoform is also reflected in previous data quantitating mRNA levels for PLC-β isoforms in atria from Wild E12 mice (Anita Jong, unpublished data). Heterozygous adult mice atria increased gene expression of both splice variants, a and b, of the PLC-β1, PLC-β3, and PLC-β4 isoforms relative to Wild Type.
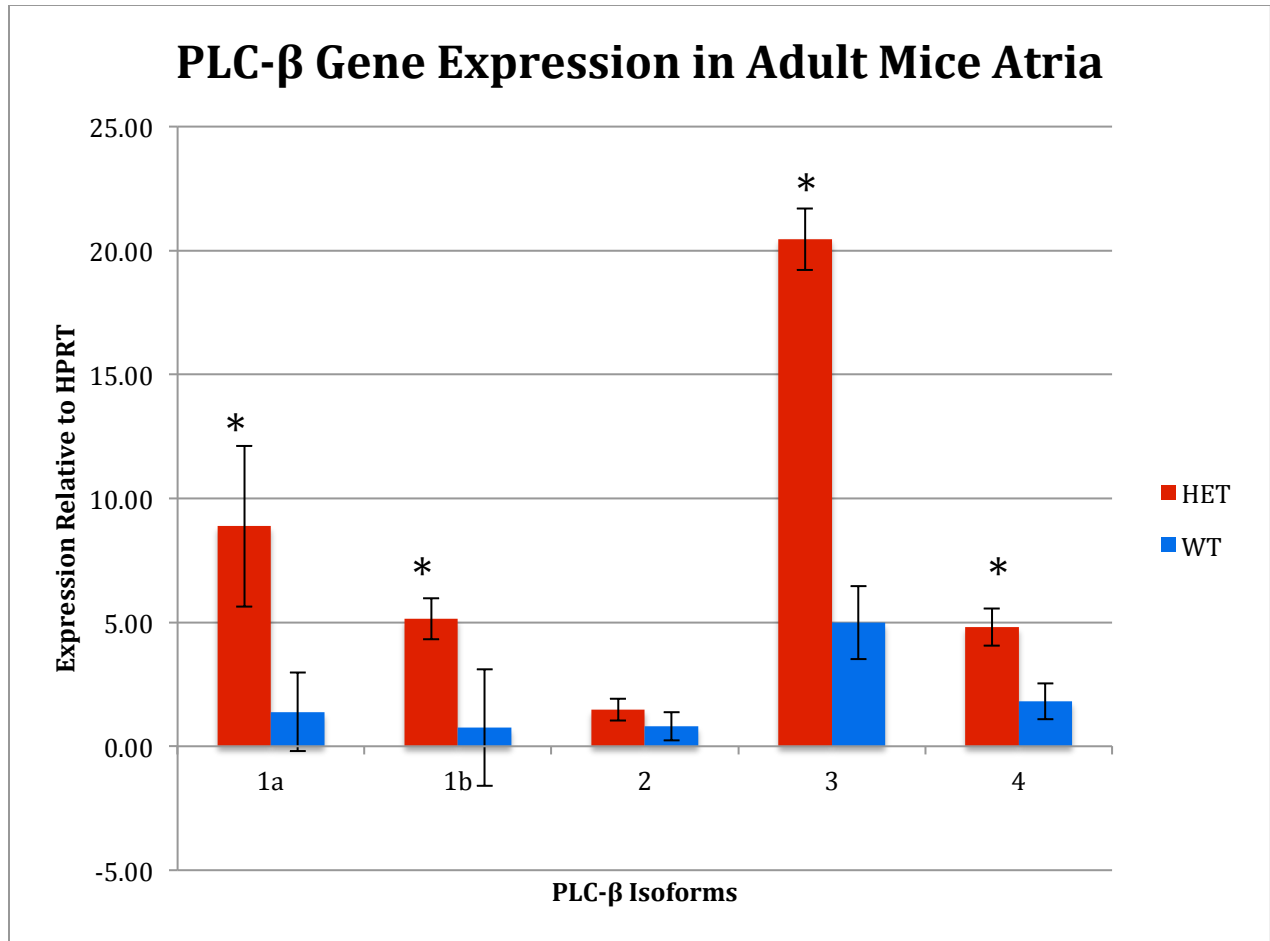
Figure 6: Quantitation of mRNA levels for PLC-β isoforms in atria from Wild Type (blue) and Pitx2$^{+/Z}$ (red) adult mouse atria as described in Methods. Shown is a representative experiment conducted in triplicate with average +/- standard deviation. All samples were normalized to HPRT as a control transcript. (* indicates significance relative to Wild Type with a $p<0.05$.)

Quantitation of PLC-β with Pitx2 Overexpression

After examining the correlation between reduced Pitx2 levels in mouse hearts and levels of PLC-β gene expression, we sought to examine the opposite relationship. H9c2 cells are a line of immortalized rat ventricular myoblasts which resemble cardiomyocytes in terms of signaling pathways and morphology. H9c2 cells undergo hypertrophy in response to stress, increasing in size, resembling cardiomyocyte hypertrophy. We chose to use this cell line to test the hypothesis that down-regulation of PLC-β would occur in the presence of increased Pitx2. To overexpress Pitx2 in H9c2 cells, we needed to find an effective transfection reagent. Using a GFP-containing

mammalian expression plasmid (pCMV-EGFP), we tested four different lipid reagents and assessed transfection efficiencies for Lipofectamine 2000, Lipofectin, Cellfectin and Metafectene. Of these, Metafectene produced the highest efficiency of approximately 20% of total cells (Figure 7) as detected by fluorescence microscopy for expression of green fluorescent protein.

We transfected H9C2 cells with the bicistronic mammalian expression vector, pIRES-GFP-Pitx2, and assessed transfection efficiency by fluorescence microscopy (Figure 8). The transfection efficiency for both experiments with Metafectene and pIRES-EGFP-Pitx2 was approximately 30%.

We sought to examine the protein levels of PLC-β in H9C2 cells by immunoblot following transfection with pIRES-GFP-Pitx2 or with the control vector pIRES-GFP. PLC-β proteins are expected to appear on immunoblots at 130-150 kDa. Whole rat brain extracts were included as a positive control tissue for the expression of PLC-β, and expression of PLC-β isoenzymes are clearly visible on each blot at approximately 130-150 kDa.
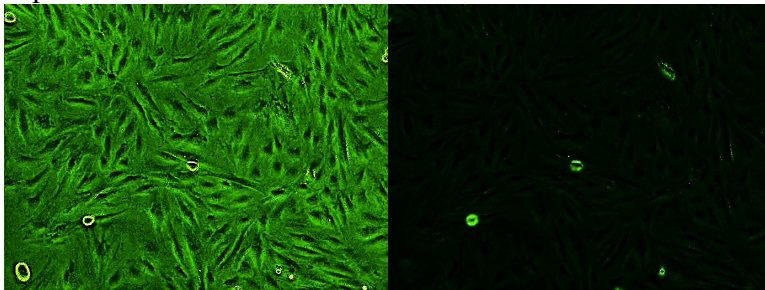
Metafectene
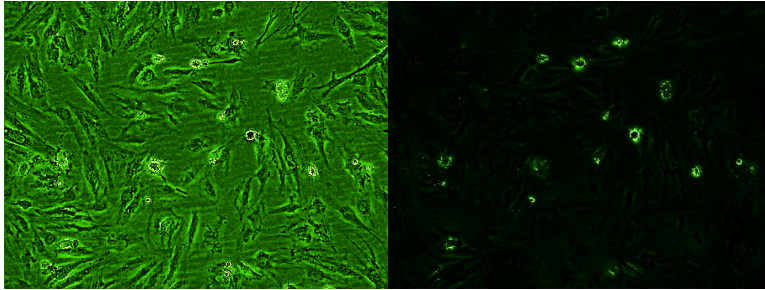
20%

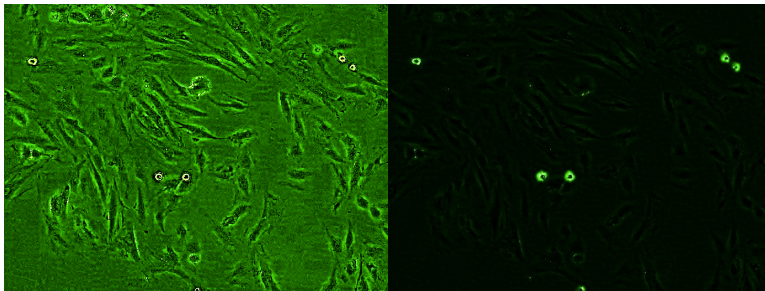Lipofectamine 2000
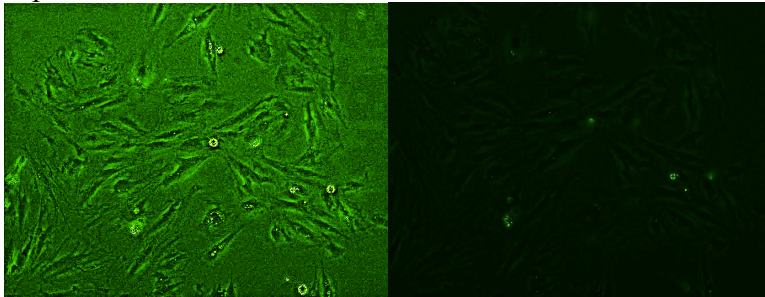
5%

Lipofectin

2.5%

Cellfectin

5%

Figure 7: Transfection efficiency of 4 different reagents in H9c2 cells. H9c2 cells were transfected with GFP cDNA in a mammalian expression vector according to manufacturer's recommended protocols by the four different lipid-based carriers listed. Shown are photomicrographs 48 hours post-transfection. Left panels are white light with green filter (total cells). Right panels are green fluorescent (GFP-expressing cells). Estimated transfection efficiency is listed to the right of the panels.
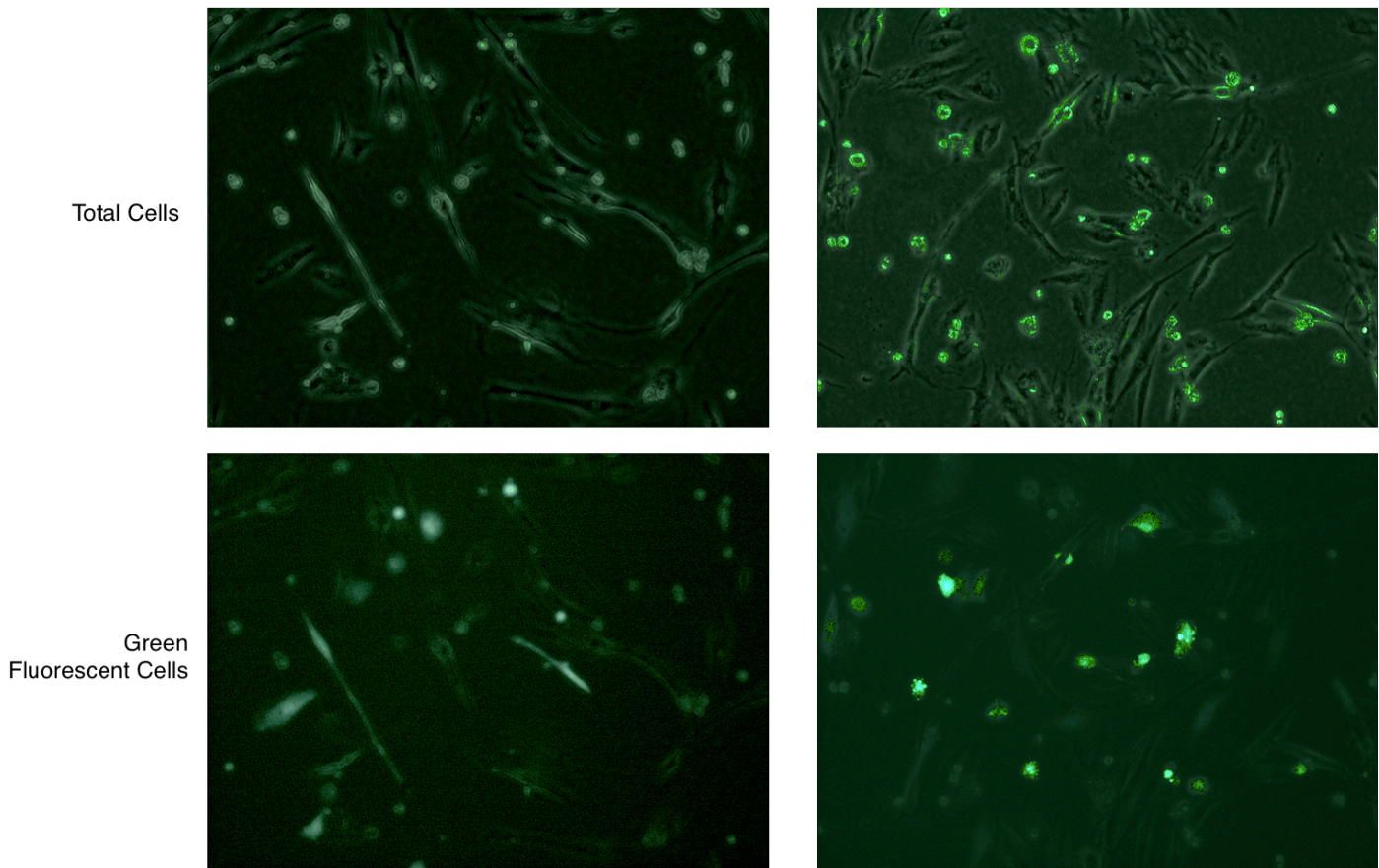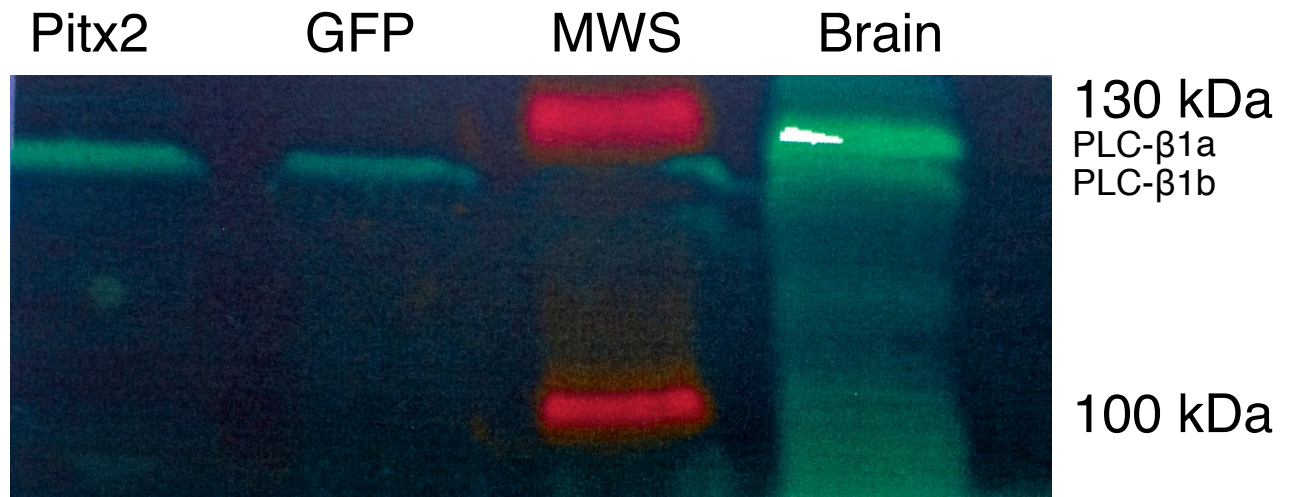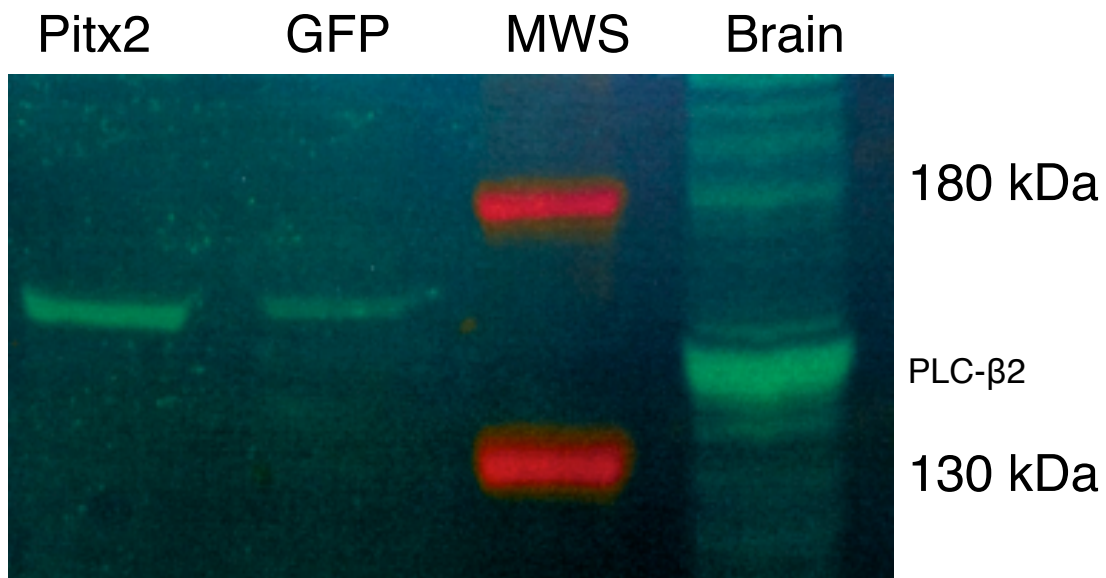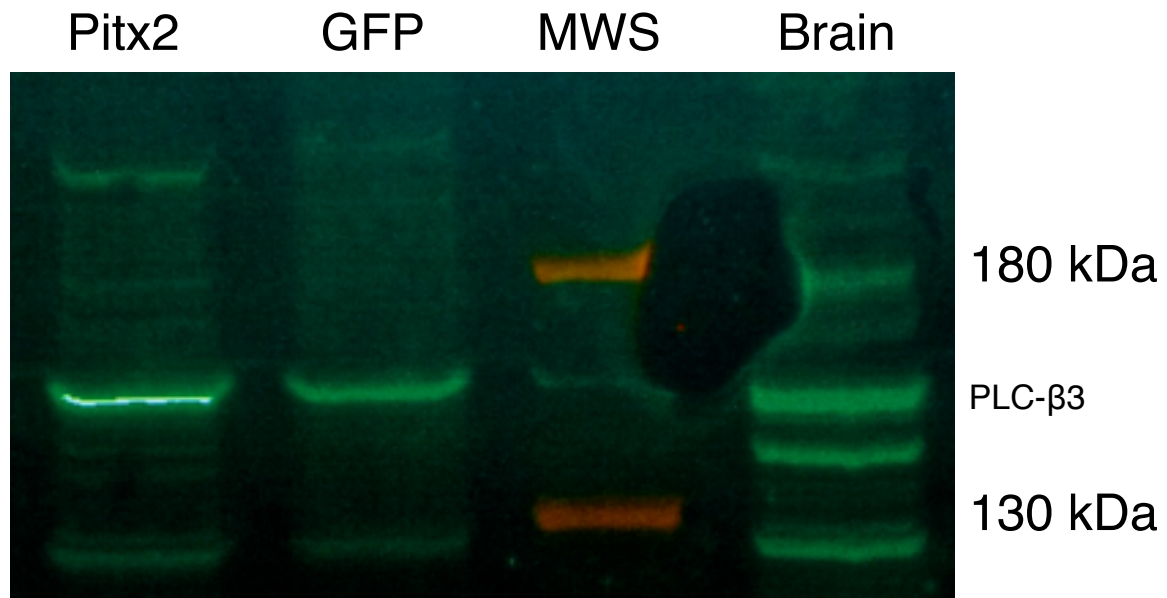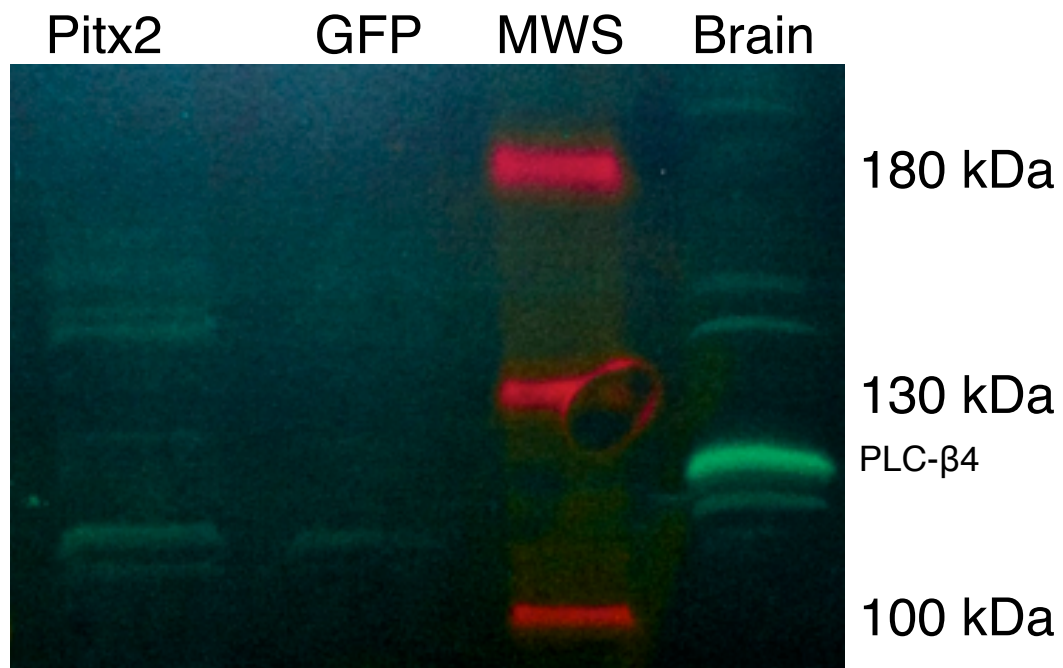
Figure 8: Transfection of H9c2 cells for expression of GFP and Pitx2. H9c2 cells were transfected with a bicistronic mammalian expression vector containing Pitx2 and GFP cDNA sequences using Metafectene. 48 hours post-transfection, photomicrographs were taken under white light with a green filter (total cells) or green fluorescent light (GFP-expressing cells). Transfection efficiency was quantitated as number of fluorescent cells over number of total cells in a field and averaged at 30%. Shown are two separate experiments (left and right columns).

Antibodies selective for the PLC-β isoforms were incubated with whole cell lysates following

SDS-PAGE electrophoresis and electrotransfer to nitrocellulose for blotting. Secondary

antibodies linked to fluorescent tags were used for quantitative detection of immunoreactive

bands with the Licor Odyssey fluorescent scanner.

As assessed by immunoblot, H9c2 cells were observed to express PLC-β1 and β3 isoforms more

highly than PLC-β2 or PLC-β4, (Figure 6) as previously shown for rodent adult hearts or

neonatal cardiomyocytes (Arthur 2001).

A) Immunoblot: PLC-β1

Pitx2	GFP	MWS	Brain	
				130 kDa
				PLC-β1a
				PLC-β1b
				100 kDa

B) Immunoblot: PLC-β2

Pitx2	GFP	MWS	Brain	
				180 kDa
				PLC-β2
				130 kDa

C) Immunoblot: PLC-β3
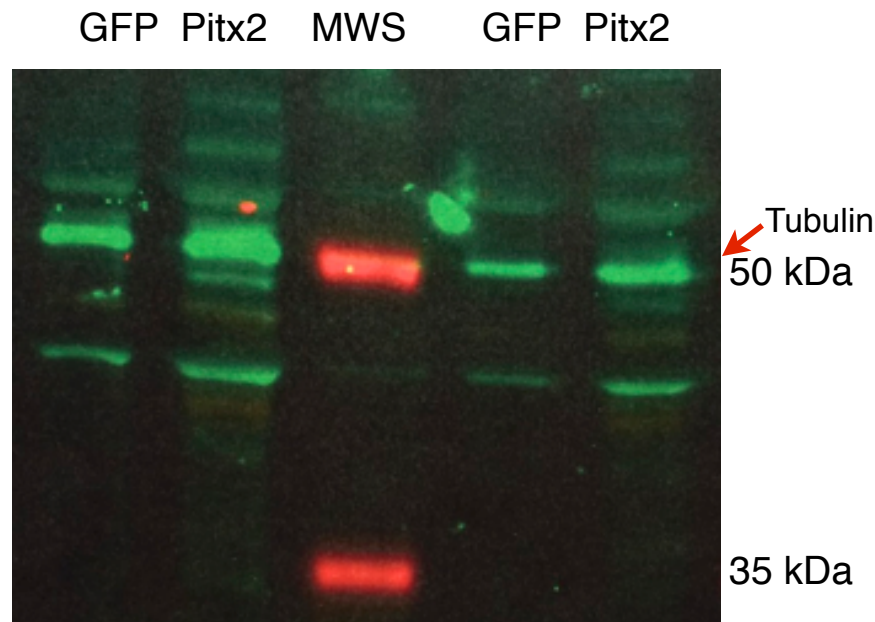

D) Immunoblot: PLC-β4

E) Immunoblot: Tubulin

Figure 9: Expression of PLC-β isoforms in H9c2 cells with overexpression of Pitx2 or GFP (negative control) via transfection of cDNAs in mammalian expression vectors, or in whole brain extracts (positive for all PLC-β isoforms). Following harvest, cell lysates were size-separated by SDS-PAGE and immunoblotted as follows: A) PLC-β1 antibody. B) PLC-β2 antibody. C) PLC-β3 antibody. D) PLC-β4 antibody. E) Tubulin antibody.

Whole rat brain extracts expressed both splice variants of PLC-β1, a and b, as expected.

However, H9c2 myoblasts appeared to mostly express the shorter splice variant, PLC-β1b,

(Figure 9A) as the PLC-β1a band was not visible on our blots. Previous studies of PLC- β1

isoforms in neonatal rat ventricular myocyte primary cultures also displayed significantly higher

levels of PLC- β1b expression relative to the β1a isoform (Filtz 2010). PLC-β immunoblot

signals are strongest for the PLC-β3 isoform in H9c2 myoblasts (Figure 9). This correlates with

mRNA transcript levels in adult rodent atria (Figure 6). Immunoreactive bands in the PLC-β2

immunoblot from H9c2 myoblast samples do not correspond with the expected size of PLC-β2

observed in control brain extracts, indicating non-specific artifacts associated with this antibody

(Figure 9B). Although quantitation depends on antibody affinity, PLC-β4 was not detected in

H9c2 myoblasts, but was present in brain control samples. This was expected as neither PLC-β2

nor PLC-β4 were detected in neonatal rat ventricular myocyte primary cultures (Arthur 2001).

Quantitation of Tubulin with Pitx2 Overexpression

Table 2: Immunoblot Intensity Values. PLC-β isoform and tubulin levels were quantitated from size-specific immuno-reactive bands shown in Figure 5 using the Licor imaging software as described in Methods.

	GFP	Pitx2	Ratio Pitx2/GFP
PLC-β1	603,000	1,850,000	3.07
PLC-β2	20,400	55,200	2.71
PLC-β3	987,000	3,910,000	3.96
PLC-β4	18,300	33,000	1.80
Tubulin	2,430,000	20,500,000	8.44

Using Imaging software to quantitate the relative levels of PLC-β isoforms in Pitx2 transfected

versus control transfected H9C2 cells, all three expressed isoforms of PLC-β, 1a, 1b and 3, were

increased in cells expressing Pitx2. However, when we normalized PLC-β protein levels to that

of a control protein, tubulin, we also found higher expression of tubulin in Pitx2-expressing

samples. Expression of Tubulin in H9c2 myoblasts appeared at the expected weight of

approximately 50 kDa (Figure 9E). Higher expression of tubulin in the H9c2 myoblasts with

overexpression of Pitx2 appeared to indicate that the increased PLC-β levels were simply due to

more cells in the samples with Pitx2 (Table 2). Another explanation we construed was that Pitx2

overexpression was affecting cell growth, indicating that tubulin levels are affected by Pitx2

levels.

Personal communication following the data collection for this project revealed that Pitx2 has

been shown to regulate cytoskeletal protein levels. High levels of Pitx2 most likely upregulate

Tubulin levels, which means another control protein is needed to accurately normalize protein

levels between samples before comparing PLC-β levels.

A further complication in our studies is that we were never able to detect Pitx2 by immunoblot in our samples. With a transfection efficiency of 30% based on GFP expression, we expected that Pitx2 would be detectable, but it was not. Therefore, our data our inconclusive as to whether Pitx2 overexpression in a cardiomyoblast-like cell line will affect expression of PLC-β isoenzymes, and so far, does not support our hypothesis that Pitx2 over-expression will decrease PLC- β levels.

Future Studies

In the future, further optimization of transfection conditions will be needed and may include using alternative protocols to increase exogenous expression of Pitx2 to truly test whether this factor will affect levels of PLC-β isoenzymes in cardiomyoblast cells. For accurate sample-sample normalization, it will be necessary to identify a different control protein whose expression is not affected by Pitx2. If ultimately, it can be demonstrated that Pitx2 knockdown or exogenous over-expression is linked to alterations in PLC-β levels in heart, this would provide reasons to further investigate the option of developing a treatment that could alter either PLC-β levels or PLC-β activity in patients prone to development of heart arrhythmias.

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